

Probiotics as modulators of the gut flora

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Probiotic ingestion can be recommended as a preventative approach to maintaining the balance of the intestinal microflora and thereby enhance 'well-being'. Research into the use of probiotic intervention in specific illnesses and disorders has identified certain patient populations that may benefit from the approach. Undoubtedly, probiotics will vary in their efficacy and it may not be the case that the same results occur with all species. Those that prove most efficient will likely be strains that are robust enough to survive the harsh physico-chemical conditions present in the gastrointestinal tract. This includes gastric acid, bile secretions and competition with the resident microflora. A survey of the literature indicates positive results in over fifty human trials, with prevention/treatment of infections the most frequently reported output. In theory, increased levels of probiotics may induce a 'barrier' influence against common pathogens. Mechanisms of effect are likely to include the excretion of acids (lactate, acetate), competition for nutrients and gut receptor sites, immunomodulation and the formation of specific antimicrobial agents. As such, persons susceptible to diarrhoeal infections may benefit greatly from probiotic intake. On a more chronic basis, it has been suggested that some probiotics can help maintain remission in the inflammatory conditions, ulcerative colitis and pouchitis. They have also been suggested to repress enzymes responsible for genotoxin formation. Moreover, studies have suggested that probiotics are as effective as anti-spasmodic drugs in the alleviation of irritable bowel syndrome. The approach of modulating the gut flora for improved health has much relevance for the management of those with acute and chronic gut disorders. Other target groups could include those susceptible to nosocomial infections, as well as the elderly, who have an altered microflora, with a decreased number of beneficial microbial species. For the future, it is imperative that mechanistic interactions involved in probiotic supplementation be identified. Moreover, the survival issues associated with their establishment in the competitive gut ecosystem should be addressed. Here, the use of prebiotics in association with useful probiotics may be a worthwhile approach. A prebiotic is a dietary carbohydrate selectively metabolised by probiotics. Combinations of probiotics and prebiotics are known as synbiotics.

Probiotics: Gut flora

Introduction

Bacterial populations inside the gastrointestinal tract have adapted such that numbers of each genera are fairly consistent, having their own individual growth niche. In order for the intestine to function optimally however, the 'balance' of the bacterial flora must be maintained, and this appears to be increasingly difficult as lifestyles have changed. An increase in stress and modern day living, which makes a consequential demand on the immune system, can disrupt homeostasis in the gut. Similarly, the direct effects of a change in dietary patterns and eating habits can affect overall gut functionality. Another

contributory factor includes the consumption of pharmaceutical compounds, in particular antibiotics, which by design destroy bacteria, and therefore can have a harmful effect on the balance of the gut microbiota. All of these combine to shift the balance of the gut microflora away from potentially beneficial or health-promoting bacteria such as the lactobacilli and bifidobacteria, towards an increase in harmful or pathogenic micro-organisms, like the clostridia, sulphate-reducers and proteolytic *Bacteroides* species. Predominance of the latter may pre-dispose towards a number of clinical disorders, including bowel cancer and inflammatory bowel diseases such as ulcerative colitis, whilst making the host more susceptible to infections by

Abbreviations: AAD, antibiotic-associated diarrhoea; FOS, fructo-oligosaccharides; GOS, gluco-oligosaccharides; SCFA, short-chain fatty acids; TOS, transgalacto-oligosaccharides.

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transient enteropathogens such as *Salmonella*, *Campylobacter*, certain species of *Escherichia coli* and *Listeria*. It is of considerable benefit to the host, therefore, to maintain a good community structure, through increased levels of bacteria such as lactobacilli and bifidobacteria, preferably at the expense of more harmful organisms. This gives justification to the use of probiotics as a way forward for the prophylactic treatment of enteropathogenic infections, as well as contributing towards protection against various intestinal diseases and disorders — especially those mediated by individual pathogens. What remains to be established is the extent to which probiotic organisms can be beneficial, to determine how any benefits may be manifest and to recognise any limitations.

Definition of a probiotic

Early attempts to classify probiotics were not generally accepted (Lilley & Stillwell, 1965; Sperti, 1971). Parker (1974) proposed one early definition as organisms and substances that influence intestinal microbial balance. This definition was subsequently modified when Fuller (1989) redefined probiotics by removing the reference to ‘substances’, like microbial stimulants — which later became known as prebiotics (Gibson & Roberfroid, 1995). Fuller’s revised definition was: ‘A live microbial feed supplement which beneficially affects the host animal by improving its intestinal microbial balance.’ This modified version stressed the need for the supplement to be composed of viable micro-organisms. A recent formal definition of probiotics was agreed by a working party of European scientists and is given as ‘a live microbial feed supplement that is beneficial to health’ (Salminen *et al.* 1998). This emphasised the importance of definitive improvements in health.

A probiotic effect can therefore be manifest via the gut microflora by ingestion of viable micro-organisms, either in the form of specific preparations such as powders, tablets or capsules, or through yoghurts and other fermented foods. They can contain only one, or several different species of micro-organism.

Development of the probiotic concept

The large bowel harbours a nutritionally and physiologically diverse range of bacteria. This microflora offers the host protection against disease, and promotes normal intestinal function (Salminen *et al.* 1998). If the gastrointestinal flora becomes disrupted due to pathogens (Bartlett *et al.* 1987; Gorbach *et al.* 1987), dietary antigens (Salminen *et al.* 1998) or other harmful substances (Borgia *et al.* 1982; Colombel *et al.* 1987; Siitonen *et al.* 1990), this can lead to dysfunction of the intestine, and subsequently a number of disease states. Certain bacterial genera, namely *Lactobacillus* and *Bifidobacterium*, which have a long and safe history in the manufacture of dairy products, are therefore traditionally included in probiotic products to protect against such effects (Vaughan & Mollet, 1999).

The first significant introduction of the probiotic concept was by Metchnikoff at the beginning of the 1900s. He believed that the complex microbial population in the colon was adversely affecting the host through so-called

Table 1. Examples of microbial species used as probiotics

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| Lactobacilli: e.g. <i>L. acidophilus</i> , <i>L. casei</i> , <i>L. rhamnosus</i> , <i>L. reuteri</i> , <i>L. plantarum</i> |
| Bifidobacteria: e.g. <i>B. longum</i> , <i>B. bifidum</i> , <i>B. breve</i> , <i>B. infantis</i> |
| Gram-positive cocci: e.g. <i>Lactococcus lactis</i> , <i>Enterococcus faecium</i> |
| Yeast: e.g. <i>Saccharomyces cerevisiae</i> , <i>Saccharomyces boulardii</i> |
| Fungi: e.g. <i>Aspergillus oryzae</i> |

‘auto-intoxication’, and reported that Bulgarian peasants, who consumed large quantities of fermented milk, experienced longevity (Metchnikoff, 1907). This was attributed to the health-promoting values of the live organisms. He therefore abandoned his practice of surgical removal of the colon, and began modification of the activity of the colonic microflora by the ingestion of soured milks. A Gram-positive rod, which he called the Bulgarian bacillus and later *Bacillus bulgaricus*, is likely to be the organism later known as *Lactobacillus bulgaricus*. This is now called *L. delbrueckii* subsp. *bulgaricus*, which together with *Streptococcus salivarius* subsp. *thermophilus* is responsible for the fermentation of milk to form traditional yoghurt.

Subsequent research looked to confirm that the consumption of lactic acid bacteria was having a beneficial effect on health. For example, preparations containing *Lactobacillus acidophilus* were used to alleviate constipation (Rettger *et al.* 1935), whilst concomitantly, in Japan, Shirota selected beneficial strains of lactic acid bacteria which could survive passage through the intestine, and subsequently used them to develop fermented milk drinks (Shortt, 1999). It was soon established that there were many species of lactic acid bacteria in the intestine, and these have subsequently been incorporated into many probiotic preparations. Table 1 shows lactic acid bacteria and other micro-organisms which are currently being used as probiotics either singly or in combination. Lactobacilli and bifidobacteria are the most frequently used genera.

Lactobacilli are Gram-positive, non-spore forming rods, catalase negative, usually non-motile and do not reduce nitrate. As glucose fermenters, they can be divided into biochemical subgroups on the basis of the metabolic route by which glucose is metabolised, either homofermentative or heterofermentative. According to 16S rRNA sequencing data, lactobacilli may further be divided into three distinct 16S rRNA groups (Collins *et al.* 1991). The *Lactobacillus* population of the human gastrointestinal system consists of various species, subspecies and biotypes of the genera, with the most frequently isolated lactobacilli belonging to six species, *L. acidophilus*, *L. salivarius*, *L. casei*, *L. plantarum*, *L. fermentum* and *L. brevis* (Mikelsaar *et al.* 1998). Lactobacilli have GRAS (Generally Regarded As Safe) status (Salminen *et al.* 1998).

Bifidobacteria have also accumulated a high research profile as probiotic bacteria, largely due to their proposed health benefits. Species belonging to this genus were first isolated from the faeces of breast-fed infants by Tissier (1906). In the past, early strains of bifidobacteria had been variously assigned several taxonomic genera including *Bacillus*, *Bacteroides*, *Tissieria*, *Nocardia*, *Lactobacillus*,

Actinomyces, *Bacterium* and *Corynebacterium* (Mitsuoka, 1984). However, bifidobacteria were classified into a separate genus (Rogosa, 1974) on the basis of their characteristic morphology, biochemistry, cell-wall constituents and DNA base composition (Scardovi *et al.* 1971). Bifidobacteria are Gram-positive, non-spore forming rods, with distinct cellular bifurcating or club-shaped morphologies. Since bifidobacteria constitute up to 25% of the gut microflora in some adult individuals, they make a significant contribution to carbohydrate fermentation in the colon. Hexoses are fermented by the fructose-6-phosphate, or 'bifidus' shunt (de Vries & Stouthammer, 1968), which is characterised by the presence of the key enzyme fructose-6-phosphate phosphoketolase.

Ecological studies have demonstrated that bifidobacteria can be isolated from various microbial systems (Modler, 1994). For example, they are resident in the colons of humans and animals, and can also be found in the bodies of insects. Of the twenty-four species of *Bifidobacterium* so far recognised, nine are isolated predominantly from humans, *B. bifidum*, *B. longum*, *B. infantis*, *B. breve*, *B. adolescentis*, *B. angulatum*, *B. catenulatum*, *B. dentium* and *B. pseudocatenulatum* (Ballongue, 1998). The most common species found in the infant gut are *B. infantis*, *B. breve*, and *B. longum*, whilst *B. adolescentis* and *B. longum* are thought to predominate within the adult colon (Modler *et al.* 1990).

Newborn infants are firstly colonised with lactobacilli and bifidobacteria primarily via the birth canal during delivery, a hypothesis supported by evidence that infants born via the genital tract are colonised more rapidly than those delivered through caesarean section (Mitsuoka *et al.* 1974; Bezirtzoglou, 1985). The numerical differences in the lactobacilli and bifidobacterial populations between breast-fed and bottle-fed infants is significant. In healthy breast-fed infants, lactobacilli may account for up to 10^7 per gram of faeces, with bifidobacterial levels at up to 10^{11} per gram, which are generally much higher than those of enterobacteria, and bacteroides within the first week of life (Mitsuoka, 1982; Yuhara *et al.* 1983; Benno *et al.* 1984). In contrast, in bottle-fed infants, lactobacilli numbers are only up to 10^6 per gram faeces, with the bifidobacterial population at around 10^{10} per gram, this is coupled with increased numbers of enterobacteriaceae, streptococci, bacteroides, *E. coli* and clostridia (Yuhara *et al.* 1983).

Numbers of bifidobacteria fall significantly upon weaning, and this occurs concomitant with an increase in populations of bacteroides, clostridia, enterobacteria and eubacteria, streptococci and even lactobacilli, such that the colonic bacterial profile typically resembles the adult pattern. These populations usually remain more or less stable throughout the adult population, until a decrease in bifidobacterial numbers is accelerated in the elderly (Mitsuoka, 1984). It is recognised however that throughout adult life, the homeostatic state of the gut can be altered by the factors described earlier and this may predispose towards acute and chronic disorders. One line of thinking as a means of combating this problem is to administer probiotics, to re-establish (for example) lactobacilli and/or bifidobacterial populations, and thereby restore the balance

of the microflora, avoiding possible further infection, or subsequent sequelae which can result from a disrupted gut flora.

Probiotic effects

The gastrointestinal microflora has been identified as being involved in a number of disease states. These can include lactose intolerance, chronic diseases such as Crohn's disease and ulcerative colitis, acute gastroenteritis, food hypersensitivity and allergies, and colon cancer (Gibson & Roberfroid, 1999). Primarily, these are thought to result from an imbalance of the normal microbial balance of the large intestine. This has highlighted the importance of so-called 'microbial interference treatment', i.e. the use of probiotics, as a means of infection control.

Intestinal infections

A number of clinical studies have used probiotics as dietary supplements for the prevention and treatment of gastrointestinal infections. These include infantile diarrhoea, travellers' diarrhoea, antibiotic-associated diarrhoea (AAD) and foodborne pathogenic diarrhoea.

Infantile diarrhoea. Rotavirus is a very common cause of infantile diarrhoea, and is frequently characterised by increased intestinal permeability (Jalonen *et al.* 1991) and higher serum levels of β -lactoglobulin containing immune complexes. Several studies have highlighted the ability of different probiotics, including various lactobacilli and bifidobacteria, to reduce the duration of rotavirus and watery diarrhoea (Table 2).

Antibiotic-associated diarrhoea. *Clostridium difficile* is thought to be an aetiological agent of pseudomembranous colitis, one of the most common causes of AAD. Infection is commonly associated with prior or concomitant antimicrobial therapy, which can disrupt the normal flora balance allowing *C. difficile* to establish and produce at least two types of toxins (Salminen *et al.* 1998). These form a pseudomembrane composed of fibrin, mucin and epithelial cells that may eventually occlude the bowel. Probiotic bacteria have been found to have beneficial effects on both the prevention and treatment of AAD (Table 2). Both *B. longum* and *Lactobacillus rhamnosus* GG have been shown to decrease the course of erythromycin-induced diarrhoea. Two trials have found that *Saccharomyces boulardii* treatment significantly reduced the incidence of AAD (Surawicz *et al.* 1989; McFarland *et al.* 1994, 1995).

Overgrowth of candida in the gut is also a frequent consequence of antibiotic therapy. Studies in hamsters have shown that the gut microflora is involved in suppression of *Candida albicans* (Kennedy & Volt, 1985). In gnotobiotic mice, *S. boulardii* protected against colonisation of the gut by *C. albicans* (Ducluzeau & Bensaada, 1982). One human trial in patients undergoing chemotherapy treatment for leukaemia showed that a milk preparation containing *L. acidophilus* and a *Bifidobacterium* sp. was effective in reducing the count of candida in faeces (Tomoda *et al.* 1983).

Travellers' diarrhoea. A common aetiological agent in travellers' diarrhoea is enterotoxigenic *E. coli*. A number

Table 2. Examples of microbial infections and the effects of probiotics

| Disorder | Subject | Probiotics | Effect | Reference |
|---------------------------------------|------------------|---|--|--|
| Infantile diarrhoea | Human | <i>Lactobacillus</i> GG | Reduced duration of diarrhoea | Isolauri <i>et al.</i> (1991) Isolauri <i>et al.</i> (1994) Kaila <i>et al.</i> (1992) Majaama <i>et al.</i> (1995) Raza <i>et al.</i> (1995) Pant <i>et al.</i> (1996) |
| | Human | <i>Lactobacillus reuteri</i> | Reduced duration of diarrhoea | Shornikova <i>et al.</i> (1997) |
| | Human | <i>B. bifidum</i> + <i>S. thermophilus</i> | Prevented rotavirus diarrhoea | Saavedra <i>et al.</i> (1994) |
| Antibiotic-associated diarrhoea | Human | <i>B. longum</i> | Decreased course of erythromycin-induced diarrhoea | Hotta <i>et al.</i> (1987) |
| | Human | <i>Lactobacillus</i> GG | Decreased course of erythromycin-induced diarrhoea, and other side-effects of erythromycin | Colombel <i>et al.</i> (1987) |
| | Human | <i>Streptococcus faecium</i> | Decreased diarrhoea associated with antitubercular drugs administered for pulmonary TB | Siitonen <i>et al.</i> (1990) |
| | Human | <i>S. boulardii</i> | Reduced incidence of diarrhoea | Borgia <i>et al.</i> (1982) Surawicz <i>et al.</i> (1989) McFarland <i>et al.</i> (1995) Elmer <i>et al.</i> (1996) |
| Relapsing <i>C. difficile</i> colitis | Human | <i>Lactobacillus</i> GG | Improves/terminates colitis | Gorbach <i>et al.</i> (1987) Bartlett <i>et al.</i> (1987) |
| | Human | <i>Lactobacillus</i> GG | Eradicated associated diarrhoea | Bennet <i>et al.</i> (1990) Biller <i>et al.</i> (1995) |
| Travellers' diarrhoea | Human | <i>L. acidophilus</i> + <i>B. bifidum</i> | Decreased frequency, not duration of diarrhoea | Black <i>et al.</i> (1989) |
| | Human | <i>Lactobacillus</i> GG | Decreased incidence of diarrhoea | Oksanen <i>et al.</i> (1990) Hilton <i>et al.</i> (1996) |
| Foodborne pathogen exclusion | Male BALB/c mice | <i>L. casei</i> Shirota | Increased resistance to lethal infection with <i>Salmonella</i> , <i>E. coli</i> and <i>L. monocytogenes</i> | Nomoto <i>et al.</i> (1989) |
| | Male rat | Yoghurt bacteria | Increased resistance to salmonellosis infection | Hitchins <i>et al.</i> (1986) Bovee-Oudenhoven <i>et al.</i> (1996) |
| | <i>In vitro</i> | <i>L. acidophilus</i> + <i>Lactobacillus</i> GG | Inhibited growth of <i>Salmonella</i> | Brassart <i>et al.</i> (1995) |
| | Human | <i>Lactobacillus</i> GG | Decreased shigellosis-associated diarrhoea | Sepp <i>et al.</i> (1995) |

of studies have assessed the potential of probiotics to prevent this form of diarrhoea, with varying results depending upon the species used, vehicle and dosage schedule (Black *et al.* 1989; Oksanen *et al.* 1990). Nevertheless, a number of probiotic bacteria have been reported to result in beneficial effects, including *Lactobacillus* GG and *B. bifidum* (Table 2).

Foodborne pathogen associated gastrointestinal infection. Disruption of the normal balance of the resident gastrointestinal microbiota can allow establishment and growth of transient enteropathogens like *Salmonella*, *Campylobacter*, *E. coli*, *Listeria* and *Shigella* spp. Studies using animal models have established the ability of certain probiotics to inhibit pathogen growth (Table 2).

Helicobacter pylori associated gastritis

Helicobacter pylori has been associated with various gastric complaints such as stomach carcinoma and gastritis (Clayton & Mobley, 1997). Although the target organ of probiotic organisms is usually the large intestine, their oral ingestion dictates their passage through the stomach.

Probiotic strains should be resistant to both gastric acid and bile salts, and may persist in the stomach for longer than other bacteria. *In vitro* investigations have established the ability of *L. acidophilus* and *L. casei* subsp. *rhamnosus* to inhibit *H. pylori* (Brassart *et al.* 1995; Midolo *et al.* 1995; Lambert & Hull, 1996), whilst lactic acid bacteria generally were found to produce anti-*Helicobacter* factors (Luo *et al.* 1994). An animal model using gnotobiotic mice found *L. salivarius* to be effective in inhibiting *H. pylori* (Kabir *et al.* 1997), whilst in a human trial, *L. acidophilus* was effective at inhibiting colonisation of the organism (Michetti *et al.* 1995). However, given the rapid transit time of organisms in the stomach and the physical location of *H. pylori* therein, it is debatable whether probiotics can exert any major effect against the pathogen.

Immunomodulatory and immunostimulatory functions

Some sections of the human population may have a dysfunctional immune response, e.g. infants, the elderly, surgical patients, trauma victims, the immunocompromised, and stressed individuals. Conventional immunostimulatory

Table 3. Immunostimulatory effects of probiotics

| Disorder | Subject | Probiotics | Effect | Reference |
|-------------------|---------|-------------------------|---|-------------------------------|
| Food allergy | Rodent | <i>Lactobacillus</i> GG | Highlighted the importance of the route of antigen absorption in determining the subsequent immune response to that antigen | Isolauri <i>et al.</i> (1993) |
| | Human | <i>Lactobacillus</i> GG | Modulated the immune response to dietary antigen in milk-hypersensitive adults | Pelto <i>et al.</i> (1996) |
| Atopic dermatitis | Human | <i>Lactobacillus</i> GG | Degraded food antigens, leading to down-regulation of T-cell responses. Possible release of tolerogens from allergens | Sütas <i>et al.</i> (1996) |

approaches have been either vaccines, which are not only ineffective in immunocompromised individuals but also incur high costs from widespread use, or immunostimulatory pharmaceuticals, which are generally effective but cause adverse effects such as fever, headache, vomiting and hypertension. In man, probiotic bacteria have been found to have immunostimulatory properties for the treatment of viral diarrhoea but also with application to food allergies/hypersensitivity and atopic dermatitis (Table 3) either of which can be attributed to, or associated with, an excess of dietary allergens. In a human trial, twenty-four subjects were fed 450 g of yoghurt daily for four months, and at the end of that period, T-lymphocytes isolated from the volunteers showed significant increase in the production of γ -interferon (Halpern *et al.* 1991).

Mechanisms of probiotic activity

The exact manner in which probiotics may achieve their effect(s) is still uncertain. However, some mechanisms can be speculated upon, such as chemical inhibition or stimulation, competition for nutrients, immune clearance and competition for adhesion receptors.

Biochemical effects

One mechanism by which organisms may inhibit one another is via the production of bacteriocins. These are proteins, or protein complexes, produced by certain strains of bacteria, which can have antagonistic action against species that are closely related to the producer bacterium (Meghrouh *et al.* 1990). Gibson & Wang (1994) suggested that some strains of bifidobacteria could inhibit a variety of pathogenic bacteria through a non-pH related effect. Furthermore, *B. bifidum* strain NCFB 1454 was found to excrete a bacteriocin which was antagonistic to potent enteropathogens and food-spoilage bacteria, such as *Listeria*, *Enterococcus*, *Bacillus*, *Lactobacillus*, *Leuconstoc* and *Pediococcus* spp. (Anand *et al.* 1984, 1985; Yildirim & Johnson, 1998). Other bacterial species known to produce bacteriocins include lactobacilli and lactococci (Talarico & Dobrogosz, 1989).

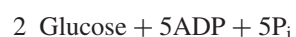
Short-chain fatty acids (SCFA) are also produced in varying quantities as metabolic end products by lactobacilli and bifidobacteria, and it is thought that these may induce an antagonistic effect against other organisms. Furthermore, the accumulation of such metabolites acts to reduce the pH of their surrounding environment, and it is

likely that this may act directly to inhibit the growth of harmful and pathogenic organisms, by producing an undesirable environment.

Both lactobacilli and bifidobacteria are capable of preventing the adherence, establishment, replication, and/or pathogenic action of specific enteropathogens (Saavedra, 1995). A number of mechanisms by which these antagonist properties may be manifest have been proposed, such as decreasing the luminal pH via the production of SCFA, rendering specific nutrients unavailable to pathogens, and/or producing specific inhibitory compounds such as bacteriocins (Sanders, 1993).

Both lactobacilli and bifidobacteria are saccharolytic in their fermentation strategies. Lactobacilli are divided into biochemical subgroups on the basis of the metabolic route by which glucose is metabolised, either homofermentative or heterofermentative species. Homolactic fermentation involves splitting of hexoses into C_3 moieties by fructose-1,6-bisphosphate via the glycolytic pathway, to yield two pyruvates, which are then converted to two lactates. Concomitant with this is the generation of 2 mol ATP/mol glucose. Heterolactic fermentation dissimilates hexoses via the pentose phosphate pathway to produce lactate, CO_2 and ethanol, generating 1 mol ATP/mol glucose. The key enzyme in this pathway is phosphoketolase, which hydrolyses xylulose-5-phosphate to acetyl-CoA and glyceraldehyde-3-phosphate. In addition, pentoses are fermented via this pathway by heterofermentative lactic acid to equimolar amounts of lactate and acetate. Some homofermentative species possess inducible phosphoketolase and can thereby also ferment pentoses to equimolar amounts of lactate and acetate (Lindgren & Dobrogosz, 1990).

Since bifidobacteria are a numerically significant bacterial population in the colonic microflora, they play a vital role in carbohydrate fermentation in the colon. Bifidobacteria ferment hexoses by the fructose-6-phosphate, or 'bifidus' shunt (de Vries & Stouthammer, 1968), as characterised by the presence of the key enzyme fructose-6-phosphate phosphoketolase. This enzyme cleaves fructose-6-phosphate into acetyl-1-phosphate and erythrose-4-phosphate, the latter of which is converted to pyruvate. The principal end products of fermentation are acetate and lactate, produced in a 3:2 ratio, as follows:



Antimicrobial compounds produced by lactic acid

bacteria, lactobacilli and bifidobacteria include fermentation products, such as organic acids, hydrogen peroxide, and diacetyl, but many lactic acid bacteria also produce antimicrobial peptides (De Vuyst & Vandamme, 1994). These compounds have an important role in inhibiting the growth of spoilage organisms, and for this reason have attracted attention as potential fermented food preservatives (Ouwehand, 1998). Two groups of antimicrobial substances have been identified, low molecular weight antimicrobial substances, for example, reuterin, produced by *L. reuteri*; and bacteriocins (Ouwehand, 1998). The latter have been most thoroughly investigated, and are defined as biologically active proteins, or protein complexes, of high molecular weight, which act bactericidally against Gram-positive bacteria, usually closely related to the producer strain (Klaenhammer, 1988). Bacteriocins are divided into four classes: Class I – lantibiotics; Class II – small hydrophobic, heat-stable peptides (<13 kDa); Class III – large heat-labile proteins (>30 kDa); and Class IV – complex bacteriocins: proteins with lipid and/or carbohydrate (Nes *et al.* 1996). Class I and Class II bacteriocins are currently the most widely studied (Jack *et al.* 1995; Nes *et al.* 1996; Sahl & Bierbaum, 1998). Most of the bacteriocins of *Lactobacillus* spp. belong to the Class II bacteriocins. One *Lactobacillus* sp., *L. plantarum*, is most often associated with bacteriocin production. A number of bacteriocins have been isolated from various *L. plantarum* strains. The production of bacteriocins from bifidobacterial strains is less well documented, but two different *B. bifidum* strains were found to produce a bacteriocin (Kebary, 1995; Yildirim & Johnson, 1998), whilst another commercial, bifidobacterial strain was reported to produce a bacteriocin (Meghrous *et al.* 1990). Bifidobacteria can also exert direct inhibitory effects against Gram-negative pathogens like campylobacters, salmonellae and *E. coli* (Gibson & Wang, 1994).

Competition for nutrients

The ability to compete for limiting nutrients is an important factor that determines composition of the gut flora, with species that are unable to compete being effectively eliminated from the system. Bacteria in the large intestine are subject to a range of substrate availability; species in the proximal colon have a large supply of nutrients, provided by dietary residues transiting from the small intestine, whilst those occupying the distal region of the colon have more limited substrate availability. Increasing lactobacilli/bifidobacterial numbers by way of a probiotic may thereby decrease the substrate available for other bacterial populations.

Immune clearance

The potential of the immune response to control growth of micro-organisms in the gut is an important consideration. It has been suggested that surface immunoglobulin A attached to the mucosal membrane may limit or inhibit adherence of enteropathogens invading the gastrointestinal tract (Perdigon *et al.* 1990). Stimulation of systemic components of the immune system, in particular the cell-mediated response, may help to regulate changes in the

gut microflora, for example by increasing macrophage phagocytic activity using lactic acid bacteria (Hatcher & Lambrecht, 1993).

Attachment

One possible mechanism for the action of probiotics is their ability to adhere to the intestinal mucosa (Jacobsen *et al.* 1999). In this way, they may resist peristalsis which would otherwise flush them from the gut. As well as occupying a niche at the expense of potentially harmful organisms, they may specifically block the adherence of enteropathogens. The CaCo-2 cell line is a suitable model for investigating adherence mechanisms, in particular since such cells display typical features of enterocytic intestinal cells (Holzapfel *et al.* 1998). This cell line has been used in a number of studies, to demonstrate that *L. acidophilus* (Coconnier *et al.* 1993; Bernet *et al.* 1994) and *L. casei* (Hudault *et al.* 1997) adhere in relatively high numbers. This may be at the expense of enteropathogens such as *Salmonella typhimurium*, *Yersinia enterocolitica* and enteropathogenic *E. coli*.

Prebiotics

Since the viability of live bacteria in food products and during transit through the gastrointestinal tract may be variable, the prebiotic concept has been developed. Here, the selective growth of certain indigenous gut bacteria is proposed. A prebiotic is a 'non digestible food ingredient that beneficially affects the host by selectively stimulating the growth and/or activity of one or a limited number of bacteria in the colon, that can improve the host health' (Gibson & Roberfroid, 1995). Thus, the prebiotic approach advocates the administration of non-viable entities and therefore overcomes any viability problems in the upper gastrointestinal tract. Certain oligosaccharides which cannot be digested, except through bacterial activity, are prebiotics. Those that contain fructose (e.g. inulin) can alter the composition of the human gut flora, by a specific fermentation, towards a community predominated by bifidobacteria (Gibson *et al.* 1995).

Criteria which allow the classification of a food ingredient as a prebiotic, include:

- (1) It must be neither hydrolysed, nor absorbed in the upper part of the gastrointestinal tract.
- (2) Selective fermentation by one or a limited number of potentially beneficial bacteria in the colon.
- (3) Alteration of the composition of the colonic microbiota towards a healthier composition.
- (4) Induction of effects which are beneficial to the host's health.

Any dietary ingredient that reaches the colon, e.g. non-digestible carbohydrates, some peptides and proteins, as well as certain lipids, is a candidate prebiotic. Non-digestible carbohydrates, like oligosaccharides, are the most likely prebiotics (Gibson *et al.* 2000). Fructo-oligosaccharides (FOS) are β -D-fructans with degrees of polymerisation varying between 2–60 (inulin) and 2–20 (oligofructose). They have several $\beta(1-2)$ - or $\beta(1-6)$ -linked fructose

residues, which may be linked to a glucose residue. A number of other non-digestible oligosaccharides have now been developed, for which there is evidence for their prebiotic effect. These include gluco-oligosaccharides (GOS), galacto-oligosaccharides, transgalacto-oligosaccharides (TOS), isomalto-oligosaccharides, xylo-oligosaccharides, and soyabean-oligosaccharides (Kohmoto *et al.* 1988; Hayakawa *et al.* 1990; Ito *et al.* 1990; 1993; Imaizumi *et al.* 1991; Saito *et al.* 1992; Roberfroid *et al.* 1999).

Of all the prebiotics tested, the inulin type fructans have been the most thoroughly investigated. The fermentability of various dietary components has been studied *in vitro* using mixed faecal culture, with the most predominant culturable bacterial groups, including bacteroides, clostridia, lactobacilli and bifidobacteria being enumerated (Wang & Gibson, 1993). Bifidobacteria selectively fermented the inulin-type fructans, in preference to other carbohydrate sources such as starch, fructose, pectin and polydextrose. This was subsequently confirmed in a human trial, which examined the bifidogenic effect of FOS (Gibson *et al.* 1995). Human volunteers were fed a strictly controlled diet, which in the test group was supplemented with 15 g/d of FOS. In comparison to the placebo group in which sucrose was administered, a bifidogenic effect was observed, with a significant increase in bifidobacterial numbers, concomitant with a decrease in the numbers of bacteroides, fusobacteria and clostridia. More recently, a study used a continuous culture system to examine the bifidobacterial ecology and FOS metabolism, and found that bifidobacterial numbers increased during 48 h fermentation (McBain & Macfarlane, 1997). Such data have also been confirmed in other human studies (Buddington *et al.* 1996; Kleesen *et al.* 1997).

GOS have as yet not been so extensively investigated. Whilst the bifidogenic effect of this sugar has been reported (Ahn *et al.* 1997), another study which fed gnotobiotic rats a diet of 40 g/d of GOS, found little effect on bacterial groups, although modification of numerous glycolytic activities was observed. In particular, β -galactosidase and α -galactosidase were increased, whilst a decrease in levels of β -glucuronidase was observed (Djouzi & Andrieux, 1997). Beta-galactosidase and α -galactosidase have been reported to improve the fermentation of resistant starch and lactulose, leading to increased production of SCFA and lactate (Macfarlane & Cummings, 1991). Furthermore, β -glucuronidase is involved in generation of toxic and carcinogenic metabolites in the distal colon, and therefore reduction of the activity of this enzyme could help to prevent development of cancer.

TOS are manufactured from lactose by transglycosylation reactions and consist of galactosyl derivatives of lactose with β 1–3 and β 1–6 linkages. Bifidobacterial numbers were significantly increased in the faeces of rats fed TOS (Djouzi & Andrieux, 1997), confirming data from earlier experiments (Tanaka *et al.* 1983; Mitsuoka, 1990). One study used TOS in a human volunteer trial to determine their effect on faecal flora. In keeping with findings from other studies, bifidobacteria and *Lactobacillus* numbers were increased, whilst decreasing the numbers of *Bacteroides* spp. and *Candida* (Ito *et al.* 1993). The

bifidogenic nature of TOS has been related to the linkage specificity of the *Bifidobacterium* β -galactosidase, which specifically cleaves β 1–3 and β 1–6 linkages, instead of β 1–4 linkages (Dumortier *et al.* 1994).

Isomalto-oligosaccharides such as isomaltose, panose and isomaltriose, have α 1–6 glucosidic linkages. Their effects on the faecal flora have been examined, and were shown to be fermented by bifidobacteria and *Bacteroides fragilis*, but not by *E. coli* or other bacterial populations (Kohmoto *et al.* 1988). Other studies have examined the soyabean-oligosaccharides, raffinose and stachyose (Hayakawa *et al.* 1990). All bifidobacterial species tested fermented this carbohydrate source, with the exception of *B. bifidum*, whilst *L. salivarius*, *Bact. fragilis* and *Mitsuokella multiacida* metabolised the substrate, but to a lesser degree.

These results suggest that all of these oligosaccharides have the potential for being used for human ingestion, to enhance numbers of bifidobacteria, without increasing numbers of other bacterial populations which may otherwise have harmful effects on host health. Since there is evidence for their purported effects, prebiotics can therefore justifiably be used as dietary supplements to alter the gut flora composition.

Inhibition of growth of some human enteropathogens such as salmonellae has been reported in the presence of FOS (Oyarzabal & Conner, 1995), whilst others report bifidobacterial antagonistic activity against Gram-negative species, such as *Salmonella*, *Campylobacter* and *E. coli* (Gibson & Wang, 1994). Since both salmonellae and campylobacters are very common aetiological agents in gastroenteritis and diarrhoeal disease, there is clearly a market potential for the prophylactic or direct treatment of such infections.

Synbiotics

A further possibility in microflora management procedures is the use of synbiotics, whereby probiotics and prebiotics are used in combination (Gibson & Roberfroid, 1995). The live microbial additions may be used in conjunction with a specific substrate for growth; for example, FOS with a *Bifidobacterium* strain. The end result should be improved survival of the probiotic, which has a readily available (and specific) substrate for its fermentation, as well as the individual advantages that each may offer.

The approach could have particular application in infants and the elderly. An accumulation of literature has reported the benefits of breast-feeding infants, as compared to bottle-feeding, in terms of the predominance of different bacterial groups (Bennet & Nord, 1987; Benno *et al.* 1984; Baquero *et al.* 1988; Kay *et al.* 1990). Administration of a bifidobacteria and/or a bifidogenic prebiotic could increase numbers in the intestine. In individuals above the age of about 55–60 years, faecal bifidobacterial counts are thought to show a marked decrease (Mitsuoka, 1990; Kleesen *et al.* 1997, 2001). This may be relevant in the susceptibility of these individuals to pathogenic infection. As nearly half of the UK population is comprised of children and adults over the age of 60, there is clearly a substantial target population for gut flora interactions.

Conclusions

The area of gut flora modulation seems to hold much promise for the prophylactic management and/or treatment of gut disorders, as mediated by pathogens. For probiotics, many promising studies have been published. However, it is relevant to note that studies on particular strains may not necessarily be extrapolated to all probiotic microorganisms. Each should be assessed on their individual merits. Moreover, it is important that reliable labelling information on microbial contents and numbers are given. For prebiotics, the generation of forms that act at the species, rather than genus, level offers further refinement to the approach (Rabiu *et al.* 2001). Such species-specific oligosaccharides may be used in combination with proven probiotics to enhance their survival, i.e. as synbiotics.

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