

# The Intestinal Microflora

Understanding the Symbiosis



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NUTRITION AND HEALTH COLLECTION

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# The Intestinal Microflora. Understanding the Symbiosis

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# THE NORMAL MICROFLORA AND COLONISATION RESISTANCE

The intestinal microflora represents an enormous biomass of up to  $10^{14}$  bacterial cells comprised of an estimated 400 species. This densely populated microbial environment can deliver an intense metabolic activity, particularly in the colon, making it of considerable physiological importance to the host.

## DEVELOPMENT OF THE MICROFLORA

*In utero* the intestine ought to be sterile, but at birth the baby acquires bacteria such as bifidobacteria and lactobacilli from the maternal birth canal. Babies delivered by caesarean section are initially colonised by bacteria picked up from the hospital environment thereby affecting the overall composition of their microflora. There is some evidence to suggest that babies delivered by natural birth benefit from their intestinal microflora by a reduced risk of necrotising enterocolitis.

The infant microflora tends to be quite simple with respect to the types of organisms present. Breast fed babies traditionally have colonic populations that are dominated by bifidobacteria and lactic acid bacteria, with very few bacteroides, clostridia, and coliforms. More variation occurs in the microflora of formula-fed babies that tend to contain larger numbers of bacteroides, clostridia and enteric bacteria. However, the size of these populations can vary between studies.

The next major stage of bacterial succession occurs during weaning to solid food whereupon the intestinal microorganisms of both sets of babies begin to converge and a more complex, adult-like microflora starts to develop. Bacteroides and other Gram negative bacteria begin to dominate and a wider range of genera is seen (*Table I*).

**Table I.A. The climax flora dominant genera (from Hill)**

Bacteroides	10-11
Bifidobacteria	10-11
Fusobacteria	7-10
Eubacteria	7-10
Lactobacilli	7-9
Streptococci	6-8
Clostridia	6-10
Enterobacteria	6-8

**Table I.B. The climax flora minor genera (from Hill)**

Veillonella	5-7
Enterococci	5-7
Bacilli	ND-5
Micrococci/Staphylococci	ND-4
Methanogens	
Sulphate-reducing bacteria	
Anaerobic cocci	
Many others	

Expressed in Log<sup>10</sup> bacteria per gram faeces.

Once this climax microflora is achieved, we generally maintain the same pattern of bacterial genera for the rest of our lives, although it will vary tremendously at strain level throughout this time. The climax microflora is dominated by non-spore-forming, strictly anaerobic bacteria. Of these, bacteroides and fusobacteria represent the predominant Gram-negative genera, whilst bifidobacteria tend to be the principal Gram-positive populations, with fewer numbers of clostridia, lactobacilli, and Gram-positive cocci. However, the numbers of these bacteria vary considerably between individuals.

## METABOLIC ACTIVITY OF THE MICROFLORA

This vast array of bacterial species represents an extremely complex community with many diverse interactions. For example, veillonella are end-chain fermentors that utilise the lactic acid produced by other genera such as bifidobacteria and lactobacilli. For this reason, lactic acid is not detected in the faeces of healthy subjects, although it is sometimes detected in the stools of infants who lack a fully developed microflora. Methanogenic bacteria and sulphate-reducing bacteria do not tend to be detected in faeces until later in childhood and are another example of organisms that utilise fermentation end products as growth substrates. Therefore, the intestinal microflora comprises a remarkably interconnected ecosystem where factors that modulate one aspect can have many consequences downstream of the initial event.

The presence and metabolic activities of microorganisms inhabiting the gut play an important role in the balance between host health and disease. The ability of bacteria to modulate immune function, metabolise carcinogenic agents and provide a direct barrier to invasion of the gut by pathogenic organisms are examples of the diverse functionality the microflora confers. Other functions are listed in *Table II*.

**Table II. Some health-related activities associated with bacteria growing in the large intestine**

(from : MacFarlane GT, McBain AJ. The human colonic microbiota. In: Roberfroid MB, Gibson GR, eds. *Colonic microbiota, nutrition and health*. Dordrecht: Kluwers, 1999)

Processes	Examples	Effects on host
Carbohydrate fermentation	Digestion of starches, non-starch polysaccharides and oligosaccharides that escape digestion in the small bowel	SCFA formation supports epithelial cell growth, energy reclamation and other aspects of large bowel function. Increased bacterial growth and faecal output. Reduced absorption of toxic products of protein fermentation. Gas production
Proteolysis and amino acid fermentation	Recycling of C and N in proteins and peptides in dietary residues and pancreatic secretions. Production of ammonia, amines, HS <sup>-</sup> , thiols, phenols and indoles	Toxic metabolites associated with hepatic coma, other neurological symptoms, cytotoxicity and colon cancer. Gas production
Hydrogen disposal	Methane production, acetogenesis, HS <sup>-</sup> -formation	Reduction in colonic gas volume, HS <sup>-</sup> cytotoxicity and ulcerative colitis?
Bile acid metabolism	Deconjugation and dehydroxylation of bile acids, desulphation of bile acid sulphates	Absorption of secondary bile acids. Possible promoting activity in colon cancer
Mutagen production	N-nitrosation of secondary amines	Large bowel cancer
Metabolism of neural steroids	Chemical modification of cholesterol, plant sterols and steroid hormones	Reabsorption and recycling of reduced corticosteroids, progesterone, oestrogens, role in breast cancer?
Transformations of xenobiotic substances	Desulphation of cyclamate to produce cycloberylamine Desulphation and deconjugation of drugs excreted in bile $\beta$ -glucuronidase methylazoxy methanol formation from cycasin Conversion of azo bond in sulfazalazine to produce the active drug 5-aminosalicylic acid	Formation of acutely toxic substances, prolonged enteropathic circulation of foreign compounds
Metabolism of lignans and phytoestrogens	Conversion to enterodiol, enterolactone and equol by the microflora	Oestrogenic and antioestrogenic effects: related to fertility and breast cancer
Immune system development and modulation	Shown in probiotic studies, investigations with probiotic animals	Enhanced resistance to infection
Colonisation resistance	Barrier effect of nature microflora against invading species. Degraded during illness or antibiotic drug treatments	Resistance to disease

These activities will vary depending on the composition of the microflora. Like many accounts, the above description of the climax microflora relates to bacterial populations found in faeces, which is then taken to represent microbial composition and activity in the large intestine. However, it is important to remember that microorganisms can establish throughout the length of the gastrointestinal tract. Although sparse relative to the colon, the gastric and small bowel microfloras also require consideration because these populations are involved in various host-microbial interactions and immunomodulatory events. Even within the colon itself, the microflora is known to vary between proximal and distal areas, and there are also the differences between luminal and mucosal associated populations to consider. Indeed regions of inflamed mucosa that occur in conditions like inflammatory bowel disease (IBD) are likely to have a different microflora than non-inflamed regions. Thus, much more information is needed to complement the available data on the faecal microflora which does not represent bacterial populations throughout the gastrointestinal tract.

### MOLECULAR APPROACHES

The culture and isolation of organisms are still perceived as the gold standard in microbial analyses. However, when applied to strictly anaerobic populations these methodologies have drawbacks in that they can be slow, laborious and relatively subjective. Also, microscopic analyses demonstrate that a large proportion of bacterial cells contained within intestinal samples cannot be cultured using existing techniques. Recent estimates of culturability range from 15-58% of all cells present. As a result, molecular techniques are used increasingly to add to our knowledge of the microflora, and in particular these tend to concentrate on analysis of 16S rRNA. This molecule is important in the classification of organisms for several reasons:

- it is present in all cellular life forms;
- its essential function in these life forms results in a highly conserved genetic sequence;
- mutations in the rRNA gene create variable regions within these conserved domains that can be used to establish evolutionary relationships between organisms.

After isolation of the nucleic acids, the rDNA can be cloned and the resultant libraries sequenced to identify prominent bacterial populations. A recent study of this type demonstrated that only 24% of clones corresponded to sequences in the databases, thereby highlighting the huge gap in our knowledge of the unculturable microflora. Gel based techniques like DGGE/TGGE allow rapid comparisons of 16S rRNA from different samples but are semi-quantitative. An alternative is to immobilise the RNA on nylon membranes for use in slot-blot hybridisations. This technique enables the abundance of a particular 16S RNA sequence to be quantified relative to another but requires prior knowledge of the sequence for probe design. The use of fluorescent labels in *in situ* hybridisations also requires probe design but this method gives the population size in the form of cell numbers and so is directly comparable to viable count data. These molecular studies have

demonstrated that rRNAs belonging to the groups bacteroides, fusobacteria, lactobacilli, bifidobacteria, clostridia and eubacteria tend to dominate in faeces. However, the proportions of these are significantly different in caecal samples and differ considerably from the population sizes estimated using viable count techniques.

Flow cytometry and real-time PCR (polymerase chain reaction) are methods that show promise for the high throughput analysis of bacterial populations and could also provide a better correlation with cell number than some of the above techniques. Another up and coming method is MALDI-TOF MS which can be used for rapid DNA sequencing and combined with high throughput DNA array technology. These offer great opportunities for studying intestinal ecology in the near future.

Substantial parts of this previously unrecognised microflora will produce SCFA, carcinogens or other toxins and so it is important to establish the activities of these populations within the intestinal ecosystem. This new perspective raises many other questions. Do animal models maintain the unculturable organisms alongside the cultivable organisms when associated with a human faecal microflora? Does not having a complete unculturable microflora contribute to disease? Do we still get good immune education if some of these species are not present?

### MAC/GAC CONCEPT

Bacterial interaction within the gut has considerable impact on host systems as well as those of other bacterial populations. Studies of these complex interactions have benefited from the use of germ-free animals and the MAC/GAC concept based on the models for determining animal influence. Microflora-associated characteristics (MAC) are defined as any physiological, biochemical, immunological or anatomical structure or function in a macroorganism that is influenced by the presence of microbes. These are ascertained by comparison to the corresponding structure and functions in germ-free animals (GAC) which have no associated microorganisms.

The existence of germ-free animals demonstrates that the gut microflora is not a necessity for the host to survive. However, comparison with conventional animals demonstrates intestinal populations affect important physiological processes, including effects on:

- the anatomy and histology of the digestive tract. Germ-free animals have a distended caecum with a thin mucosa and the speed of cell renewal is reduced;
- the speed of transit which is lower in germ-free animals;
- large bowel biochemistry. Germ-free animals lack many bacterial enzymes and chemical transformation processes conferred by the microflora, *e.g.* SCFA production, bile acid metabolism, conversion of bilirubin to urobilinogen, conversion of cholesterol to coprostanol;
- modulation of the immune activity. Various factors of macrophage function are influenced by the microflora including chemotaxis, phagocytosis, cytokine production and intracellular killing. Germ-free animals also have reduced CD4+ cells and IgA-producing cells in the lamina propria and mucosa, respectively.

## COLONISATION RESISTANCE OR BARRIER EFFECT

The above-mentioned physiological and immunological changes are part of a phenomenon called colonisation resistance, which can be defined as the ability of microorganisms belonging to the normal gut microflora to impede the implantation of pathogens. This function of the microflora is also known as the barrier effect. The non-specific resistance to bacterial pathogens is mediated by direct and indirect mechanisms.

Amongst the **indirect mechanisms** of colonisation resistance are some of these physiological microflora-associated characteristics impacts. For example, the faster rate at which the digester passes through the intestine of conventional, compared to germ-free animals, is important in preventing the establishment of pathogens or potential pathogens in the gut by a wash-out effect. The immune system is also known to regulate the composition of the microflora. For example, organisms we are initially exposed to after birth seem to determine the balance of the microflora later in life. Issues relating to immune balance and tolerance to the microflora are discussed in subsequent chapters.

The **direct effects** of the microflora in colonisation resistance occur when the indigenous bacterial community makes it extremely difficult for newly introduced organisms to establish. A striking example of this was observed back in the 1960s. When mice were dosed with *Salmonella typhimurium* intragastrically, the lethal dose for germ-free mice was approximately 100 million times less than the conventional animals. Unfortunately, even after several decades, the direct mechanisms that make it so difficult for exogenous organisms to establish in this ecosystem are still unclear. In general terms, four mechanisms can be considered:

- **Competition:** two or more microbial types in rivalry for a factor in the ecosystem that is not present in sufficient quantity to satisfy the demands of all of the inhabitants. This could be competition for nutrients, for space, etc. For example, the forestomach of a mouse has large populations of lactobacilli living in an adherent layer on the non-secretory epithelium (approx  $10^{8-9}$  per gram epithelium). Adding 0.3 g/l penicillin to the drinking water of conventional mice removed the lactobacilli from this part of the digestive tract, thereby allowing yeast cells (*Candida*), normally confined to the corpus, to also colonise the forestomach.

- **Amensalism:** the inhibition of one or more microbial types by the production of a toxic substance by another type. A good example is the short chain fatty acids produced by obligately anaerobic bacteria inhibiting populations of facultative anaerobes and pathogenic bacteria such as *Salmonella* under conditions of appropriate pH and Eh.

- **Predation:** one microbial type consumes another, smaller type. Protozoa can ingest and digest bacterial cells, particularly in the rumen, although this is unlikely to be a major factor in monogastric animals.

- **Parasitism:** one microbial type consumes a larger type. Bacteriophage infecting bacteria could be important in this respect but again there are no good examples of its relevance in the human intestinal microflora literature.

## REDUNDANCY

*Escherichia coli* has been used in experiments to try to understand colonisation resistance in mice. Various organisms were gradually allowed to colonise the mouse caecum and their ability to suppress *E. coli* populations assessed. It was found that changing the diet of the animals changed the intestinal environment and a population of 55 strains that could previously inhibit *E. coli* growth was no longer sufficient. An additional 45 strains had to be added to give the same suppressive effect under the new conditions. This work shows there are likely to be many active mechanisms by which the intestinal microflora regulates the population of *E. coli*. Therefore, there is a degree of redundancy as alternative suppression mechanisms come into effect when conditions in the intestinal environment change.

A situation with more relevance to the human intestine is the regulation of *Clostridium difficile* populations in the gut. This bacterium is a member of the normal microflora in a small percentage of humans. However, it is also a major cause of nosocomial infection when it establishes in the gut of patients treated with antibiotics, resulting in diarrhoea and sometimes pseudomembranous colitis. Trying to breakdown the complex intestinal community to just a few strains that will suppress *C. difficile* to the same extent as the gut microflora is an impossible task, and despite years of research, the mechanisms involved in colonisation resistance of *C. difficile* by the microflora remain unknown.

Thus, mechanisms affecting the balance of the microflora and the colonisation resistance functions that it imparts are poorly understood. A pertinent question is that of how we can exploit these influences in health and disease? Knowledge of its composition and activities are central to this, as is a better understanding of how the microflora is tolerated by the host. A great deal of research is currently focused on enhancing colonisation resistance by dietary changes and probiotic therapy. Issues relating to this are discussed in the next chapter.

# EFFECTS OF THE ENVIRONMENT ON THE MICROFLORA

Macronutrients are an important determinant of the microflora in the caecum but because nutrients quickly become depleted as the digester passes through the colon, the effects of these can be difficult to show in the faecal populations. When considering the effects of exogenous factors on the gut microflora, it is important to remember that there are two distinct entities to modify. One is the composition of the bacterial populations and the other is the metabolic activity of these populations. The latter is perhaps the more amenable to effects from the environment.

The interaction of intestinal bacterial populations with dietary components has been studied extensively. Fermentation of carbohydrate by anaerobic bacteria produces SCFA and these are responsible for several physiological effects. These fermentation products have been demonstrated to affect colonic epithelial transport, colonic metabolism, hepatic control of lipids and carbohydrates, provide energy for muscle, kidneys, heart and the brain. Other beneficial aspects of bacterial nutrient metabolism include lipid hydrolysis, protein degradation with production of peptides and amino acids, and vitamin production. However, the metabolic activity of the microflora also has potent detrimental effects such as the inactivation of drugs, and the production of carcinogenic or toxic metabolites.

The effects of dietary components on the microflora has been the subject of many studies, although a considerable number of these were done in animal models. The approaches tend to compare institutional diets to free diets, liquid diets or vegetarian diets. The use of fluids taken from ileostomy patients provides a good working model for activity in the human digestive tract but these samples are not easily available. Dietary components that have been addressed for their effects on the microflora in human feeding studies include protein sources like beef, fat, and of course, dietary fibre. However, one of the most studied effects of diet on the microflora is the intake of milk in the newborn infant,

whether bovine or human. There are many differences in these two types of milk and it is difficult to pin-point the exact components responsible for altering the microflora, but various studies have examined the effects of buffering capacity, lactose content, calcium and phosphate content, casein and whey proteins, bovine lactoferrin, iron, and nucleotides.

When compared to formula-feds, breast milk is often stated as the gold standard in infant and neonatal nutrition and is associated with protection from a range of infections including sepsis, gastroenteritis, otitis media, pneumonia and necrotising enterocolitis. Such conditions are a considerable cause of morbidity in the Western world and life-threatening in developing countries. Numerous studies have identified protective components of breast-milk which have complex interactions with the host. These defence agents include:

- direct-acting antimicrobial agents (including the non-nutrient fractions of carbohydrates, proteins and lipids) such as oligosaccharides, lactoferrin, lysozyme, immunoglobulins and fatty acids;
- growth promoters for selecting bacteria such as bifidobacterial factors;
- immuno-competent cells, including macrophages and lymphocytes;
- anti-inflammatory and antioxidant agents;
- immunostimulating agents such as cytokines.

The combined antimicrobial effect of breast-milk can competitively prevent colonisation of the gut by pathogenic bacteria and may improve the strength of the host mucosal defences. One of the major microbiological differences observed between breast-fed and formula-fed infants in numerous studies using viable count methodologies is the increased ratio of bifidobacteria to clostridia. The latter group of organisms include a range of potential pathogens such as *C. difficile* which is known to be one of the causative agents of antibiotic-associated diarrhoea. Studies using probiotics to increase the intestinal concentrations of bifidobacteria and lactic acid bacteria have shown positive effects for these organisms in aiding the outcome of antibiotic-associated diarrhoea and infant rotavirus diarrhoea, although the mechanisms involved are unknown. Commensal bacteria are known to drive the development of intestinal immune system and these effects on colonisation resistance could be due to bacterial interaction with the infant intestinal mucosa. However, further work is necessary in order to understand how the components of breast milk are driving these protective mechanisms and how these factors could be manipulated to improve infant formulas.

## PROBIOTICS

Elie Metchnikoff was interested in the link between fermented foods and longevity over a century ago and is credited with being the first to introduce the idea of a beneficial gut microflora. Fermented milks and yoghurts are consumed worldwide and research has shown them to be associated with various physiological benefits such as regulation of intestinal peristalsis, alleviation of the symptoms of lactose intolerance, and many others. Study of the organisms contained in fermented foods led to the concept of

probiotics, which can be defined as a live microbial food ingredient that, when consumed in sufficient quantities, exerts health benefits on the host beyond basic nutrition. Yoghurts and other fermented milk products remain one of the favoured modes of delivery for probiotic organisms although they can also be obtained in the form of enteric capsules.

Many claims regarding the health-promoting effects of these probiotic preparations have been suggested and include stabilisation of inflammatory conditions like IBD, prevention of colorectal cancer, and a reduction in hypertension (see Table III). Probiotic therapy has also been implicated in enhancing the colonisation resistance of the gut microflora against intestinal pathogens such as *Helicobacter pylori*, *Salmonella* sp., *C. difficile* and rotavirus infection. However, there are varying amounts of data relating to each of the above claims, with some requiring much more research before any health benefits can be confirmed. The general effects of probiotics on the microflora as a whole are discussed in this section, whilst information pertaining to their effects on the outcome of clinical conditions will be presented in subsequent chapters.

**Table III. Healthfull effects (from Hill)**

Improved absorbability of certain nutrients	Anticancer effects
Alleviation of lactose intolerance symptoms	Provides antagonistic environment for pathogens
Metabolism of some drugs	Blocking adhesion sites from pathogens
Serum cholesterol reduction	Inactivating enterotoxins
Improvement of intestinal motility	Alleviating constipation
Stimulation of immune system	Relieving vaginitis

Given that many experimental factors are difficult to control in human feeding studies, it is not surprising that a good example of a probiotic preparation enhancing colonisation resistance of the gut microflora should be validated in an animal model. Chickens are normally raised under hygienic conditions with no contact with the mother hen. As such, the young chicks do not acquire a normal microflora, thereby leaving them vulnerable to colonisation by *Salmonella*. The *Salmonella* sp. involved are not pathogenic for poultry but are for humans and this results in a public health problem. Studies have shown that when newly hatched chicks were dosed with a probiotic product containing 29 bacterial species, significant levels of protection against colonisation by *Salmonella* could be induced within hours of inoculation. The strength of these findings is evidenced by the fact that this product is currently the only probiotic preparation to be approved by the Food and Drug Administration.

However, the day old chick is probably an optimum for experiments manipulating the intestinal microflora because it is similar to working with germ-free models. It is more difficult to manipulate the microflora in humans; one problem being that the composition

of the intestinal microflora is quite different from one human to another, which is an immediate obstacle to manipulating it. Despite this, there is a lot of interest in establishing probiotic therapies for humans and even raises the question as to whether an artificial microflora could be designed to benefit babies delivered by caesarean section.

Many of the probiotic products on the market at the moment contain lactic acid bacteria, the populations of which tend to be a relatively small proportion of the total gut microflora. Bifidobacteria tend to be the more numerous populations of potentially probiotic genera in the colon, accounting for approximately 1% of the total microflora. Populations of lactobacilli can be several orders of magnitude smaller than this. The relative sizes of these populations and the ability of comparatively low doses of a probiotic organism to directly manifest effects in the intestine remain an issue of debate. Various factors must be kept in mind when considering this. If a low dose is given often and the organism is able to persist in the gut, then levels of the bacterium will increase, thereby enhancing any effect it might have by altering the composition of the microflora. These effects could include those exerted by direct mechanisms of colonisation resistance. However, consensus thinking is that the importance of the lactic acid bacteria as probiotic agents lies more in the indirect mechanisms such as immunomodulation. Indeed, the positive effects of probiotics on viral diarrhoea indicates an immunological mechanism is important because the virus must replicate in the enterocytes lining the small intestine which is away from the major site of bacterial colonisation in the gut. However, the lactobacilli pass through the small intestine where they could have some beneficial impact on the immune system.

If probiotics have an adjuvant effect, there might be an enhanced production of sIgA antibodies which could neutralise virus particles as they are released from destroyed enterocytes, thereby aiding recovery and reducing shedding time. These results relate to serum antibodies, not to those present in the intestinal contents and, unfortunately, most studies done with probiotics on humans include only a very small number of subjects.

Do probiotics offer a way to displace potentially harmful bacteria in the gut instead of searching for new antibiotics to inhibit organisms in a world of ever increasing antibiotic resistance? In order to overcome colonisation resistance, probiotic strains must be robust enough to reach the intestine in significant numbers as they need to be able to grow at sufficiently rapid rates to compete with the resident related species. Recognised actions by which probiotics may act in defending our enterocytes include binding to some glycoproteins, thereby out-competing potentially pathogenic bacteria, or tightening the mucosal physical barrier to microorganisms. This raises further questions of adherence and colonisation of probiotic organisms in the intestine which also need to be addressed.

## PREBIOTICS AND SYNBIOTICS

Another approach to altering bacterial populations within the gut is through the use of prebiotics. These are dietary ingredients that are not hydrolysed nor absorbed in the upper intestine and so are available for fermentation in the large bowel by a limited number

of bacteria. Short chain carbohydrates with recognised prebiotics effects include galacto-oligosaccharides, gluco-oligosaccharides, xylo-oligosaccharides, fructo-oligosaccharides and inulin. The ability of prebiotics to modulate glucose absorption, serum cholesterol levels, SCFA production, faecal bulking and colonisation resistance can, in part, be related to their ability to selectively increase bifidobacterial populations in the gut. However, the literature supporting the use of these compounds therapeutically is not as well developed as that of probiotics and varies considerably between the different carbohydrate products.

A recent concept is that of synbiotics which is a combination of probiotics and prebiotics in one preparation. The rationale is that the presence of a selective substrate will aid the survival of the probiotic organism in the intestine and various synbiotic yoghurts are currently available on the market. Human trials investigating the clinical effectiveness of these preparations are currently underway, including a large European study on cancer prevention.

### **OTHER FACTORS**

With the exception of diet, relatively little is known about other environmental influences on the microflora. Diarrhoea in itself is a determinate of the microflora. The greater the transit time, the greater the ratio of strict anaerobes to facultative anaerobes. Also, where an infection causes inflammation, the mucosal associated microflora and the way it interacts with the mucosa will be affected. Chemotherapy can have a direct effect on specific organisms and can have a general effect on the colonisation resistance of the microflora leading to conditions such as antibiotic-associated diarrhoea.

Very few data exist with regard to temperature and climate and the relevance of these is unclear. Likewise, the effects of physical activity, environmental pollutants, age and stress are poorly documented. These could be of indirect importance given their known effects on host well-being.

# HOST PHYSIOLOGY. FACTORS AFFECTING COLONISATION

The intestinal ecosystem can be considered as comprising of three major components which are in permanent contact and have complex interactions; host cells, bacterial cells, and nutrients.

In a normal individual the transit time through the gastrointestinal tract is between 55-72 hours. Passage through the caecum is rapid, taking only 4-6 hours whereas the digesta can then reside in the colon for an average of 56 hours. This slower movement is one factor that allows such an enormous biomass to colonise the large gut. Opinions differ as to whether the bacteria adhere directly to the enterocytes under normal circumstances. However, it is generally accepted that a large proportion of the microflora exists in biofilms, either in association with food particles, mucus or exfoliated cells. The interactions that exist between these components need to be examined in order to understand how such a large population of bacteria can colonise the gut.

## MUCUS

Mucus is continually produced by goblet cells (*see Figure 3*) to lubricate and protect the intestinal epithelium from the effects of bacteria and digestion. There are two forms of mucus: a water soluble gel adherent to the mucosa, and a water soluble viscous layer covering the gel. The mucus is composed of mucins, which are native glycoproteins whose structure is responsible for the formation of the gel. This mucus layer is able to trap bacteria either selectively or non-selectively.

## THE CARBOHYDRATE REPERTOIRE

Mucin polymers form the structural basis of mucus. The oligosaccharide side chains of these glycoconjugates contain different sugar residues such as fucose, galactose, N-acetyl-glucosamine, N-acetyl-galactosamine and sialic acids. Their structure can be extremely varied depending on the links between these moieties, which include end-to-end sulphide bonds. The polymerised mucin forms a tangled network, thereby increasing resistance to hydrolysis by bacterial enzymes. Intestinal mucins are in direct contact with gut bacteria and it is well established that carbohydrates play an important role in the cell-cell recognition process. The carbohydrate moieties of these glycoproteins not only act as nutrients for the intestinal microflora but also as a binding site for biomolecules such as microbial toxins and surface proteins. Therefore, the presence or absence of such receptors has an important role in the host's susceptibility or resistance to intestinal infections. Carbohydrate structures present in the lumen or on the surface of the mucosa are termed the "carbohydrate repertoire" of the host and these will be recognised by specific structures such as bacterial adhesins (see Figures 1 and 2).

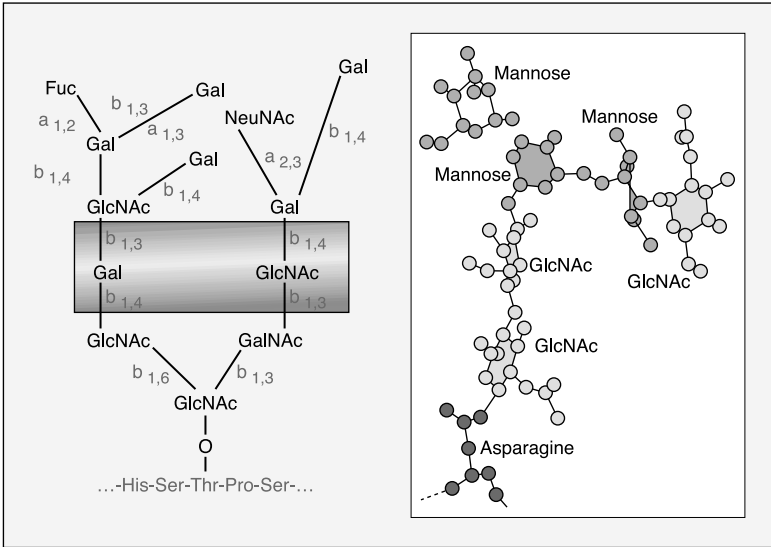
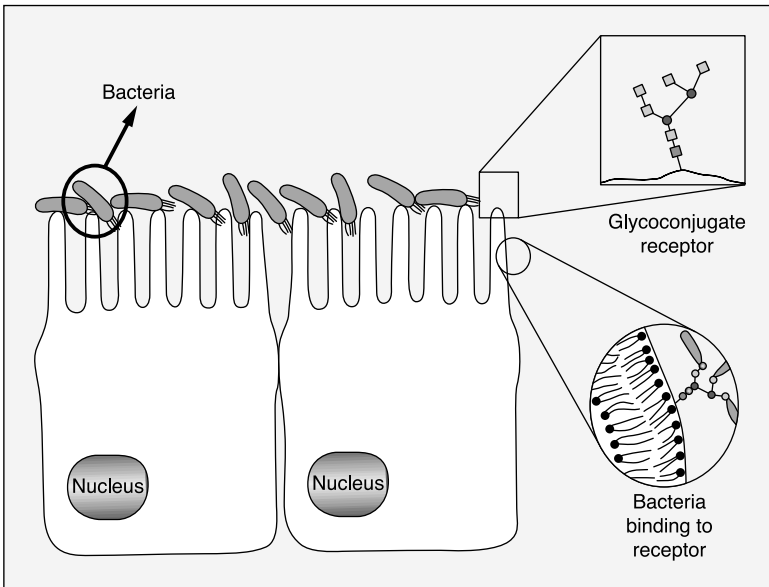


Figure 1. Simplified structures of glycoconjugates (from Freitas).

Glycoconjugates anchored to the cell membranes, which contain the same carbohydrates as mucin along with mannose, are also part of this repertoire. If the mucus has disappeared for some reason, then the bacteria can find additional adhesion sites on the cell surface. This carbohydrate repertoire is genetically controlled by the host and it is one way in which the host's genes can control gut microbial behaviour, *i.e.* adhesion of bacteria.



**Figure 2.** Microbial attachment to the glycoconjugates on the cell (from Freitas).

Three processes are known to regulate this repertoire:

- biosynthesis, which is genetically controlled from inside the cell and can produce different structures of carbohydrates forming an innate repertoire,
- partial or total degradation of these structures by glycosidases produced by the resident bacteria, thereby forming a modified carbohydrate repertoire,
- dialogue between bacteria and cells, which results in an altered innate repertoire.

## BACTERIAL ADHESION

The nature of the carbohydrate repertoire plays a major role in microbial adhesion because bacterial adhesins link to specific carbohydrates present on the cell surface. This can have a beneficial role if the adherent bacteria contribute to the host's intestinal defence, or a detrimental role in the case of adhesion of pathogenic organisms.

This ability of bacteria to adhere to epithelial components coupled with the long colonic transit time contribute to the establishment and maintenance of the large bacterial communities observed in this ecosystem. Moreover, the process of primary bacterial colonisation in the infant gut is initially dependent on the nature of the individual's innate carbohydrate repertoire. If these first bacteria possess glycolytic activities, they will modify the carbohydrate repertoire, thereby encouraging new bacteria to establish and others to be lost. This also provides another explanation as to why each individual possesses his or her own unique microflora.

Given the presence of carbohydrate components in the diet and those associated with the host, it is hardly surprising that a form of competitive interaction occurs between the two. Experiments using mono-associated mice have shown that manipulation of dietary carbohydrates administered in drinking water can modify the adherence of the bacteria to the intestinal wall. This presents another possible mechanism for modulation of the microflora using prebiotics.

With regard to probiotics, colonisation is an important issue. It is not considered essential that probiotic strains adhere to the mucosa to elicit their effects. However, the organisms should show signs of colonisation whereby they form persistent populations beyond that of a mere wash-out effect. A good definition of colonisation is required in order to clarify these matters and more studies should include inert particles as comparators for bacterial transit times through the intestine. Indeed, it is a considered opinion that beneficial effects of a probiotic would be lost if a probiotic strain was able to colonise the gut as its immunomodulatory properties would be compromised. However, some form of host-bacterial interaction would still be required.

### **FACTORS OF COLONISATION RESISTANCE**

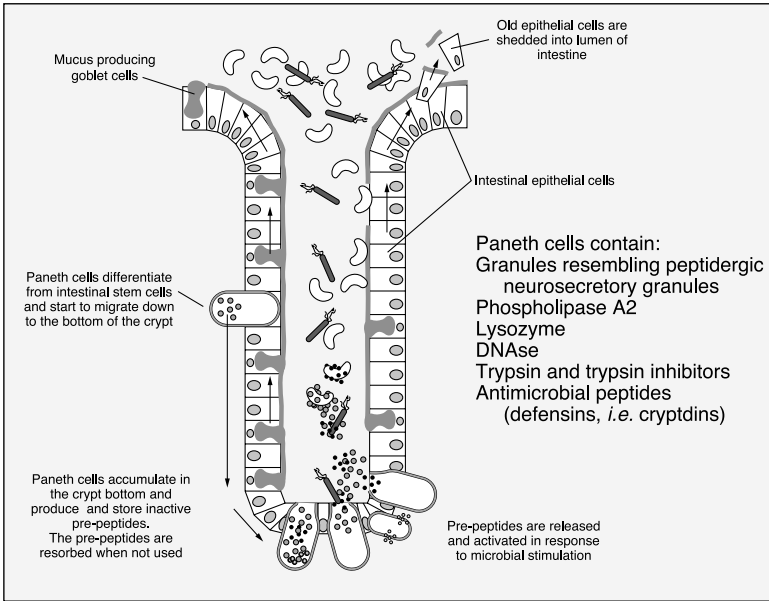
Bacterial-host interaction at the level of the small intestine is frequently overlooked despite being a site of considerable importance with regard to immune modulation. This point is particularly relevant to probiotic therapy for rotaviral diarrhoea. The small bowel microflora is often thought of as secondary to that of the large bowel due to the greatly reduced populations that reside there. However, a better knowledge of all gastrointestinal tract populations and their activities would provide much needed insight into host-bacterial interactions.

There are many factors known to contribute to the low numbers of organisms in the small intestine: the motility of the gut, the bile salts that enter the small intestine, and also the contribution of the small intestinal mucosa itself. The physiology of the intestinal epithelium includes exfoliation of enterocytes within 2-3 days of them being generated in the crypts of the villi. As these cells migrate, they can differentiate into goblet cells that secrete the mucins associated with the mucus barrier, and there are M cells which are associated with macrophages and lymphocytes that can sample antigens within the lumen. There are also dendritic cells that infiltrate this monolayer, and the enterocytes themselves which secrete vast amounts of IgA into the lumen directed against bacteria and viruses.

### **PANETH CELLS**

Another cell population, however, differentiates in a downward migration into crypts. The hallmarks of this cell are granules that are delivered apically into the space of the crypt lumen. Amongst the products of these cells are lysozyme, defensins and secretory

phospholipase A2. Thus, the crypt lumen is distinctly different from that above the crypt-villus junction. These Paneth cells produce  $\alpha$ -defensins which can also be found in neutrophils where they are involved in non-oxidative cell killing after phagocytosis.  $\beta$ -defensins, widely expressed by epithelial cells including the colonic epithelium, are not found in the small intestine (see Figure 3).



**Figure 3.** Schematic presentation of a small intestinal crypt depicting Paneth cell turnover and its production and release of antimicrobial substances (from Axelsson LG, Mahida Y. Flora: role in colonisation resistance and other effects; production of antimicrobial peptides. *Microbial Ecol Health Dis* 2002, Suppl. 2: 216-22.)

*In vitro*,  $\alpha$ -defensin peptides are highly bactericidal in sub-micromolar concentrations. Experiments with knockout mice that lack functional  $\alpha$ -defensins (matrilysin-null mice) showed that they were less effective at clearing orally administered, non-invasive *E. coli* and they succumbed more rapidly, and to lower doses, of virulent *Salmonella typhimurium* than wild-type mice. Thus, eliminating these molecules from the gut has an influence on the epithelium and small intestinal ability to regulate the populations of bacteria that exist in the lumen.

Paneth cells can release these molecules under cholinergic stimulation and on a constitutive basis, so stimulation by bacterial antigen is not required in order to elicit these secretions. However, additional *in vitro* experiments demonstrated that crypts not exposed to bacterial antigen did not secrete these molecules indicating that the secretory granules in the Paneth cells are discharged into the medium on exposure to bacteria. Upregulation of these host defences can afford significant protection against enteroinvasive organisms,

although the mechanisms of Paneth cell secretion in response to microbial antigens are not established. The determinants of  $\alpha$ -defensin bactericidal activity also remain to be elucidated, as does any differential sensitivity of Paneth cells to pathogenic and commensal bacteria.

# THE HOST'S KNOWLEDGE OF THE MICROFLORA

## **INTESTINAL IMMUNE SYSTEM AND THE ROLE OF THE MICROFLORA**

This chapter will focus on the specific immune responses generated at the level of the gut associated lymphoid tissue (GALT) and proposed mechanisms of interaction with the colonising microflora.

Along with cellular responses, immune function in the gut centres on two important specific responses:

- protective/suppressive role, preventing immune responses to dietary proteins and avoiding hypersensitivities, especially allergies and IBD;
- induction of the specific immune responses through secretory IgA (sIgA) antibodies which are secreted into the lumen and are proposed to play an important role in the protection against pathogen colonisation.

These two immune functions are of particular importance in infants because a major pathology in the first two years of life is dietary hypersensitivity. Although no causative agent has been identified, a disturbance of the normal intestinal microflora may be involved, according to the hygiene hypothesis. Other important pathologies include infant rotavirus diarrhoea and allergy. Several independent studies have shown the composition of the microflora to be important in these conditions. With respect to rotavirus diarrhoea, the curative effect of various strains of bacteria is correlated with an increase in the anti-rotavirus IgA in infants.

One of the major questions concerning intestinal immunity is how our immune system makes the distinction between suppressive and pro-inflammatory responses to the vast numbers of antigens present in the intestine, thereby maintaining homeostasis. To address this question, the role of the GALT in oral tolerance and the T lymphocyte Th<sub>1</sub>/Th<sub>2</sub> cytokine balance needs to be considered.

### OVERVIEW OF THE GALT

Peyer's patches and intestinal epithelium can be regarded as separate compartments within the GALT. Peyer's patches are inductive sites covered by a specialised epithelium that samples antigen and presents it to the underlying immune cells. The epithelium is regarded more as an effector site responding to pathogen invasion. The immune system underlying the epithelium, the lamina propria, is full of mature intestinal immune cells that are ready to respond to foreign antigen. There is a further immune compartment composed of intra-epithelial lymphocytes that reside between the tight junctions of the enterocytes. These are mainly CD8 positive cells and are proposed to play an important role in the maintenance of epithelial integrity.

The synthesis of antibodies requires the cooperation of three types of cells. Antigen presenting cells (dendritic cells, B cells and macrophages) that are able to capture antigen, digest it and present antigenic moieties to helper T (Th) cells. The activated helper T cell signals B lymphocytes *via* secreted cytokines. The composition of this signal from the Th cell then determines which secreted antibody isotype is selected by the B cell, either IgG, IgA or IgE.

There are several kinds of Th cells. Th<sub>1</sub> and Th<sub>2</sub> cells differ by their secreted cytokine profile. Th<sub>1</sub> cells after activation secrete a cytokine profile mainly represented by IFN $\alpha$ . The activation of Th<sub>1</sub> cells leads to ineffective antibody production by B cells mainly represented in the subclass IgG<sub>2</sub>. Th<sub>1</sub> cells are primarily involved in cellular immunity, they have a relatively insignificant influence on antibody production. Th<sub>2</sub> cells secrete a different profile of cytokines that directly influence antibody production by B lymphocytes inducing IgG<sub>1</sub>, the main effector subclass of the IgG family. The involvement of Th<sub>1</sub> and Th<sub>2</sub> is mutually exclusive.

Specific signals lead to the differentiation of T lymphocytes into Th<sub>1</sub> or Th<sub>2</sub>. Macrophages and dendritic cells are involved in the differentiation of Th<sub>0</sub> cells to Th<sub>1</sub> cells by the production of IL-12, and natural killer cells play a role through IFN $\alpha$  production. Much research is currently focused on the role of dendritic cells. These cells originate in bone marrow and can populate tissue, including intestinal tissue, in an immature state. When they meet pathogens, the contact leads to maturation of the dendritic cells. The mechanisms of uptake are poorly understood, but after uptake they can migrate to the draining lymph nodes where they mature and present antigenic moieties to T cells. Thus, they have an important role as sentinels that recognise pathogens and control the presentation of antigen to T lymphocytes, thereby initiating antigen specific immune responses.

So how does this complex system respond to microbial and dietary antigens and what is the role of specific bacteria? There are two pertinent concepts - oral tolerance and Th<sub>1</sub>/Th<sub>2</sub> balance.

## Th<sub>1</sub>/Th<sub>2</sub> BALANCE

IL-10 has important immunomodulatory effects on the development of Th<sub>0</sub> to Th<sub>1</sub> or Th<sub>2</sub>. Disruption of the Th<sub>1</sub>/Th<sub>2</sub> balance can result in allergic disease. To avoid rejection of the foetus during pregnancy, the foetus is maintained in a Th<sub>2</sub> context and must develop Th<sub>1</sub> after birth. In allergic subjects, the switch to Th<sub>1</sub> is very difficult. The mechanisms controlling this switch to Th<sub>1</sub> during the neonatal period are unclear, but it is thought that intestinal bacteria could be very important factors. Evidence for this is provided by work using splenic macrophages from conventionally reared mice with a normal intestinal microflora. These cells produce IL-12, whereas splenic macrophages isolated from germ-free mice with the same genetic background cannot produce IL-12. Furthermore the same conventionally reared animals present with a spontaneous inflammatory condition due to secretion of inflammatory cytokines IL-1, IL-6, and TNF $\alpha$  by peritoneal macrophages. Germ-free mice under the same conditions fail to present with this inflammation and spontaneous levels of these three cytokines are very low. In addition, germ-free mice that have been mono-associated with *Bifidobacterium bifidum* do not spontaneously produce these inflammatory cytokines, unlike animals mono-associated with *E. coli* which was found to induce this secretion. Thus, the presence of a balanced bacterial population in the gut could be important for healthy development and function of the GALT.

## ORAL TOLERANCE AND ALLERGY

This is a mechanism of immune regulation in the induction of systemic immunological hyporesponsiveness (tolerance) to fed protein antigens; it is a long lasting process.

Induction of a tolerant state can be demonstrated in animals by feeding one group with protein, such as ovalbumin (OVA), and a second control group with buffer. Seven days later the two groups are systemically challenged with the same protein and peripheral immune responses are assessed. If oral tolerance is established, a decreased immune response in OVA fed group compared with the buffer group is observed. Thereby, a local and systemic immune suppression towards that particular protein is induced. In order to induce oral tolerance, it is imperative to give high doses of antigen by the oral route, or repetitive low doses. Other influencing factors include the age and genetic make-up of the host.

### The inductive site for oral tolerance

For many years it was thought that Peyer's patches were the inductive site of oral tolerance; however, a recent paper using B cell knock-out mice which are devoid of M cells (the specialised epithelium which provides the luminal surface or dome of the patch) and Peyer's patches themselves are able to become orally tolerated. This suggests that the intestinal epithelium can function as the inductive site for oral tolerance. Since both the Peyer's patches and the lamina propria have resident dendritic cells that are capable of capturing antigen either directly or *via* epithelial cell produced antigen-containing liposomes, it is possible to imagine that both sites may be involved.

There has been much recent research on the different populations of dendritic cells present in the immune compartments of the gastrointestinal tract. There are reported to be several subsets of dendritic cells in the lamina propria and Peyer's patches. CD11b positive myeloid dendritic cells are found especially in the dome area of the Peyer's patches and under the epithelial villi. Another subset of dendritic cells are CD8a+ located in the anterior follicular region of the germinal centre in Peyer's patches and double negative DC which are found in both Peyer's patches and lamina propria. After antigenic stimulation, CD11b+ dendritic cells secrete IL-10 that activates CD4+ naïve T cells present in the M cell pocket, enabling secretion of the anti-inflammatory cytokines IL-4 and IL-10. In contrast, antigenic stimulation of the same subset of T cells present in the spleen leads to the production of the inflammatory cytokine IFN $\gamma$ . The CD8a+ dendritic cells in the anterior follicular region are able to secrete IL-12 that leads to the differentiation of naïve CD4 T cells to Th1 cells and subsequent secretion of IFN $\gamma$ .

### Role of gut microflora in oral tolerance

In germ-free mice the suppression of the anti-OVA specific IgG antibody response is much shorter lived (15 days) than that in conventionally raised animals (up to 4 months), suggesting a role for the normal microflora in maintenance of the tolerant state. On recolonisation of these animals with selected organisms it was found that the introduction of bifidobacteria had no effect, but that *E. coli* and bacteroides allowed longer periods of suppression of the IgG response. Furthermore, it is impossible to induce a suppression of the IgE antibody response in germ-free mice. However, if these mice are colonised with *Bifidobacterium infantis* from birth, suppression of the IgE response can be detected.

Current data indicates that the first bacteria to colonise the gut during the post-natal period have considerable impact on the oral tolerance process and it is possible that both Gram-negative and Gram-positive bacteria are involved.

### Mechanisms of oral tolerance

Dose of antigen is crucial for inducing tolerance by the oral route but dose also affects the method by which tolerance is induced. High dose preferentially elicits clonal anergy while a low dose of antigen elicits bystander suppression.

The activation of T cells requires presentation of antigenic peptide on antigen presenting cells (APC) in the context of the major histocompatibility complex (MHC) which is recognised by T cell receptors on the responding T cell. Ligation with co-stimulatory molecules present on the same APC is also essential. Clonal anergy is a specific mechanism that can produce oral tolerance. It involves a specific subset of APC that do not express the co-stimulatory molecules necessary for T cell activation. This leaves the antigen specific T cell in an unactivated and anergic or tolerant state.

Bystander suppression involves regulatory T cells designated Tr1 and Th3 that control the oral tolerance process after normal APC stimulation. The particular APC involved in this process would be the CD11b+ dendritic cells specific to the intestine. After antigen presentation there is secretion of anti-inflammatory cytokines TGF $\beta$ , IL-4 and IL-10 by the regulatory T cells leading to suppression of T cell activation.

### **ALLERGY**

In order to understand the role of the microflora in allergic disease, we must address the presence of this immense number of bacteria in terms of tolerance by the host. In this view, the gut microflora as a whole cannot be deemed a good or bad microflora by evaluating all the organisms present, nor should the presence of high numbers of bifidobacteria in our faeces necessarily be taken as an indicator of a good microflora. It is more important that the host accepts all these bacteria as normal and this can be assessed by inflammatory responses in the gut. Current techniques to study this balance in clinical practice are limited but some progress has been made. For example, *in vitro* tests of immune response to certain bacteria in the microflora show that immune cells from patients with Crohn's disease react to the bacteria but no response is observed with cells from the healthy host.

### **ATOPIC DISEASE**

Atopic disease can be defined as a familial or personal tendency to develop allergen-specific IgE on exposure to environmental allergens, and to suffer typical allergic symptoms, such as asthma, eczema or hay fever. This is an area where evidence is accumulating that an altered microflora could be something of primary importance in causing the disease state, rather than simply a secondary effect of inflammatory responses.

Recent studies have shown alterations in the gut microflora may precede the expression of atopy. Infants from birth were followed for 1-4 years and it was found that those who had skin reactivity to common environmental allergens at the age of 12 months had a different microflora at the age of 3 weeks when compared to infants who did not develop atopy. Modulation of gut microflora with probiotics was found to be effective in preventing some manifestations of atopic eczema.

A randomised placebo-controlled trial of 159 pregnant women from high risk groups found the incidence of recurring atopic eczema in the infant at two years of age

could be halved if the mother took a probiotic. Again, the probiotic showed specificity in that the expression of atopy was comparable between groups. In a subgroup of this study, 62 pregnant women received either probiotic or placebo before delivery and during breastfeeding. Results showed that TGF $\beta$ 2 concentrations in breast milk were higher in the group of mothers who consumed probiotics. As discussed previously, TGF $\beta$  is an important anti-inflammatory cytokine associated with oral tolerance. Breast milk delivers sIgA and TGF $\beta$  from the mother to the baby while the baby's own sIgA production is very low. Interestingly, previous data have shown that the concentration of TGF $\beta$  in breast milk is associated with the infant risk of developing atopic disease early in life. But again specificity was observed in that levels of cows milk allergy was not affected. Thus probiotic therapy is not a universal treatment to everything early in life. This study used only one probiotic bacteria; it may be necessary to design mixtures of different probiotic organisms in order to treat different disease states.

These results pose many new questions. Allergologists frequently present that IgE sensitisation is the cause of atopic disease but good data on the Th2 type immunity of Australian newborns has shown that Th2 type immunity was stronger at birth in infants that did not become atopic. It was actually lower during the neonatal period in infants that became atopic. So obviously Th2 type immunity cannot be the cause of allergy but it happens to be a strong dominator of allergy. Adults that have allergic disease have strong Th2 type immunity compared to healthy subjects, but this does not seem to be the case early on in life.

Similar results have been obtained for respiratory allergens in that the more an infant is exposed to environmental allergens like dust mite allergens, the higher the IgE levels to these allergens. However, this was not associated with asthma later in life, so more must be learnt in allergic disease about the sequence of phenomenon associated with sensitisation, food allergy and atopic disease.

Thus, the present consensus is that there does not appear to be one single mechanism behind all types of allergies, and more studies are needed on the mechanisms behind atopic eczema, asthma, and food allergies. Also, if probiotic therapies are to be developed to treat these diseases, then we need to know more about the probiotic organisms. We know that different probiotic strains have different immunological effects, so we need to identify strains that can help the host with diarrhoeal diseases, inflammatory diseases or other affections and how these effects are manifested in healthy and diseased intestines. Probiotics are not something that simply changes the balance of gut microflora. Colonisation of the gut is not necessary as this could lead the healthy host to become tolerant to this strain, thereby removing its capacity to be a probiotic.

### **Inflammatory disease affects the microflora**

Chronic disease affects the microflora by inducing inflammatory responses. For example, in IBD, tolerance is lost and gut microflora changes, leading to inflammation. It has also been shown that infants with allergic disease have a different microflora to healthy

infants. From this point of view, it is less relevant to document this altered bacterial community because any kind of disease inducing inflammatory responses will secondarily affect the microflora. What is unclear is if it is efficacious to use probiotic strains to control the disease situation.

A vicious circle exists - if an antigen (e.g. rotavirus) induces an inflammatory response and the gut microflora becomes more aberrant, then this leads to more inflammation that locally affects gut barrier function resulting in a more leaky mucosa. New antigens are absorbed, leading to more inflammatory responses, and so on. Thus, these points can be seen as the targets for probiotic activity in disease to re-establish the balance even if the antigen causing the effect is unknown. Bacterial strains from the intestine are known to degrade antigens and it has been documented *in vitro* as well as *in vivo* that specific strains of the microflora may control inflammatory cytokine production. The classical probiotic effect is to modulate the microflora towards a more beneficial balance. Specific bacterial strains have been shown to tighten the mucosal barrier, enhance IgA production and to control intracellular permeability.

### **Microflora and inflammatory diseases**

Very little data exist on the whether anomalies or certain bacteria in the healthy human gut microflora cause disease. However from animal models of IBD the evidence for the role of the microflora in induction of disease is compelling. None of the animals with the genetic potential to develop IBD showed any symptoms in a germ-free environment. They only showed disease when their gut was colonised. Could factors such as antibiotic therapy in the first few weeks of life lead to an altered microflora later in life that somehow enhances the risk of allergic disease or IBD by inducing early inflammatory responses? It is certainly a possibility.

The control of cytokine production helps us to maintain the healthy microflora. In all humans there is a strong Th<sub>1</sub> type immunity producing IFN $\alpha$  and other inflammatory cytokines in the intestine, known as physiological inflammation. Subjects with allergic disease typically have a strong systemic Th<sub>2</sub> type immunity, but recent data have shown that Th<sub>2</sub> type immunity also predominates in the intestine of the allergic host and might be inflammatory.

So the classical Th<sub>1</sub>/Th<sub>2</sub> type parody that was the immunological rationale of the hygiene hypothesis of allergy is too simple. If skin biopsies are taken from patients with atopic eczema that have strong Th<sub>1</sub> type immunity, they can also have strong Th<sub>2</sub> type immunity in blood samples. Also, the incidence of both Th<sub>1</sub> and Th<sub>2</sub> type diseases, such as type 1 diabetes and allergic disease have increased in Western countries. This would not be expected if the processes were counter-regulatory. The involvement of regulatory cell types (Th<sub>3</sub>, Tr<sub>1</sub>) that produce anti-inflammatory cytokines like TGF $\beta$  and IL-10 needs to be assessed. It has been documented that specific strains of intestinal bacteria seem to be strong inducers of these cytokines, and therefore we need to know more about the immu-

nomodulatory effects of gut microflora to answer the question " Could an altered microflora cause disease?"

It has been suggested that the disease free state in the gut of normal individuals is caused by the colonising microflora directly influencing the host and not by dysregulation of sophisticated immunoregulatory circuits. This could occur through either direct binding of members of the microflora to the host epithelium or by secretion of signalling molecules from the bacteria towards the host. A combination of these mechanisms is also possible. The recognition molecules of these interactions on the host are now being elucidated. Over the last 4 years a new group of human receptors called toll-like receptors (TLR) have been identified. There are now 10 human TLRs that are directly involved in recognition of bacterial, viral and fungal products and surfaces. These molecules signal through NF $\kappa$ B and can induce a myriad of downstream responses in the host. These receptors can exist in homo and hetero-dimers giving the human host more than 500 different receptor combinations for bacterial recognition at the epithelial surface. This also begs the question of the importance of the innate immune response in probiotic recognition and downstream responses initiated by the epithelium. The introduction of an immune modulating probiotic could directly affect the functioning of the gut and underlying immune response. It would then follow that the correct selection of a single probiotic or combination of probiotics could be used to resolve an inflammatory condition either locally in the gut or systemically.

### ANTI-ROTAVIRUS RESPONSES

Modulation of the intestinal microflora can affect the outcome of acute rotavirus diarrhoea in infants. Experiments with mice have shown bifidobacteria can have an adjuvant effect on rotavirus IgA antibody responses, unlike *E. coli* that can actually suppress this kind of response.

But how does this effect come about? We have already seen that the IgA response is induced in the Peyer's patches, where luminal antigen is sampled and presented to T cells. This allows activation of antigen specific B cells that migrate to mesenteric lymph nodes. The B cells can then return to the intestine *via* the blood stream by the way of  $\alpha$ 4 $\beta$ 7 integrins which are recognised by madcam1 in the lamina propria of the intestine where they differentiate and produce antigen specific sIgA. These Peyer's patches derived B cells are called B2 cells and are directed against protein antigens. There is another subset of LP B cells, the B1 cells which are derived from a population in the peritoneal cavity and do not appear to require T cell help to become activated. These cells are specific for bacterially derived antigen such as LPS. The control of activation and recruitment of these two types of B cell appears to be dependant on the composition of the microflora.

Indeed, the immunomodulatory effect of bacteria on the anti-rotavirus IgA response has been shown to be strain specific, whereby two bifidobacterial strains (one *B. bifidum* isolated from a baby and one commercial strain) were able to stimulate the anti-rotavirus specific IgA response, whereas three bifidobacterial strains from a human

adult were unable to stimulate this response. The latter three strains actually suppressed this response, as did *Bacteroides vulgatus* and *E. coli*.

On examination of cytokine expression involved in the Th1/Th2 balance by splenic macrophages, the infant strain of *B. bifidum* suppressed IL-4 production but stimulated IFN $\gamma$  production. However, the *Bifidobacterium sp.* isolated from the adult did not affect IL-4 production and down regulated IFN $\gamma$  production. Investigating cytokine production in the intestine is the next step in these experiments.

Thus, the microflora affects the development and activation of the immune system leading to the modulation of its functions. Questions remain about the specificity of strain dependant effects, and the influence of regulatory cells Tr1 cells and Th3 cells on such immunomodulation. What is clear is that there is a delicate balance between the microflora and the immune response within the intestine.

# BACTERIAL COMMUNICATION AND CROSS-TALK

## BACTERIA – BACTERIA COMMUNICATION

There is currently a great deal of interest in the processes bacteria use to communicate, both with each other and with their host. A phenomenon of communication between microorganisms called quorum sensing is known to control a diverse range of cell-density dependent factors. The best-characterised quorum sensing systems involve N-acylhomoserine lactone signals in Gram-negative bacteria. The signal molecules accumulate in the extracellular environment, and once a critical threshold level is reached, gene transcription is affected *via* the LuxR family of response regulators. This process of auto-induction is known to be involved in regulating antibiotic production, biofilm formation, and pathogenicity in numerous bacterial systems. The large populations of bacteria present in the intestine and great ecological competition that exists here indicate quorum sensing could be an important aspect in the bacterial colonisation of the gut. However, very little is known regarding these processes in the human intestine at present, although LuxR homologues have been described in organisms such as salmonella and *E. coli*.

An additional quorum sensing system based on the *luxS* gene has been described in a wide range of organisms that can inhabit the colon, including both Gram-negative and Gram-positive species. For example, highly conserved *luxS* homologues have been identified in *E. coli*, *Helicobacter pylori*, *Campylobacter jejuni*, *Clostridium perfringens* and *C. difficile*. The reduced specificity of this signalling system has led to the hypothesis that it plays a role in inter-species communication, unlike the N-acylhomoserine lactones that are intra-species signals. However, the bacterial processes controlled by these LuxS autoinduction systems outside *Vibrio harveyi* remain to be determined.

Gram-positive organisms are known to employ other quorum sensing systems, the signal molecules of which are typically in the form of small post-translationally modified peptides. These communication systems have been shown to regulate competence in *Bacillus subtilis* and *Streptococcus pneumoniae*, conjugation in *Enterococcus faecalis*, virulence in *Staphylococcus aureus*, and bacteriocin production in certain lactic acid bacteria. Again, little is known of the relevance of these signalling systems to microbial ecology in the gastrointestinal tract but it is an intriguing thought that these regulatory mechanisms could be exploited to modify bacterial composition and activity within the microflora.

### **BACTERIA – HOST COMMUNICATION**

It has been demonstrated recently that resident bacteria can modulate the host's intestinal glycosylation process by sending a signal interfering with cellular glycosyl transferase activity or expression. This results in a modified carbohydrate repertoire. Dialogue such as this between bacteria and host corresponds to "cross-talk" and is essentially a new discipline, "cellular microbiology". Very little is known of the communication between commensal bacteria and the host and this partly relates to the fact that the effects of tolerated organisms on the host are less easily identified compared to pathogenic bacteria, and so are much harder to study. However, the development of new technologies over the past decade has provided us with another view of intestinal ecology by studying genomic information.

### **MOLECULAR ECOLOGY**

#### **Bioinformatics**

Part of understanding the molecular basis of the stability of this ecosystem is to know to a certain degree what kind of organisms there are. A significant example of how additional information can be gained about bacterial populations present in the gut is by analysing the large amounts of genetic data that already exist using informatics. In a very recent analysis of 3 million ESTs from publicly available data libraries, scientists subtracted mitochondrial, human and mouse sequences, and then increased the stringency to give a final result of approximately 65,000 sequences in human EST libraries that are of microbial origin. Many of these sequences were viral, but nevertheless it demonstrates a method of finding new markers for the microflora.

#### **Genomics**

The recent application of molecular techniques to the study of microbial ecosystems has revolutionised our ability to properly identify the organisms present. Charac-

terisation of bacteria at the genetic level allows a greater understanding of activity within the gastrointestinal tract and the contribution of a particular organism to the microflora as a whole.

The term microbiome has been used to describe the collective genome of bacterial populations in the normal microflora. The collected gene products assembled by all the components of the normal gut microflora has been estimated to be well over 400,000, considerably more than the 35,000 genes found in the human genome. Indeed, there are ongoing discussions about forming a consortium to sequence the microbiome of the gastrointestinal tract, thereby allowing the genes of commensal organisms to be included in our genetic view of the human species.

Numerous genome sequencing projects have been completed within the past few years, and many more are currently underway. These sequences are listed on various databases such as The Institute for Genomic Research, The Sanger Centre and The US Department of Energy - Joint Genome Institute. The website of the European Bioinformatics Institute currently lists 76 bacterial genomes that have been completed. Research has typically concentrated on pathogenic and model organisms, although a number of commercially relevant strains are also present in the databases. The genomes of five lactic acid bacteria, including *Lactococcus lactis* and *Lactobacillus acidophilus* have been sequenced, as have those of other probiotic candidates such as *Bifidobacterium longum* and *E. coli*. Comparison of this genetic information allows housekeeping and highly conserved genes to be identified and has also indicated that significant amounts of horizontal gene transfer occurs between microorganisms.

Identification of key genes involved in processes of adherence, production of antimicrobials, stress tolerance, transport systems, and cell signalling will dramatically improve our knowledge of bacterial colonisation within the intestine and the active role of microbes in host pathology and well-being.

### Gene expression

The predicted proteins of an astonishingly high number (approximately 40%) of genes in these microbial genomes are currently classified as unknown. Analysis of gene expression is essential in order to understand the biological significance of this genetic code. Studies using microarrays and proteomic analyses provide a holistic view of a particular system from which validation using gene knockouts, overexpression and complementary experiments can progress to establish the functional properties of genetic differences.

Aspects of functional genomics include:

- the transcriptome - the complement of mRNAs transcribed from the entire genome and their relative expression levels under defined conditions;
- the proteome - the complement of proteins encoded by the genome;
- the metabolome - quantification of all low molecular weight molecules present in a cell under various physiological conditions.

Knowledge gained using these strategies should prove particularly useful in isolating beneficial aspects of the gut microflora and modulating these processes to the advantage of the host. For example, assessing bacterial activity in food systems compared to various conditions found in the gastrointestinal tract has relevance to the selection of probiotic genotypes delivered in food products.

Various studies have been published over the last two years on the effects of gut bacteria on host physiology and pathophysiology. Most of these have shown effects using mixtures of organisms, so it is unclear as to which organism is of consequence and thus the mediator of the effects remains unknown. However, considerable advances are being made. For example, a study on IBD describes the NOD2 frameshift mutation. This gene encodes a protein that responds to LPS and further clarifies the link between bacteria and diseases that are described as iatrogenic.

Molecular signatures can be included in a version of Koch's postulates to identify mechanistic pathways in health or disease. The reductionist approach of using germ-free animals is often employed thereby allowing control over microbial factors affecting function or dysfunction. The genetic aspect of these models can be isolated allowing other components to be introduced to the system under controlled conditions and any changes to the genome or proteome baselines can be used to identify biomarkers of bacterial - host interactions. It is then necessary to validate the importance of the markers either in the animal model or a cellular system. This validation step is of particular importance given the complexity of the intestinal ecosystem.

### ***Bacteroides thetaiotaomicron* model**

An example of this research is the communication between *Bacteroides thetaiotaomicron* with the mouse model host. Lectins are carbohydrate-binding proteins and these can be used to characterise the peripheral sugars of carbohydrates in the intestine by observing variability in staining. In experiments with germ-free mice no staining with lectins that detect fucose in the intestine was apparent, whereas in conventional adult animals a fulminant production of fucosylated glycoconjugates in the epithelial cells was observed. Further investigations with conventional mice demonstrated that there are signals by the microflora that increase the glycoconjugates as weaning occurs. This raised the problem that these effects were mediated by a component of the gut microflora that was as yet unknown.

By studying a selection of intestinal, fucose-consuming bacteria, a fucose-utilising operon in *B. thetaiotaomicron* was identified which contained genes for enzymes to digest fucose once it was taken up by the cell, and also for sensors that regulated these enzymes. Thus, when no fucose was available the enzymes were not expressed but some form of signal to the host must be generated to induce production of fucose. Therefore, a cross talk existed where the bacterium adapts its own metabolism to the environment and also influences the host at the expression level of fucosyltransferase.

Hence, one of the factors that leads to a stable ecosystem is the utilisation of nutrients. Bacterial succession is important because pioneer colonisers utilise the nutrient foundation as it is initially presented, but these colonisers can then reshape this niche where they, and perhaps a select group of other bacteria, can have a competitive advantage compared to others.

### **BARRIER FUNCTION**

Mucosal integrity relates to the prevention of inappropriate epithelial translocation of microbes and macromolecules across the intestinal mucosa. Components of mucosal integrity range from very specific and well described molecules that regulate cell-cell interactions, to proliferative regulation and turnover, mucus production, peristalsis, and immune exclusion and elimination. Regulation of these processes is of interest to many research groups due to its implications in conditions such as IBD.

A hypothesis free approach was taken using Affymetrix chips covering 26,000 genes of the mouse genome. cDNA was prepared from total ileal RNA extracts of the *B. thetaiotaomicron* model to determine which genes were up-regulated 10 days after colonisation with this bacterium. The cut off point of up/down regulation was twofold. It is pertinent to note that the biological significance of any altered expression of this magnitude is unclear; twofold was purely a mathematical choice. The results showed that over 110 mouse genes were up or down regulated compared to the germ-free controls. These included genes associated with mucosal barrier function such as expression of the polymeric Ig receptor, mucus associated genes, and those for other proteins involved in cell-cell interactions.

After predicting what the functional phenotype would be, the next step was to validate that these changes actually occurred. By studying the mucosal barrier in the presence of *B. thetaiotaomicron*, an upregulation of polymeric immunoglobulin, mucin, and acute phase response proteins were confirmed. Thus, the mucosal barrier function as a whole was effectively increased and further studies are underway to validate this in germ-free mice.

### **SIGNAL SPECIFICITY**

This system was also used to compare species specificity of these effects. Interestingly, there appear to be certain activities that are more or less generic between bacteria but there are also some very unique response mechanisms. For example, colonisation with *B. thetaiotaomicron* resulted in the upregulation of small proline-rich protein 2, which contributes to the mucosal integrity of the epithelium, but no upregulation of this gene was observed with colonisation by other organisms including *E. coli* and bifidobacteria. Such species specificity of host - bacterial interaction has enormous consequence for the selection of probiotics whether for use in health or disease.

The *B. thetaiotaomicron* genome has also been sequenced recently thereby allowing future investigations into response mechanisms of the bacterial genome and identification of the signalling molecule. This should facilitate future work identifying bacterial agents that influence human physiology. Such technology offers exciting insights in to the world of microbial communication but many questions still remain. Current data indicate that *B. thetaiotaomicron* does not adhere to the epithelium and yet it signals the host to up-regulate these genes. Therefore, soluble factors are likely to be involved in the mechanism. Most soluble factors induce the NF $\kappa$ B pathway, although data from the transcriptome indicates little upregulation of this pathway. Consequently, the details of this interaction in the mouse model still remain elusive.

With such additional knowledge, manipulation of the microflora offers an attractive method for preventing and alleviating disease by selecting for a bacterial phenotype containing specific components. Alternatively, bacteria could be used as natural vehicles as has been previously demonstrated with the *Lactococcus lactis* delivery system. This type of therapy would result from a more mechanistic approach to probiotic therapy.

# PROBIOTICS AND DIARRHOEA

## ROTAVIRAL DIARRHOEA

Rotavirus infection is one of the most frequent causes of diarrhoea in children. The condition is usually self-limiting with symptoms lasting several days. Oral rehydration is the conventional treatment for infection but does not shorten the period of illness. However, it is now established that certain strains of probiotic can reduce the duration of diarrhoea and rotavirus shedding.

Numerous randomised controlled trials have demonstrated *Lactobacillus rhamnosus* GG to be effective in this manner and this has been confirmed recently in a double-blind, placebo-controlled multi-centre European trial on acute diarrhoea caused by rotavirus and other pathogens. An oral rehydration solution supplemented with either *L. rhamnosus* GG or placebo was given to 287 children (aged 1-36 months) with acute diarrhoea. No significant difference was observed between the two treatment groups in the 186 children who were rotavirus negative, but the duration of diarrhoea due to rotavirus infection was significantly reduced in the probiotic group (56 hours) compared to the placebo group (77 hours).

The probiotic is thought to have an adjuvant effect and elevates the rotavirus specific IgA response. Studies have also shown heat inactivated *L. rhamnosus* GG can reduce the duration of diarrhoea. However, it is generally considered that live organisms offer more potential as probiotic agents. Indeed, live *L. rhamnosus* GG was found to have a more pronounced adjuvant effect on the amount of anti-rotavirus IgA compared to the heat inactivated preparation.

Lactobacilli are not the only strains that have been found to alleviate infant rotaviral diarrhoea. In a double-blind placebo-controlled trial following 55 hospitalised infants, a combination of *Bifidobacterium bifidum* and *Streptococcus thermophilus* was used as the

probiotic. The infants were randomised to receive either standard infant formula or the same formula supplemented with the two probiotic organisms. Results showed 7% of infants receiving the probiotic formula experienced diarrhoea during the follow-up period compared to 31% of the control subjects. The prevalence of rotavirus shedding was also significantly lower in the group of infants taking the probiotic supplemented formula.

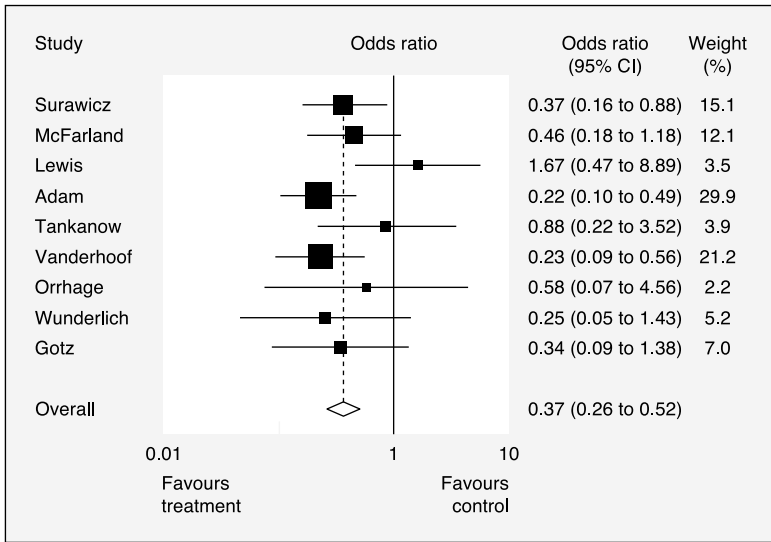
## ANTIBIOTIC-ASSOCIATED DIARRHOEA

One of the side effects of antibiotic therapy is the induction of diarrhoea, although the likelihood of this varies with the antibiotic being used and other risk factors such as institutionalisation. Along with acting at the site of infection, these drugs also influence the intestinal microflora, and the ensuing imbalance can lead to the overgrowth of potentially pathogenic organisms such as *C. difficile*. Diarrhoea caused by intestinal overgrowth of this organism is a particular problem in the hospital setting where between 10-20% of all antibiotic-associated diarrhoea can be attributed to *C. difficile*. Prevention of antibiotic-associated diarrhoea would reduce the duration of hospital stay for some patients and many studies have examined the ability of probiotic agents to stabilise the gut microflora against antibiotic-induced imbalance.

A recent meta-analysis re-evaluated the results of nine double-blind placebo-controlled trials that investigated the role of various probiotics in the prevention of antibiotic-associated diarrhoea. Information from these trials (four using yeasts as the probiotic and five using bacterial strains) were combined and the summary odds ratios and 95% confidence intervals were calculated. In clinical trials, the odds ratio estimates the relative odds of being cured by the treatment compared to placebo. An odds ratio of one indicates that there is no association, whilst a ratio below one favours the active treatment compared to the placebo.

Various statistical methods were employed to analyse these data for publication bias and a funnel plot showed the larger studies found a beneficial effect of the probiotic, whilst the smaller trials gave results ranging from good to no benefit. The combined odds ratio for the nine trials was 0.37 with 95% confidence intervals of 0.26-0.53 signifying a benefit of probiotic treatment over placebo in the prevention of antibiotic-associated diarrhoea. The calculated benefits of the individual trials are shown in *Figure 4*.

The combined odds ratio for the trials using *Saccharomyces boulardii* (0.39) was found to be similar to that for the bacterial probiotic trials (0.34) with 95% confidence intervals of 0.25-0.62 and 0.19-0.61, respectively. Thus, both calculations favoured active treatment over the placebo. Variation in the efficacy of a probiotic common to several of these trials can be explained partly by factors at the level of study design, such as duration of treatment and dose. Also, the different antibiotics taken in the trials will have a large effect on the outcome. Despite all of these factors, it was concluded that the evidence is strong for the role of probiotics in preventing antibiotic-associated diarrhoea, although no conclusion could be reached for the use of such agents in treatment of this condition.



**Figure 4.** Plot of the log of odds ratios for the proportion of patients free of diarrhoea in treatment groups compared with control groups (from D'Souza A *et al. Br Med J* 2002; 324).

A second meta-analysis of the published literature also supports the potential benefit of probiotics in preventing diarrhoea during antibiotic therapy. However, additional placebo controlled trials are needed to establish whether probiotics could be used to reduce the length of hospital stay by alleviating antibiotic-associated diarrhoea, and the relationship between different antibiotics and probiotic strains needs to be determined.

## TRAVELLER'S DIARRHOEA

Diarrhoea can be a common health problem experienced by international travellers. The condition is thought to be caused by indigenous intestinal populations being displaced by ingested strains from the new environment. Several controlled trials have investigated the use of probiotics to stabilise the gut microflora and prevent traveller's diarrhoea. Results of these studies tend to be conflicting.

One study found the yeast *S. boulardii* had a small, dose-dependant protective effect for tourists. Another double-blind placebo-controlled trial showed *L. rhamnosus* GG could reduce the incidence of diarrhoea but this effect was destination-dependant. Conversely, four other trials using lactobacilli found no significant effect of the probiotic therapy in preventing diarrhoea.

The number of factors affecting the outcome of these trials tend to obscure any potential benefit the probiotics might have. Changes in diet and newly ingested bacterial strains will vary greatly depending on the tourist's destination. This, combined with the degree of specificity between probiotic strains and potential antagonists in gut, lends a great deal of ambiguity to the procedure of selecting an appropriate probiotic strain in a

traveller's diarrhoea study. A trial using a combination of four organisms (two *Lactobacillus* sp, one *Bifidobacterium* sp and one *Streptococcus* sp) found that tourists to Egypt could reduce their risk of diarrhoea from 71% to 43% and indicates that probiotic preparations containing multiple strains might prove more useful in protecting tourists against diarrhoea.

At present, evidence for the use of probiotics in the prevention of traveller's diarrhoea is currently weak. Data from many more trials is needed before a beneficial effect could be substantiated and study design would benefit hugely from a better understanding of the organisms and mechanisms involved in this type of diarrhoeal disease.

## INFLAMMATORY BOWEL DISEASE

Crohn's disease and ulcerative colitis are the two main forms of chronic relapsing inflammatory bowel disease. Both genetic and environmental factors are associated with the pathogenesis of these disorders although the mechanisms involved in the initiation and maintenance of the inflammatory state are not yet fully understood.

Various transmissible agents have been implicated in the aetiology of IBD, although at present, most evidence indicates that these inflammatory conditions result from an abnormal immune response to the commensal gut bacteria. Organisms growing on the mucosal surfaces of the gut are likely to have greater interaction with the host immune system and there is evidence that these bacterial communities are altered in patients with IBD. For example, examination of large bowel biopsies indicated that the frequency of isolation of bacteroides was higher in ulcerative colitis patients. Some authors also reported variation in other commensal bacterial populations such as bifidobacteria and lactobacilli, although these results have yet to be corroborated. Recent data using PCR techniques have revealed the presence of the potential pathogen *Mycobacterium paratuberculosis* in 50-70% of Crohn's patients. It should be noted, however, that it is not known at present whether any detected shift in the mucosal microflora drives the inflammation or if changes in these populations are a consequence of the inflamed mucosa itself.

The presence of the normal microflora is known to be essential for the development of IBD and this has been demonstrated in a wide range of animal models. This provides a rationale for using pre- and probiotics to modulate the microflora and its engagement with the mucosal immune system.

Several studies have investigated the impact of probiotic therapy on IBD in animal models, and there have been a few, relatively small studies conducted on human patients. Mesalazine is a standard treatment used to maintain remission of ulcerative colitis and randomised controlled trials have shown *E. coli* to be equally effective as this 5-ASA drug in preventing relapse. Another probiotic preparation containing eight organisms of three different genera has been shown to aid remission of colitis, although this trial was uncontrolled. However, in a randomised controlled study, 17 out of 20 pouchitis patients maintained remission whilst taking this probiotic mixture. Interestingly, all 17 of these patients relapsed after the probiotic therapy was withdrawn.

An important issue regarding the use of probiotic therapy in IBD is that of strain specificity and this could account for differences in the outcome of various investigations. Experiments with the severe combined immunodeficiency (SCID) mouse model which lacks both B and T cells showed that a particular bifidobacterial strain could reduce weight loss whilst a lactobacillus strain could not. A notable point is that this lactobacillus strain showed protective effects in a different animal model, the IL-10 knockout mouse, demonstrating differences between models. Another interesting observation was that when these two organisms were simultaneously administered to the SCID mouse, their ability to reduce weight loss was found to be less than that of the bifidobacterial strain alone. Thus, antagonism can exist between probiotics and careful consideration should be given to the selection of strains if they are given in combinations.

In an Irish study, feeding a lactobacillus strain to human volunteers showed a modulatory effect on the gut microflora in that lactobacillus and bifidobacterial populations were increased. Although no IgG antibodies specific to the probiotic strain were found in the serum of these individuals, 20% of the fed volunteers secreted sIgA against the bacterium in saliva. This demonstrates that the probiotic could indeed engage with the mucosal immune system, modulation of which is a target for probiotic therapy in IBD.

An interesting area is that of toll-like receptors (TLR). These type 1 transmembrane proteins are present on epithelial surfaces and macrophages and they have the ability to trigger NF $\kappa$ B and cytokine production. TLR are a pattern recognition system and homo- or hetero-dimers can give more than 500 different pattern recognition abilities. Their role in the recognition of pathogens and commensal organisms is unknown at present, but the diversity of recognition they offer to the innate immune system suggests the enterocyte should not be overlooked as an important cell in gut immunity (see *Figure 5*).

Modulation of the immune response has been achieved using genetically engineered organisms containing the mouse IL-10 gene. When fed to mouse models of IBD, these organisms secrete IL-10 which can then be detected in the gut lumen and provide complete resolution of the inflammatory disease. This offers an exciting method to treat severe inflammatory diseases, particularly if probiotic organisms with other proven beneficial effects can be genetically manipulated in a similar manner.

However, the use of GMOs to treat human diseases is a matter of serious debate and could take some time to resolve. Thus, current options for probiotic therapy in IBD are limited to conventional strains. Despite some encouraging probiotic data, more double-blind placebo-controlled trials are necessary to establish a definite role for these organisms in the treatment of IBD and additional studies are required to determine the mechanisms behind these conditions.

## COLORECTAL CANCER

Colorectal cancer is a major cause of morbidity and mortality in the Western world and the gut microflora is an important factor in promoting this carcinogenic process.

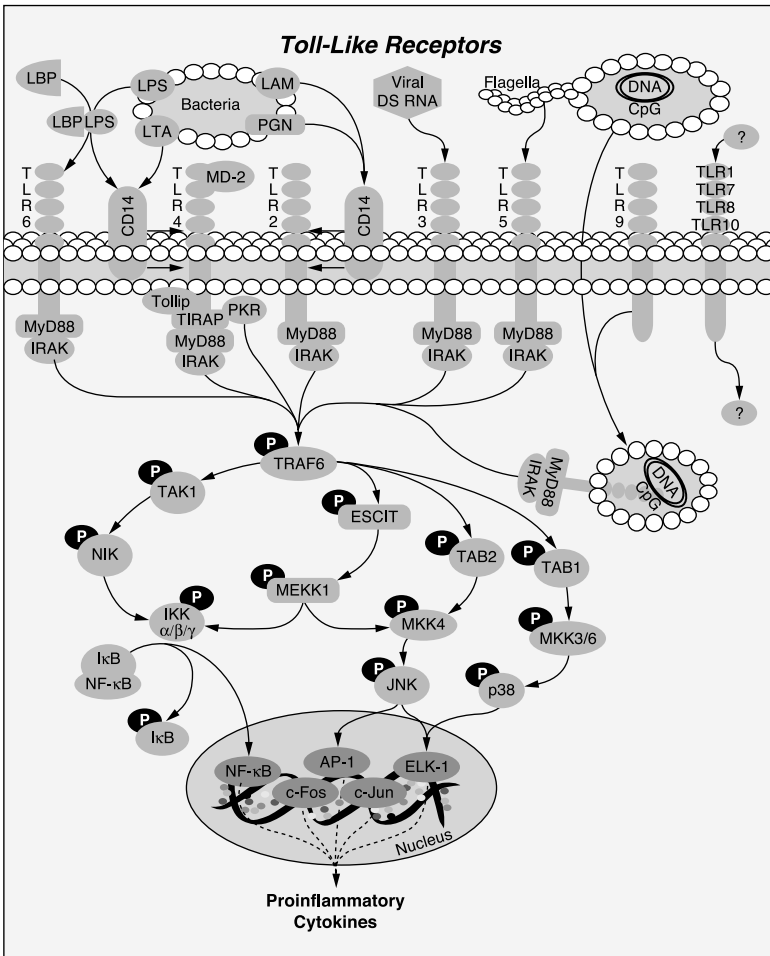


Figure 5. Human toll-like receptors 1 to 10 - stimuli and signalling cascade (from Collins).

Bacteria are known to metabolise numerous carcinogens in the intestine and many studies have investigated methods of modulating microbial activity to give a tangible reduction in cancer risk. There is very strong data from animal and *in vitro* studies to support a role for probiotics in the prevention of colon cancer but the clinical and human epidemiological data lag far behind this. Thus the anticancer activity of probiotics remains one of the most controversial aspects of these organisms.

Potential mechanisms by which probiotics could protect against colon cancer include:

- quantitative and qualitative changes in the intestinal microflora. Consumption of fermented milk products containing lactic acid bacteria can significantly reduce the viable populations of faecal putrefactive bacteria such as coliforms. Suppressed populations of putrefactive organisms could lead to a reduction in the tumour promoting factors they produce;

- altered activity of the microflora. Administration of lactic acid bacteria (LAB) can decrease the faecal activity of enzymes such as  $\beta$ -glucuronidase, nitroreductase and azoreductase which are involved in the conversion of procarcinogens to carcinogens. However, the role of these enzymes in human cancer is still unclear;
- altered physicochemical conditions in the colon. Evidence indicates that LAB can decrease caecal pH and possibly reduce concentrations of cytotoxic bile acids in faecal water. An interesting study from Italy showed administration of LAB to adenoma patients reduced the high colonic proliferative activity seen in these patients. This increased proliferation of stem cells and their movement up the crypts is one of the early signals of colon cancer development and it is thought that the reduced proliferation was due to the effects of LAB on pH and bile acid concentration in faecal water.

One of the problems with the data pertaining to these mechanisms is the reliance on animal models whose intestinal microfloras are known to be different from those of humans. Studies have benefited from germ-free animal models associated with a human faecal microflora. For example, heterocyclic amines are found in meat cooked at very high temperatures and are regularly consumed in the Western diet. These compounds are part of a range of food mutagens found in the diet which can increase the formation of colonic DNA adducts in mice associated with a human faecal microflora compared to germ-free animals. Thus, the activity of the human microflora is an important factor in generating these pre-cancerous lesions. Interestingly, supplementing the diet with LAB significantly reduced the level of DNA adducts in these animals.

Despite the accumulation of data, epidemiological studies have yet to show that administration of bifidobacteria or lactobacilli have a significant role in the prevention of colon cancer. It is possible that this discrepancy could be related to differences in bacterial strains used in the studies, although this is unlikely to be elucidated without better knowledge of the mechanisms. However, steps are being taken to address these issues and a large European trial investigating the effects of synbiotics on colon cancer should provide some answers to the role of pre- and probiotics in cancer prevention in the not too distant future.

### CLINICAL USE OF PROBIOTICS

In conclusion, whilst evidence exists for the use of probiotics in a range of diarrhoeal diseases, current scientific knowledge would limit their usage to rotaviral and antibiotic-associated diarrhoea. However, differences between bacterial strains make the selection of probiotics for a patient difficult. Also, discrepancies in dosage and duration of treatment between studies add to this uncertainty. Therefore, a standardised protocol needs to be established before health-care workers can tangibly apply probiotic therapy in clinical practice.

# GENERAL CONCLUSION

The intestinal microflora is an extremely complex ecosystem and is closely linked to many aspects of host health. However, the mechanisms involved in these systems are poorly understood, particularly with regard to colonisation resistance. Advances in molecular techniques should help to increase our knowledge of this microbial community and its myriad of interactions with the host.

Despite a wealth of studies to date, many questions still remain as to how modulation of the microflora is able to exert a beneficial influence on the host in either healthy or diseased states. Knowledge of its composition and activities are central to this, as is a better understanding of how the microflora is tolerated by the host. One of the best studied methods is that of probiotic therapy and the choice of probiotic strain is known to be very important in this regard. Colonisation of the gut is not an absolute necessity since this could lead to the strain becoming tolerated by the healthy host, thereby removing its immunological capacity to be a probiotic.

Since different probiotic strains are known to have different immunological and microbiological properties, molecular studies should enable specific components of a bacterial phenotype to be assessed, and so identify strains that can benefit the host with diarrhoeal or inflammatory diseases. Modulation of the microflora with probiotics has been shown to be beneficial in rotaviral and antibiotic-associated diarrhoea, and whilst there is encouraging evidence for their usage in other conditions such as ulcerative colitis and colon cancer, the evidence for these needs to be interpreted with caution until further validation studies are completed. Alongside differences in bacterial strains, discrepancies in dosage and duration of treatment between studies add further ambiguity to the outcome of such treatments. This makes it difficult for clinicians to prescribe probiotic therapy in general practice and a standardised protocol needs to be established before it becomes widespread. As such, the safety and efficacy of these preparations are important questions for future research.

## List of Abbreviations

APC	Antigen presenting cell
CDNA	Complementary DNA
DC	Dendritic cell
DGGE	Denaturing gradient gel electrophoresis
Eh	Redox potential
EST	Expressed sequence tag
GAC	Germ-free-associated characteristics
GALT	Gut associated lymphoid tissue
GMO	Genetically modified organism
IBD	Inflammatory bowel disease
LAB	Lactic acid bacteria
LP	Lamina propria
LPS	Lipopolysaccharide
MAC	Microflora-associated characteristics
MALDI-TOF MS	Matrix-assisted laser desorption/ionisation-time of flight mass spectrometry
MHC	Major histocompatibility complex
OVA	Ovalbumin
PCR	Polymerase chain reaction
PP	Peyer's patches
RDNA	Ribosomal DNA
RRNA	Ribosomal RNA
SCFA	Short chain fatty acids
SCID	Severe combined immunodeficiency
SlgA	Secretory IgA
TGGE	Temperature gradient gel electrophoresis
Th cells	Helper T cell
TLR	Toll-like receptor



Danone Vitapole

# The Intestinal Microflora

## Understanding the Symbiosis

*Over the years, Danone Vitapole, the R&D division of the Danone Group, has focused its interests on the effects of probiotics on gut function and the body's natural defences. Evidence for various aspects of probiotic functionality has been steadily accumulating and this literature-base emphasises the vital implications of gastrointestinal ecology and physiology for the human body. At present, three major components of this system are recognised: the microflora, the mucosa, and the intestinal immune system.*

*During the summer of 2002, Paris hosted two prestigious events dedicated to this subject. Firstly, a workshop entitled "The Intelligent Intestine", which provided a forum for leading physicians from 22 countries. Secondly, at the Xth International Congress of Bacteriology and Applied Microbiology, part of the joint IUMS meeting, Danone Vitapole organised a scientific session on the role of lactic acid bacteria in barrier defences.*

*This booklet, eleventh in the collection "Health and Nutrition", collates the discussion and ideas raised at these symposia and completes the information provided in our previous summaries in the fascinating area of lactic acid bacteria. It offers a synthesis of the most pertinent data available and discusses the topics for ongoing research in the field of probiotics and their benefits for human health.*



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