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Characterization of food-isolated strains of *Lactobacillus fermentum* with potential probiotic activity

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Lactobacilli are normal inhabitants of the human gastrointestinal (GI) tract and among the dominant bacteria in the small bowel and colon. Together with other bacteria of intestinal origin, some specifically isolated strains of lactobacilli are commonly used in the preparation of products with claimed beneficial effects on human health (Vaughan *et al*. 1999). A substantial amount of literature has been generated supporting the use of some *Lactobacillus* species as probiotics. Strains of *L. rhamnosus* (LGG), *L. johnsonii* (La1), *L. reuteri* and *L. plantarum* have been shown to survive transit through the GI tract, adhere to epithelial cells and colonize the human GI tract. Cell-wall associated proteins have been, in some cases, identified as adhesion factors (Vaughan *et al.* 1999).

In order to identify new strains potentially useful as probiotics, we screened a collection of food-isolated lactobacilli for their ability to survive acidic pH and bile salt concentrations lethal for non-intestinal bacteria. Two isolates, L33 and DRL36B, showed resistance to pH 2·5 and bile salts significantly higher than that observed with the well characterized probiotic strains LGG and La1 and were then selected for further analysis. Both strains did not produce exopolysaccharides but secreted antimicrobial molecule(s) active against strains of *Salmonella* and *Staphylococcus* (L33 and DRL36B) and of *Escherichia coli* (L33). Based on the alignment of their 16S rDNA sequences, L33 and DRL36B were both classified as *L. fermentum*, a species commonly isolated from dairy products and from the oral, intestinal and genital microflora of animals.

We then analysed the adhesion properties of L33 and DRL36B to enterocyte-like differentiated Caco-2 cells, comparing them with those of the probiotic LGG. As shown in Fig. 1, all three strains were able to adhere *in vitro* to Caco-2 cells with comparable efficiencies and significantly better than other isolates of our collection (not shown). Interestingly, adhesion to Caco-2 cells was completely abolished when L33 and DRL36B cells were washed in conditions that allow extraction of proteins non-covalently bound to the cell wall. Two predominant polypeptides of 48 and 65 kDa, detected by SDS-PAGE analysis of the extraction buffer, are currently under investigation as potentially important adhesion factors.

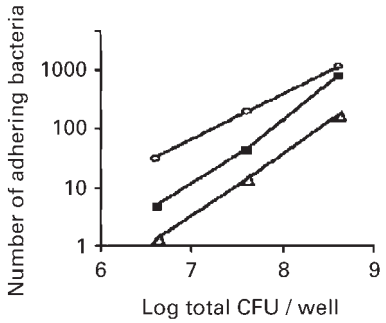

Fig. 1. Concentration dependence of adherence to Caco-2 cells for strains LGG (squares). L33 (triangles) and DRL36B (circles). Adhesion assays were performed in duplicate and numbers reported are the average of adherent bacteria in twenty randomly selected microscopic fields.

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The influence of bifidobacteria on pathomorphological pattern and microflora of gastrointestinal tract in non-infected and *Salmonella*-administered rats

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This research is a continuation of our study on probiotic properties of bifidobacteria strains isolated from the human and animal gut. Strains of *B. longum* KN29·1 and KNA1, and *B. animalis* KSp4 were selected for an *in vivo* experiment and were characterised by their resistance to low pH and bile, adhesion to colon epithelium, and by their high antagonistic

activity against food-borne pathogens (Bielecka *et al.* 1998, 2000, 2002). The experiments were carried out on four groups of Wistar rats (ten per group), fed a casein diet for 15 days. Three experimental groups (B1, B2, B3) were administered orally $\geq 10^9$ *Bifidobacterium* cells, as a suspension in physiological saline and the control group (C) received only the physiological saline. Additionally, five rats in each group of animals were administered orally *Salmonella enteritidis* 458 (subgroups: SC and SB1, SB2, SB3). After 15 days, the mean number of bifidobacteria in the control group of rats (C) was 8·89 log cfu/g of faeces, whereas in groups receiving *B. longum* KN29·1 and KNA1 (B1 and B2) the populations were significantly higher by about 0·6 log cycle ($P \leq 0{\cdot}05$). *B. animalis* KSp4 (B3) did not affect the number of bifidobacteria. The other groups of colonic bacteria analysed: spore-forming aerobic and anaerobic proteolytic and saccharolytic bacteria, mesophilic aerobic and facultatively anaerobic bacteria, as well as coliforms showed no significant changes in comparison with controls (C). Administered *S. enteritidis* 458 effected great changes in the microflora. In the control group with *Salmonella* (SC), the number of bifidobacteria decreased by 1·58 log cfu/g ($P \leq 0{\cdot}01$), and spore-forming bacteria increased by 2·1–3·5 log cfu/g ($P \leq 0{\cdot}001$) in comparison to group C. The other groups of microflora did not significantly change. In groups of rats administered bifidobacteria and *Salmonella* (SB1, SB2, SB3) the numbers of bifidobacteria were significantly higher by 1·65 log cfu/g ($P \leq 0{\cdot}01$), 3·20 ($P \leq 0{\cdot}001$) and 2·12 ($P \leq 0{\cdot}01$), respectively, whereas the numbers of spore-forming bacteria were significantly lowered by 1–3 log cfu/g in comparison to the control (SC). The results indicate the preventive role of bifidobacteria for maintenance of a proper microflora balance in cases of intestinal enterobacteria disturbance. The *Bifidobacterium* strains did not cause undesirable morphological changes in the mucosa of the stomach, small or large intestine. They increased the activity of stomach mucosal endocrine cells, the number of intraepithelial lymphocytes in lamina priopria, as well as the number of plasma cells in villi. Furthermore, in groups SB1, SB2 and SB3, bifidobacteria prevented the morphological damage to the gastrointestinal tract caused by *Salmonella*. Bifidobacteria affected positively non-specific cellular immunity parameters (phagocytosing cells, mean number of bacteria cells absorbed by leukocyte – phagocytic index, and metabolic activity of leukocytes) as well as increasing the levels of lysozyme and total protein in blood serum, also in groups of rats administered *Salmonella*. The results indicate that bifidobacteria may beneficially influence non-specific cellular immunity as well as humoral immunity in rats.

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Investigation of mucin-degrading activity of bowel flora in preterm and full term infants

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The objective of this study was to describe the postnatal development of mucinase activity of the enteric flora in term and preterm infants. The mucus gel layer of the intestinal tract functions as a mucosal protective layer as well as a substrate for microbial proliferation and is in a dynamic equilibrium between mucosal cell synthesis and bacterial mucinase degradation. Mucinase activity is due to the combined action of enzymes produced by the bacteria which inhabit the gut (Roberton & Corfield, 1999). The intestinal tract is sterile immediately after birth but is subsequently colonized by normal enteric organisms which produce mucinase (Midtvedt *et al.* 1994).

Faecal extracts were assayed for sialidase, β-galactosidase, β-*N*-acetylhexosaminidase, proteinase, arylesterase, sulphatase and whole mucinase using synthetic and physiological substrates. Single samples were analysed from ten adults; forty-one hospitalised preterm babies up to twenty-five days, consecutive weekly samples from eight term babies up to twenty-seven weeks of age, and single samples from fifty-five babies at paediatric follow-up clinics.

All samples had detectable mucinase activity. In general, whole mucinase and individual enzyme activities were lowest for preterm babies and highest for adults, with adult values exhibiting a broader distribution. During the first week of life for preterm babies, whole mucinase, proteinase, sialidase and arylesterase activities increased and then remained at a steady state; whereas β-galactosidase and hexosaminidase activity gradually increased over the period of study. Results from term babies fluctuated extensively between samples. In general, enzyme levels were higher for term than preterm babies and showed no association with age, apart from proteinase activity which increased from 2–3 months of age.

This study demonstrates that mucin-degrading activity is acquired after birth and shows a characteristic developmental pattern for each individual activity. As these enzymes are produced

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by the enteric bacteria it is the composition of the gut flora that governs the effective and regulated turnover of the mucus barrier while still maintaining effective mucosal protection. Mucinase activity can be used to monitor the changes in gut flora colonisation occuring during early life and the responses to feeding practices, probiotics and antibiotics. Improved knowledge of mucinase activity contributes to the assessment of optimal mucosal protection.

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Probiotics in the management of inflammatory bowel disease and irritable bowel syndrome: a literature review

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The precise aetiologies of inflammatory bowel disease (IBD) and irritable bowel syndrome (IBS) are unknown, but the intestinal flora plays an important role in their pathogenesis (Blomquist *et al.* 2001; Camilleri, 2001). In IBD, characteristic mucolytic, enteroadhesive strains of *Escherichia coli* that produce haemolytic and necrotizing toxins have been found; some acute attacks respond to antibiotics. In IBS, abnormal intestinal flora have been observed, and the condition may begin following infectious gastroenteritis or a course of antibiotics that has modified the gut flora.

As the management of both diseases by pharmacological interventions is not entirely satisfactory, alternative strategies have been explored. Probiotics may beneficially alter the intestinal flora, and also have immunomodulatory effects, and so offer a logical approach. Results of twenty-five trials, published as full papers, abstracts and case reports in English or German, have been analysed and are summarized here.

In IBS, ten out of twelve trials, involving over 1100 patients, have reported a beneficial outcome when probiotics were used. Five of the trials were double-blind, randomized and placebo-controlled: each produced a statistically significant result. Probiotics used included *Enterococcus faecium*, heat-killed *Lactobacillus acidophilus*, *L. plantarum* and various mixtures, in different daily doses (range 8×10^6 to 3×10^{11}, median 3×10^9) for varying times (range once to twelve weeks, median four weeks).

In IBD, thirteen trials involving 498 patients (199 with Crohn's disease, 299 with ulcerative colitis) have been analysed, probiotics having been tested to maintain remission between acute attacks. In four comparative trials, a probiotic was as effective as, or improved the activity of, the standard maintenance therapy, mesalazine. The other trials also showed encouraging results. The most commonly used organism (seven trials) was *E. coli* strain Nissle; other organisms used were *L. salivarius*, *Lactobacillus* GG, mixtures, and the yeast *Saccharomyces boulardii*. Again, both the daily dose (range 10^9 to 3×10^{12}, median 5×10^{10}) and period of treatment (range once to one year, median one year) were highly variable, but notably greater than the corresponding figures for IBS.

While some of trials reported here were less than ideally planned and/or reported, the overall impression from the data available is that probiotics may have a place as adjuvant treatment for both IBS and IBD. More formal trials are required, especially in order to determine which probiotics are effective, and optimal doses and periods of treatment. In the meantime, patients with less debilitating forms of IBS and who are anxious to try a new treatment modality might be encouraged to take a course of probiotics, under supervision. Side-effects are minimal, and there is reason to be optimistic about the outcome.

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Utilisation of prebiotic carbohydrates by strains of *Lactobacillus reuteri*

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Prebiotic carbohydrates have been shown to be an interesting alternative to the application of probiotics (Gibson & Roberfroid, 1995). Moreover, synbiotic combinations containing both components have been screened and introduced (Kneifel *et al.* 2000). The intention of this study was to clarify whether strains of *Lactobacillus reuteri* can be combined with prebiotics for

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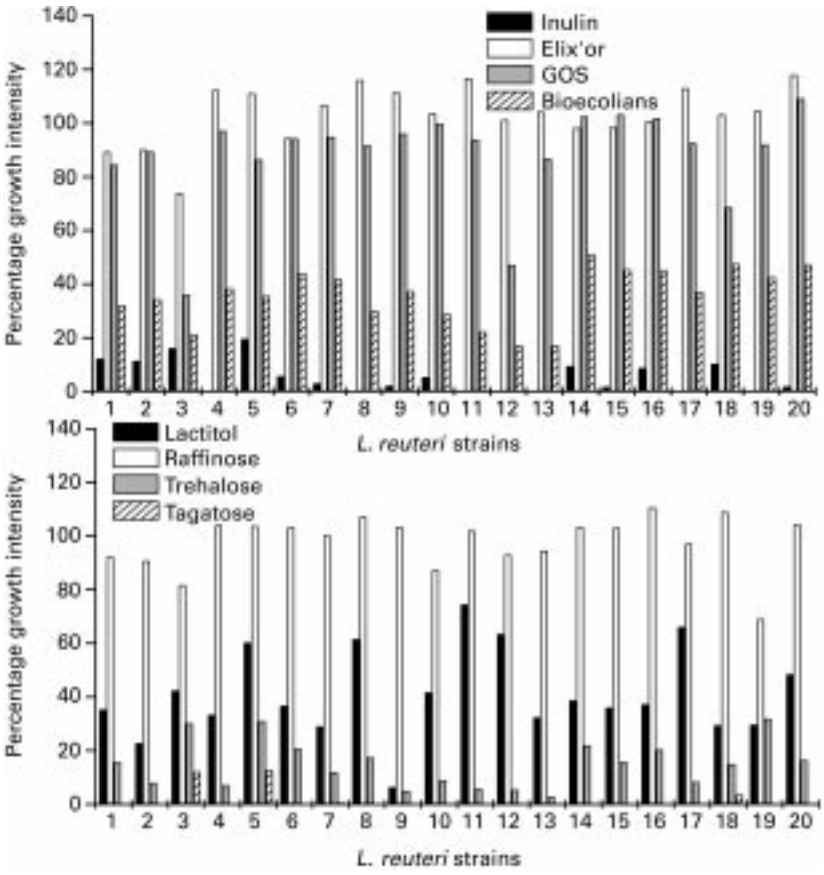

Fig. 1. Growth of strains of *L. reuteri* in MRS broth containing selected prebiotic carbohydrates (growth intensity in glucose medium = 100 %).

synbiotic applications. Twenty strains of *L. reuteri* originating from different sources and belonging to the strain collection of Biogaia Biologics AB were examined for their ability to utilise a selection of carbohydrates of presumptively prebiotic importance. For this purpose, the lactobacilli were cultured in MRS-based media each supplemented with various sugars at 2 % (w/v) and monitored for their growth properties using automated turbidity measurement in a microtitre format and under anaerobic conditions. Those sugars which basically induced a turbid medium were examined at lower concentrations and subjected to a conventional plate count culture technique. In parallel, three commercially available *Bifidobacterium* strains were also investigated and served as reference micro-organisms. Their (bifidogenic) growth performance detected in modified MRS broth was compared with that of the lactobacilli strains. Furthermore, the acidification properties of the bacteria were recorded.

In general, an excellent growth stimulation effect of the galacto-oligosaccharides (Elix'or, GOS) was observed with most of the strains. Also raffinose, lactulose, stachyose and the gluco-oligosaccharide preparation Bioecolians were utilised by many, lactitol by a few strains. Selected results are shown in Fig. 1. Fructo-oligosaccharides did not induce pronounced growth of the bacteria. The acidification potential observed was in agreement with the optical density and viable count values. The bifidobacteria exhibited a markedly strain-dependent behaviour and also indicated some preference for galacto-oligosaccharides and for the gluco-oligosaccharide products. Moreover, they also grew well in some of the fructo-oligosaccharide-containing media. In the light of functional food and feed product development, these *in vitro* data indicate that synbiotic combinations of bacterial strains of *L. reuteri* with certain carbohydrates would be sensible and should further be studied in *in vivo* studies.

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Effect of monoassociation with probiotic strain *Bifidobacterium bifidum* on enterocyte brush-border enzymes in gnotobiotic mice*

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A probiotic, *Bifidobacterium bifidum* (*B. bifidum*) is a Gram-positive anaerobic bacterium colonizing the intestine of healthy newborn mammals. The effect of the intestinal colonization of *B. bifidum* on enterocyte brush-border enzymes was studied in weaned 23-day-old and in 2-month-old gnotobiotic mice and compared with germ-free (GF) and conventional controls (CV).

The inbred mouse strain B10.BR, H-2^k, reared in GF or *B. bifidum*-associated conditions or conventionally reared, were used for our experiments. Two age groups of GF mice were associated with *B. bifidum* CCM 3762 (10^8 CFU/ml) eleven days before the end of the experiment. Specific activities of lactase, sucrase, glucoamylase, alkaline phosphatase (AP), dipeptidyl peptidase IV (DPP IV) and γ-glutamyltranspeptidase was determined in isolated jejunal brush-border membranes using substrates — lactose, sucrose, starch, *p*-nitrophenylphosphate, Gly-L-Pro-4-nitroanilide and 5-L-Glu-(4-nitroanilide), respectively (Kessler *et al.* 1978; Kozakova *et al.* 1998).

Monoassociation with *B. bifidum* was found to speed up the biochemical maturation of enterocytes resulting in a shift of specific activities of brush-border enzymes between the values found for GF and CV mice. This effect of *B. bifidum* supplementation was less pronounced for AP, sucrase, glucoamylase and DPP IV in immature gut of weaned mice than of these enzyme activities of 2-month-old ones. See Table 1.

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Table 1. Specific activities of lactase, as representative of disaccharidases, and DPP IV, as representative of peptidases, are presented

	Lactase (nkat/mg protein)				DPP IV (nkat/mg protein)			
	23-day-old		2-month-old		23-day-old		2-month-old	
	Mean	SD	Mean	SD	Mean	SD	Mean	SD
GF	11·5	1·3	8·3	0·4	7·9	0·2	4·9	0·6
B. bifidum	7·5*#	2·2	5·2+	0·2	6·3	0·9	9·0*#	0·3
CV	1·5*	0·2	1·7*	0·1	6·2	1·1	10·3*	0·2

*$P<0·001$ vs. GF controls, +$P<0·05$ vs. GF controls, #$P<0·001$ vs. CV controls.

Development of an ELISA to detect *Lactobacillus casei* Shirota in human stool samples

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The application of probiotics is revolutionising the management of diarrhoeal diseases and allergy. Since we reported a marked increase in sodium absorption in an infant with short bowel syndrome (Candy *et al.* 2001), we were encouraged to investigate other therapeutic applications of *Lactobacillus casei* Shirota (LcS). In order to optimise doses and to measure compliance with LcS therapy, an antigen-capture ELISA was developed.

A specific monoclonal antibody (mAb) raised in mice against

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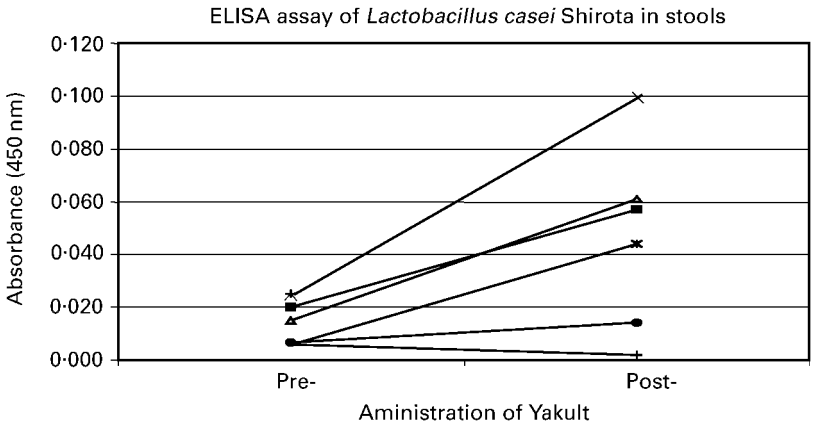

LcS (clone L8-T10) was kindly donated by Yakult Ltd. One half of the mAb was successfully biotinylated for use as the detector antibody. On the basis of various checkerboard assays the optimal concentration for antigen capture adsorbed to the solid phase was found to be 4 μg/ml and for detection (biotinylated mAb) was 5 μg/ml. Antigen labelled by biotinylated mAb was incubated with extrAvidin-HRP, followed by TMB substrate, and the absorbance read at 450 nm. Standards were prepared using formalin treated LcS and run with each assay in order to obtain the total concentration of LcS in stool samples. Six volunteers were recruited, and each provided an initial stool sample prior to consuming LcS-containing Yakult® (65 ml/day) for four days after which a final sample was obtained. Stool samples were diluted 1:256 and assayed as described above.

There was a significant difference between absorbances pre- and post-LcS consumption ($P<0{\cdot}05$). According to the standards run with the assay (not plotted), the total loading (viable and non-viable) ranged from $4{\cdot}2\times10^{7}-2{\cdot}1\times10^{8}$ LcS/g wet faeces. These results compare well with published data (Spanhaak *et al.* 1998) of viable counts of LcS in human stools.

We therefore present a method that provides data on the concentration of LcS in human stools. It bypasses the lengthy culture-based method of Yuki *et al.* (1999) and also demonstrates a novel use of the mAb L8-T10.

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Specific *in vitro* inhibition of the T-helper type 2 cytokine production by lactic acid bacteria: potential role in allergic diseases

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Lactic acid bacteria as probiotics are considered to play a role in human health, mainly by favouring the maintenance of immune homeostasis (Dugas *et al.* 1999). Indeed specific probiotic strains are described as preventing the development of allergic diseases by modifying the balance of the T helper type 1 (Th1)/T-helper type 2 (Th2) cytokine production (Murosaki *et al.* 1998; Kalliomaki *et al.* 2001). In order to verify this hypothesis *in vitro*, we have analysed the effect of some lactic acid bacteria strains (*Lactobacillus plantarum* NCIMB8826, *Lactobacillus lactis* MG1363, *Lactobacillus rhamnosus* GG and *Lactobacillus casei* ATC392) on the capacity of purified peripheral blood mononuclear cells (PBMC) to produce Th2 cytokines following appropriate stimulation. Thus purified PBMC from healthy donors or patients allergic to house dust mite *Dermatophagoides pteronyssinus* were stimulated by the staphylococcal enterotoxin A superantigen (SEA) or by specific house dust mite allergen (Dpt) in the presence or absence of lactic acid bacteria for different periods of time. The levels of Th2 (interleukin (IL)-4 and IL-5) or Th1 (IL-2 and interferon-γ (IFN-γ) cytokines were measured in the supernatants mainly after 48 h incubation. Results showed that, in both cases (stimulation by SEA or Dpt), the production of IL-4 and IL-5 was highly reduced when PBMC were incubated with lactic acid bacteria. In addition the production of Th1 cytokines (IL-2 and IFN-γ) was increased. Further experiments carried out with monocyte-derived dendritic cells indicated that the production of IL-12 involved in the polarisation of T-cell response was also affected by lactic acid bacteria. Taken together, the bacteria tested *in vitro* as probiotics appeared to have the capacity to limit the Th2 cytokine production associated with allergic diseases. These *in vitro* experiments will be further correlated with *in vivo* experiments carried out in the humanized severe combined immunodeficiency mouse model.

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Lactic acid bacteria and bifidobacteria can reduce dietary exposure to aflatoxins*

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Besides their association with liver cancer, aflatoxins have other detrimental effects on the health of humans and animals. Aflatoxin contamination of foods and feeds is a world-wide problem, particularly in the developing countries, with a significant percentage of the world's food supply being affected every year (Henry *et al.* 1999). Epidemiological evidence indicates that even complete elimination of hepatitis B virus infection leaves a significant risk in regions of high aflatoxin contamination. Hence, reduction of aflatoxin exposure needs to be targeted.

It is difficult to reduce the levels of aflatoxins in foods, but it may be possible to reduce their bioavailability. Specific strains of food and intestinal bacteria effectively bind aflatoxin B_1 (AFB_1), aflatoxin M (AFM_1) and other aflatoxin metabolites in test solutions (El-Nezami *et al.* 1998).

To reduce aflatoxin exposure we selected a number of dairy lactic acid bacteria and bifidobacteria strains for assessment of aflatoxin binding. Specific strains proved highly effective in binding AFB_1 and AFM_1 *in vitro* and *ex vivo* in an animal model. In a given genus and even within a given species the capacity for aflatoxin removal was a characteristic of only some strains, and binding efficacy varied markedly.

The ability of the most effective endogenous lactic acid bacteria, *Lactobacillus rhamnosus* GG (ATCC 53013) and *Lactobacillus rhamnosus* LC705 (DSM 7061), was further assessed for binding AFB_1 and AFM_1. Figure 1 shows the binding capacities of these specific dairy strains in aqueous solution and milk. The removal of aflatoxins by non-viable (heat-killed) bacteria indicated that the toxins are not removed from solution by bacterial metabolism but rather are bound to the bacteria.

For effective removal of aflatoxin from solution by these strains, the bacterial concentrations must exceed 10^9 bacteria/ml and the total number of aflatoxin molecules that can be bound to a single viable bacterium is estimated to exceed 10^8. The site of the binding and the types of interactions involved in the mechanism have been identified. Aflatoxin appears to bind to the bacterial surface as indicated by the accessibility of bound aflatoxin

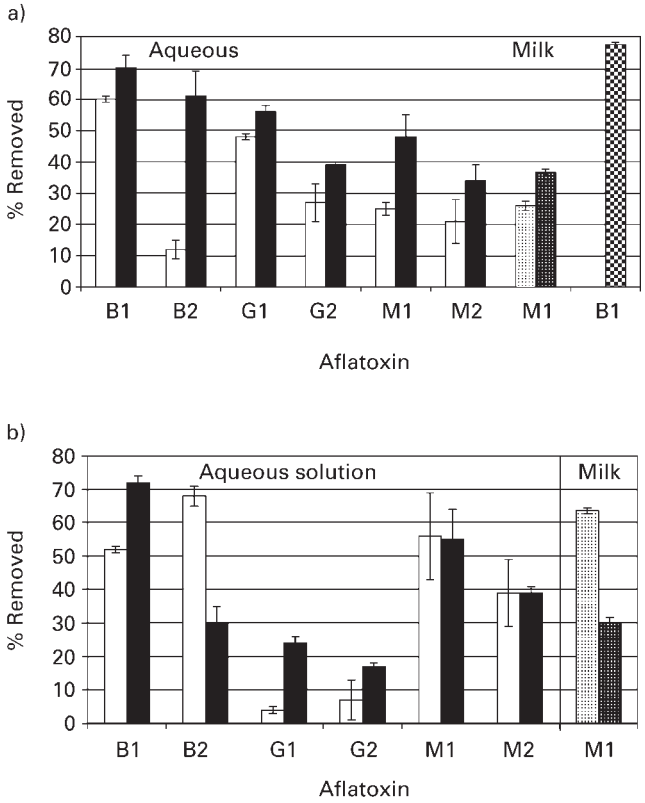

Fig. 1. Ability of a) viable V-GG (□) and non-viable heat-treated, NV-GG (■) *Lactobacillus rhamnosus* strain GG (ATCC 53013), and b) viable V-LC (□), and non-viable heat-treated, NV-LC (■) *Lactobacillus rhamnosus* LC705, to remove different aflatoxins from aqueous solution (□ and ■) and milk (▨ and ▩). The patterned bars indicate removal of aflatoxin from full-cream milk and the NV-GG bar (▪) indicates aflatoxin B_1 removal from milk using NV-GG cells immobilised to a mixture of food thickeners.

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to a polyclonal anti-aflatoxin antibody in an indirect competitive inhibition ELISA, and recovery of up to 98 % of bound aflatoxin from the bacteria by chloroform extraction (Haskard *et al.* 2001). Transmission electron microscopy has shown that exopolysaccharides are present for both strains when optimal binding occurs. The effects of pronase E, lipase and *m*-periodate on binding suggest that binding occurs predominantly with exopolysaccharides of *L. rhamnosus* strain GG and protein components of *L. rhamnosus* strain LC705. The effect of the anti-hydrophobic agent, urea, on binding suggests that hydrophobic interactions are important. Studies over a range of ionic strengths, using monovalent and divalent metal ions, and a range of pH, showed no substantial effects on bacterial aflatoxin binding, implying that electrostatic interactions and hydrogen bonding do not play a major role.

The tested bacteria offer a means for the decontamination of foods. In the case of milk, specific viable and non-viable lactic acid bacteria selectively remove aflatoxin residues. Such properties warrant studies on their dietary administration to humans to reduce the exposure to aflatoxins.

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Survival of butyrate-producing *Eubacterium* strains in a fermentor system inoculated with human faecal bacteria*

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Dietary polysaccharides that are not digested in the small intestine provide a major source of energy for the commensal obligatory anaerobic bacteria resident in the large intestine. Since some of these bacteria contribute to healthy gut function directly by producing nutrients required by the colonic mucosa and also affect pathogen exclusion, it is important to understand how diet affects microbial competition and fermentative activity. The role of butyrate in the metabolism and development of a healthy colonic epithelium and thus in the prevention of cancer and ulcerative colitis is well documented, but the ecology of butyrate-producing bacteria is little understood. Barcenilla *et al.* (2000) found that butyrate-producing bacteria isolated from human faecal samples belong to clusters XIVa and IV of the *Clostridium* subphylum of low G+C % Gram-positive bacteria (Collins *et al.* 1994). The survival of three cluster XIVa isolates, chosen as representatives of the major bacterial ribotypes, in duplicate anaerobic fermentor systems that simulate the human colon (based on Scott *et al.* 1998, fermentor medium was based on that of MacFarlane *et al.* 1989) was investigated here. The presence of different antibiotic resistances (rifampicin, tetracycline and fusidic acid) in each strain allowed precise enumeration of each of the three added strains against a background of faecal flora. One strain was also selected by its ability to utilise inulin, in combination with an antibiotic resistance marker. The fermentors were inoculated with faecal flora and allowed to stabilise in the presence of mixed substrate for one week before inoculation with the labelled bacterial strains. The three bacterial strains were then inoculated and enumerated over a seven-day period. Thereafter, the substrate was changed and strains re-inoculated every seven days, to include amylopectin starch, pectin, inulin, xylan and pre-treated shredded wheat. The three introduced strains showed differing survival against a background of human faecal flora and growth was shown to be strongly dependent on which carbohydrate substrate was added. Inulin was seen to enhance growth of two of the introduced isolates, suggesting that this substrate may encourage growth of other important commensal bacteria in addition to bifidobacterial populations.

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Quality of fermented probiotic milks in relation to claims concerning numbers and types of starter bacteria*

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A wide range of fermented probiotic products is available on the UK market. These include concentrated cell suspensions, fermented milks, soft cheeses, and non-dairy products based on soya, oats, etc. There are two areas of concern with respect to the benefits to health claimed for these products (Rowland, 1999). First, that numbers of viable cells at point of consumption may be below 5×10^6 cfu/g, the minimum number necessary to provide therapeutic effects. Second, that the bacteria actually present may not be those designated on the label and, thus, may not be probiotic strains.

A total of 43 chilled fermented probiotic products were included in this study. Preliminary identification of isolates was made on the basis of colonial morphology, cell morphology and Gram-reaction. Fermentation patterns were determined to support presumptive identification. Enzyme profiles were investigated as a means of differentiating poorly defined species. For genetic characterisation, chromosomal DNA was extracted and a fragment of the 16S rRNA gene region was amplified by polymerase chain reaction PCR-amplified and sequenced (Holzapfel *et al.* 2001).

The majority of the products contained viable counts above 5×10^6 cfu/g. Low counts tended to be associated with particular brands and, possibly, starter cultures. Very low counts ($< 10^5$ cfu/g) tended to involve bifidobacteria rather than lactobacilli. In most cases, numbers of both bifidobacteria and lactobacilli were relatively stable during storage, but reduction of numbers was usually greater with bifidobacteria than with lactobacilli. Isolation may be made both of stated strains and phenotypically distinct strains of *Lactobacillus* which do not resemble recognised species. Three soya products have been examined together with an oat-based dessert and a probiotic fruit drink. Two of these products contain a similar strain of *L. rhamnosus*, clearly distinct from *L. acidophilus* and *L. acidophilus* LA5 named on the labels; these designations are thus incorrect. In most cases sequence results confirmed the identification of lactobacilli and bifidobacteria according to phenotypic data. According to 16S rRNA analysis an isolate was identified as *Streptococcus pleomorphus* (rather than *Bifidobacterium*); furthermore, another product contained *L. fermentum* (rather than *L. acidophilus*).

In general terms, the composition of the microflora conformed with label claims. Diversity of lactobacilli present was, however, much greater than anticipated and raised questions which could only be answered by genetic analysis. Although the products examined were generally satisfactory, there were a number of products where consumer expectations were not met and, in other cases, evidence of poor process control and inconsistency of quality.

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Use of real-time polymerase chain reaction for microflora analysis

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The development of functional foods that influence the composition of the intestinal microflora is dependent on methods to properly monitor this microbial ecosystem. It is known that enumeration of bacteria by plating on selective media is only possible for a small number of bacterial groups. In contrast, molecular tools offer the potential to discriminate between almost all bacterial groups or species (Vaughan *et al.* 2000). We have studied the possibility of using DNA sequence information for enumeration of lactobacilli and salmonella by quantitative real-time polymerase chain reaction (PCR) using the Applied Biosystems Taqman 7700 system.

The potential of this method for microflora analysis was inves-

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tigated in a rat study in which five groups of eight rats received a purified diet containing either 4 % (w/w) lactulose, fructo-oligosaccharides (FOS), resistant starch, wheat fibre or cellulose. After adaptation to the diets for fourteen days, all rats received an oral dose of 10^8 cfu of *Salmonella enteritidis*. For *Lactobacillus*, a selective primer–probe combination was developed and validated with pure cultures of lactobacilli and negative control strains. For salmonella we used a commercially available kit (Nogva & Lillehaug, 1999). The numbers of salmonella and lactobacilli were enumerated in faeces four days after infection by plating on brilliant green agar and rogosa agar respectively, and by quantitative real-time PCR.

Lactulose and FOS were found to be most effective in stimulating lactobacilli and suppressing salmonella. Quantitative PCR data correlated strongly with those obtained by plating. These results open the way to develop new primer and probe combinations to enumerate other bacterial groups, species or strains (including probiotics) without cultivation.

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Lactic acid starter and probiotic bacteria: a comparative study of probiotic characteristics and biological barrier resistance

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A 'probiotic' is a live microbial feed supplement that beneficially affects the host animal by improving its intestinal microbial balance. There is some debate as to whether the concept of probiotic should include dead micro-organisms, or even bacterial fragments (Ziemer & Gibson, 1998). If we take into account that *Lactobacillus* and *Streptococcus* have traditionally been used in fermented dairy products to promote human health (Dunne *et al.* 1999), it would be interesting to determine the probiotic characteristics of lactic acid starter bacteria in comparison with the traditionally called 'probiotic bacteria' since the literature contains many conflicting observations for their proposed benefits (Chou & Weimer, 1999). The aim of this work was to determine and compare some probiotic characteristics and resistance to biological barriers to lactic acid starter and probiotic bacteria.

Four probiotic characteristics (deconjugation of bile salts, hydrophobicity, β-galactosidase activity and inhibition of pathogenic bacteria) and the resistance to biological barriers (gastric juice and bile) of twenty-four strains of lactic acid starter bacteria (*S. thermophilus*, *L. delbrueckii* subsp. *bulgaricus* and *L. lactis*) and 24 strains of probiotic bacteria (*L. acidophilus*, *L. casei*, *L. paracasei*, *L. rhamnosus* and bifidobacteria) were compared.

Among the probiotic bacteria tested, *L. acidophilus* was the most interesting species since it showed high values of resistance to gastric juice (viable cell loss was between 0·7 and 1·7 log orders at pH 3 and between 3·7 and 4·8 log orders at pH 2) and bile (growth higher than 70 % with respect to a control in the presence of 1 % bile salts), hydrophobicity (between 41·0 and 67·8 %), β-galactosidase (between 675 and 1300 Miller units) and bile salts deconjugation activities. *B. bifidum* strains showed the same behaviour, although the values of the parameters investigated were slightly lower than those obtained for *L. acidophilus*. Recent criteria have included *S. thermophilus* and *L. delbrueckii* subsp. *bulgaricus* as members of the probiotic organisms list since these are able to release, among other compounds, enzymes (such as β-galactosidase). In this work, it was demonstrated that *L. delbrueckii* subsp. *bulgaricus* was the lactic acid starter species with the best probiotic characteristics among the starter species assessed. Some of the strains assessed were quite resistant to gastric juice and bile, and showed high values for β-galactosidase and pathogen inhibition activities. On the other hand, lactic acid starter bacteria showed hydrophobicity values similar to or higher than those obtained for the strains of the *L. casei* group.

According to the results found, the total probiotic value of a fermented dairy product should take into account not only the intestinal probiotic cultures used in the formulation but also the probiotic contribution of the lactic acid starter microflora.

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Interactions among lactic acid starter and probiotic bacteria used for fermented dairy products

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In the dairy industry, the current trend is to add cultures composed of defined single strains to fermented milks and cheeses. However, microbial interactions may generate undesirable changes in the composition of the bacterial flora during the manufacture and cold storage of fermented dairy products (Bellengier *et al.* 1997). There is little information available about interactions among lactic acid starter and probiotic bacteria strains added to fermented dairy products (Giraffa *et al.* 1996). Further studies on interactions among these bacteria would be desirable (Rajagopal & Sandine, 1990) to ensure that they reach the intestinal tract in a viable form and are able to perform their probiotic function. In this work, interactions among lactic acid starter and probiotic bacteria were investigated in order to establish adequate combinations of strains to manufacture probiotic dairy products.

A total of forty-eight strains of *Streptococcus thermophilus*, *Lactobacillus delbrueckii* subsp. *bulgaricus*, *Lactococcus lactis*, *L. acidophilus*, *L. casei* group and *Bifidobacterium* (eight of each) were used. The detection of bacterial interactions was carried out using the well-diffusion agar assay and the interactions found were further characterized by growth kinetics. Cell-free supernatants (CFS) were obtained from milk and broth cultures.

A variety of interactions were demonstrated. *L. delbrueckii* subsp. *bulgaricus* was found to be able to inhibit some *S. thermophilus* strains. Among probiotic cultures, *L. acidophilus* was the sole species that was inhibited by the others (*L. casei* group and *Bifidobacterium*). In general, probiotic bacteria proved to be more inhibitory towards lactic acid bacteria than vice versa since the latter did not exert any effect on the growth of the former, with some exceptions. The study of interactions by means of growth kinetics allowed the establishment of four different kinds of behaviours between species of lactic acid starter and probiotic bacteria (stimulation, delay, complete inhibition of growth and no interactive effects). It was found that the complete inhibition of growth of *L. delbrueckii* subsp. *bulgaricus* Ab1 by CFS of *L. acidophilus* CNRZ 1881 was due to the production by the latter of a bacteriocin-like substance. Although it was stated that acetate enhanced the growth of *L. acidophilus*, the addition of sodium acetate did not reproduce the stimulation on growth of *L. acidophilus* CNRZ 1881 and A3 observed when CFS of *B. bifidum* A12 and BBI were added.

Possible interactions among strains selected to manufacture a probiotic fermented dairy product are factors that should be considered when choosing the best combinations in order to optimize their technological performance in the process and their survival in the products during cold storage.

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Enzyme activity of the vaginal microflora and its effect on the secreted cervical mucus barrier

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Bacterial vaginosis (BV) is a vaginal condition in which commensal lactobacilli are replaced with a profound overgrowth of *Gardnerella vaginalis* and mixed anaerobes (Speigel *et al.* 1983). It is characterised by an increase in vaginal pH, mucinase activity (McGregor *et al.* 1994), and in some cases a thin, malodorous discharge. It is associated with an increased risk of preterm birth, neonatal morbidity and HIV. The cause of the condition is unknown. Attempts to treat the condition with metronidazole and clindamycin have had little long-term success. Probiotics have been proposed as a potential therapy in BV to combat the loss of lactobacillus strains and stabilize the cervical mucus barrier, which is the first line of defence against bacterial colonisation of the genital tract. Certain BV strains produce glycosidases which may hydrolyse the mucin component of the cervical mucus, therefore rendering the mucosal barrier less effective in resisting bacterial colonisation.

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This study was designed:

(1) to assess the vaginal fluid glycosidase activity in women with and without BV, and
(2) to evaluate the effect of enzymatic activity on human cervical mucins in early pregnancy using a microplate assay for whole mucinase activity.

Glycosidase activity and differences in cervical mucin composition between women with and without BV were assessed in ninety-two women <14 weeks' gestational age attending the Central Health Clinic, Bristol. High vaginal and endocervical swabs were taken. Elutions from the high vaginal swabs spun in a Tris/HCl buffer were used to measure bacterial enzyme activity. Mucus from the cervical swabs was purified for agarose gel electrophoresis characterisation of the cervical mucins.

Glycosidase activity was significantly higher in BV than non-BV cases (Kruskal–Wallis test: sialidase $P<0{\cdot}0001$, galactosidase $P<0{\cdot}0008$, hexosaminidase $P<0{\cdot}049$). Whole mucinase activity was not associated with BV. *Prevotella bivia* was identified as the main sialidase-producing species. However, some strains of commensal lactobacilli were also positive for sialidase activity on culture. One sialidase-positive strain had a high level of sequence identity with *Lactobacillus gasseri* (98·5 %), one strain showed a high level of identity with *L. crispatus* (97·9 %), while three strains showed lower levels of identity with *L. amylolyticus* (91·1–92·2 %).

High glycosidase activity associated with BV may contribute to effective colonisation of the female genital tract. The inhibition of such activity may therefore be a long-term therapeutic option. The finding of sialidase activity in commensal *Lactobacillus* spp. may be indicative of role for this species in normal mucin regulation and turnover in the vagina, and requires further investigation.

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Modulation of the immune response by probiotics in chicken

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Gut flora acquisition takes place during the first few weeks of life. The development of this flora depends on host properties and environment. Interactions with host gut physiology and the host immune system result in a balanced gut flora. A balanced flora contributes to protection of the animal against pathogenic micro-organisms by specific micro-organisms, that compete for milieu factors, space and adhesion. Probiotic micro-organisms, like lactic acid bacteria, can, when taken up with food, specifically stimulate the immune response of the animal (Maassen *et al.* 1998, 2000). Young animals are highly vulnerable to infections in early life because of the immature immune system and decreasing maternal protection in combination with exposure to many new pathogens in a relatively short time. Protection of the animal by stimulating the immune response not only results in the protection of the livestock animal itself, but also of those who consume it and are thus protected from pathogenic bacteria.

	Anti-TNP antibody titre			
Lactic acid strain orally fed	Average IgM	SEM	Average IgG	SEM
Lactobacillus A	158	36	150	86
Lactobacillus B	93	41	46	20
Lactobacillus C	11	9	10	10
Lactobacillus D	20	11	19	9
Streptococcus A	41	17	9	5
No lactic acid bacteria	31	13	18	8

Since policies concerning feed additives like antibiotics will be more stringent in the future, pre- and probiotics, which support growth and health of livestock animals, might be an alternative for these additives. Our interest is focused on the immunological aspects of the use of probiotics. We investigated whether selected probiotics have a measurable effect on the immune response of poultry and whether this effect is dependent on the strain. We isolated several lactic acid bacteria strains from chickens to take advantage of qualities of host-specific strains.

Six groups of ten chickens (five weeks old) were fed with 10^9 lactic acid bacteria or buffer for five days. The chickens were immunised intravenously with 20 μg of the model antigen TNP-KLH. At day seven after immunisation IgM- and IgG-specific anti-TNP titres were measured in serum with an ELISA based on a coating with BSA-TNP.

In this experiment it was clearly shown that one of the selected strains had a statistically significant ($P=0{\cdot}02$) immune stimulating effect in poultry through an increase antigen-specific antibody response. It also became evident that different lactobacillus strains have different effects on this specific response. This immune-enhancing effect may be used to enhance the response to vaccinations and infections. In the future probiotic strains with immune stimulating properties could be added to the feed of chickens in order to protect them from pathogenic infections.

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Lactic acid bacteria and cancer: epidemiological perspective

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Oral preparations of a specific lactic acid bacteria show antitumour activity against experimental bladder tumours in rodents and inhibit some mutagenic activity in the human bladder (Hayatsu & Hayatsu, 1993). One double-blinded placebo-controlled trial using *Lactobacillus casei* (LC) strain Shirota showed a statistically significant decrease in the recurrence of superficial bladder cancer (Aso *et al.* 1995). Based on previous studies, a case–control study was conducted in Japan to determine whether there was a preventive effect of oral habitual intake of fermented milk products against bladder cancer (Ohashi *et al.* 2002). In Japan, yoghurt has not been popular until recently but a fermented milk drink containing LC strain Shirota has been widely consumed through a direct delivery system to offices and homes. More than 10 % of the whole population consumed the strain in the 1970s.

A total of 180 cases (mean age: 67 (SD 10)) were selected from seven hospitals and 445 controls matched by gender and age (within 5 years) were selected from the population. Trained interviewers who were blinded to the study objective asked subjects about eighty-one items including smoking habit and past dietary pattern using a validated questionnaire. The conditional logistic regression was used to estimate the adjusted odds ratio (OR). The adjusted OR for smoking was 1·61 (95 % CI: 1·10, 2·36). Those with a previous (10–15 years ago) intake of fermented milk products of 1–2 times/week had an adjusted OR of 0·46 (0·27, 0·77) and 0·61 (0·38, 0·99) for 3–4 or more times/week. The OR restricted to a specific product containing the Shirota strain were similar and no interaction was found between demographic factors and the protective effect. It was strongly suggested that the habitual intake of lactic acid bacteria reduces the risk of bladder cancer.

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