

Functional food science and gastrointestinal physiology and function

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Abbreviations: GALT, gut-associated lymphoid tissue; IBS, irritable bowel syndrome; Ig, immunoglobulin; ILSI, International Life Sciences Institute; IQ, 2-amino-3-methyl-7H-imidazo[4,5-f]quinoline; MTT, 3-[4,5-dimethyl-thiazol-2-yl]-2,5-diphenyltetrazolium bromide; 7-OHIQ, 7-hydroxy-2-amino-3,6-dihydro-3-methyl-7H-imidazo[4,5-f]quinoline-7-one; rRNA, ribosomal RNA; SCFA, short-chain fatty acids.

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Abstract

The gut is an obvious target for the development of functional foods, acting as it does as the interface between diet and the metabolic events which sustain life. The key processes in digestive physiology which can be regulated by modifying diet are satiety, the rate and extent of macronutrient breakdown and absorption from the small bowel, sterol metabolism, the colonic microflora, fermentation, mucosal function and bowel habit, and the gut immune system. The intestinal microflora is the main focus of many current functional foods. Probiotics are foods which contain live bacteria which are beneficial to health whilst prebiotics, such as certain non-digestible oligosaccharides which selectively stimulate the growth of bifidobacteria in the colon, are already on the market. Their claimed benefits are to alleviate lactose maldigestion, increase resistance to invasion by pathogenic species of bacteria in the gut, stimulate the immune system and possibly protect against cancer. There are very few reports of well-designed human intervention studies with prebiotics as yet. Certain probiotic species have been shown to shorten the duration of rotavirus diarrhoea in children but much more work is needed on the mechanism of immunomodulation and of competitive exclusion and microflora modification. The development of functional foods for the gut is in its infancy and will be successful only if more fundamental research is done on digestive physiology, the gut microflora, immune system and mucosal function.

Gastrointestinal function: Microflora: Immune system

1. Introduction

One of the most promising areas for the development of functional foods lies in modification of the activity of the gastrointestinal tract by use of probiotics, prebiotics and synbiotics. To understand the potential value of these functional foods and to be able to develop new approaches it is necessary to study the normal human intestinal flora, fermentation, the gut immune system, mucosal function and the principal gut-related diseases.

2. Intestinal microflora: physiology and functions

2.1. *The normal flora (Gibson & Macfarlane, 1995)*

Bacterial numbers and composition vary considerably along the human gastrointestinal tract. The total bacterial count in gastric contents is usually below 10^3 /g, with numbers being kept low due to the acid lumen pH. In the small intestine, numbers range from approximately 10^4 /ml contents to about 10^6 – 10^7 /ml at the ileocaecal region. The main factors limiting growth in the small bowel are the rapid transit of contents and secretion of bile and pancreatic juice.

The human large intestine is an intensely populated microbial ecosystem. Several hundred species of bacteria are usually present, with typical numbers of about 10^{11} – 10^{12} /g. The majority of these bacteria are strict anaerobes.

Table 1 lists bacteria commonly isolated from the human colon. Bacterial counts of individual species range over several orders of magnitude, and the nutrition and metabolic products of different bacterial groups vary considerably. Most bacteria growing in the colon are non-spore-forming anaerobes and include members of the genera *Bacteroides*, *Bifidobacterium* and *Eubacterium* among many others. Clostridia are also represented, although they are outnumbered by the non-spore-forming anaerobes, as are facultative anaerobes such as streptococci and enterobacteria. Quantitatively, the most important genera of intestinal bacteria in animals and man are the bacteroides and bifidobacteria, which can account for 30% and 25% of the total anaerobic counts respectively. The Gram-negative *Bacteroides* group (e.g. *Bacteroides ovatus*, *Bacteroides fragilis*, *Bacteroides thetaiotaomicron*) are thought to be numerically predominant. The genus contains both proteolytic and saccharolytic species. Amongst the Gram-positive, non-spore-forming rods, several genera are numerically significant. Obligate anaerobes include eubacteria and bifidobacteria, such as *Bifidobacterium bifidum* and *Bifidobacterium infantis*, which are prominent in the faeces of breast-fed infants. The genus *Lactobacillus* contains many species that occur in the gut of most warm-blooded animals. Although numerically important in the alimentary tract, their ecological significance has not been conclusively determined.

Table 1. Bacteria, their substrates and products in the human large intestine (From Macfarlane *et al.* 1995)

Bacteria	Description	Concentration (log ₁₀ /g dry wt faeces)		Substrate	Fermentation products
		Mean	Range		
Bacteroides	G- rods	11.3	9.2–13.5	Saccharolytic	A, P, S
Eubacteria	G+ rods	10.7	5.0–13.3	Saccharolytic, some amino acid fermenting species	A, B, L
Bifidobacteria	G+ rods	10.2	4.9–13.4	Saccharolytic	A, L, f, e
Clostridia	G+ rods	9.8	3.3–13.1	Saccharolytic and amino acid fermenting species	A, P, B, L, e
Lactobacilli	G+ rods	9.6	3.6–12.5	Saccharolytic	L
Ruminococci	G+ cocci	10.2	4.6–12.8	Saccharolytic	A
Peptostreptococci	G+ cocci	10.1	3.8–12.6	As for the clostridia	A, L
Peptococci	G+ cocci	10.0	5.1–12.9	Amino acid fermenters	A, B, L
Methanobrevibacter	G+ cocco bacilli	8.8	7.0–10.5	Chemolithotrophic	CH ₄
Desulfovibrios	G- rods	8.4	5.2–10.9	Various	A
Propionibacteria	G+ rods	9.4	4.3–12.0	Saccharolytic, lactate fermenting	A, P
Actinomyces	G+ rods	9.2	5.7–11.1	Saccharolytic	A, L, S
Streptococci	G+ cocci	8.9	3.9–12.9	Carbohydrate and amino acid fermenting	L, A
Fusobacteria	G- rods	8.4	5.1–11.0	Amino acid fermentation, carbohydrate also assimilated	B, A, L
Escherichia	G- rods	8.6	3.9–12.3	As for streptococci	Mixed acids

G+, Gram-positive; G-, Gram-negative; A, acetate; P, propionate; B, butyrate; L, lactate; S, succinate; f, formate; e, ethanol.

Several types of spore-forming rods and cocci are also inhabitants of the gut. The genus *Clostridium* is probably the most common: *C. perfringens*, *C. bifermentans* and *C. tetani* are regularly isolated, albeit in relatively low numbers, and are of significance in human and veterinary medicine. Facultative and obligately anaerobic Gram-positive cocci are also numerically important. The strict anaerobes include *Peptostreptococcus*, *Ruminococcus*, *Megasphaera elsdenii* and *Sarcina ventriculi*. The facultatively anaerobic streptococci are well represented by many species from Lancefield group D, including *S. faecalis*, *S. bovis* and *S. equinus*, and some from group K, such as *S. salivarius*, which is usually associated with the mouth. Gram-negative anaerobic cocci include *Veillonella* and *Acidaminococcus*.

Although not numerous, the Gram-negative facultative anaerobic rods include a number of important pathogens. For example, members of the Enterobacteriaceae, particularly *Escherichia coli*, are usually thought of as characteristic intestinal bacteria.

The large-gut microflora is acquired at birth. Initially, facultatively anaerobic strains dominate. Thereafter, differences exist in the species composition that develops and this is largely controlled by the type of diet. The faecal flora of breast-fed infants is dominated by bifidobacteria. In contrast, formula-fed infants have a more complex microbiota with bifidobacteria, bacteroides, clostridia and streptococci all being prevalent. After weaning, a pattern that resembles the adult flora becomes established (Ducluzeau, 1993).

The principal role of the intestinal microflora is to salvage energy from carbohydrates not digested in the upper gut, through fermentation. The major substrates for fermentation are dietary carbohydrates that have escaped digestion in the upper gastrointestinal tract. These include starch that enters the colon (resistant starch), as well as NSP, e.g. cellulose, hemicelluloses, pectins and gums. Other carbohydrate sources available for fermentation are non-digestible oligosaccharides, various sugars and sugar alcohols (Cummings *et al.* 1997). In addition, proteins and amino acids can be effective as

growth substrates for colonic bacteria. These include elastin, collagen and albumin, as well as bacterial protein released following cell lysis. Pancreatic enzymes represent a source of N. Bacterial secretions, lysis products, sloughed epithelial cells and mucins may also make a contribution as fermentation substrates. Total substrate availability in the human adult colon is 20–60 g carbohydrate and 5–20 g protein/d (Cummings & Englyst, 1987; Cummings *et al.* 1989).

Significant regional differences occur in bacterial activity in the colon. The right (proximal) colon is characterized by a high substrate availability (due to dietary input), low pH (from acids produced in fermentation) and rapid transit. The left, or distal, colon has a lower concentration of available substrate, the pH is approximately neutral and bacteria grow more slowly. The proximal region tends to be a more saccharolytic environment than the distal gut, the latter having higher bacterial proteolysis.

In addition to its role in fermentation the large-intestinal microflora contributes towards health in a number of other ways. The development of the intestinal microflora provides the basis for a barrier that prevents pathogenic bacteria from invading the gastrointestinal tract. The composition of the intestinal microflora together with the gut immune system allows resident bacteria to exert a protective function. In addition gut bacteria are involved in vitamin synthesis (especially vitamins B and K) and in the metabolism of xenobiotics. Thus, modification of the flora by dietary means offers one of the most effective opportunities for development of functional foods.

2.2. Fermentation and short-chain fatty acids (Binder *et al.* 1994; Cummings, 1995; Cummings *et al.* 1995)

Through fermentation, bacterial growth is stimulated (biomass), and short-chain fatty acids (SCFA) and the gases H₂, CO₂ and CH₄ are produced.

SCFA are the major end-products of bacterial fermentative reactions in the colon and are the principal anions in the hindgut of man and all other mammals. The SCFA are acetate, propionate and butyrate but other significant end-products of carbohydrate fermentation include lactate, ethanol, succinate, formate, valerate and caproate (Table 1). Branched-chain fatty acids such as isobutyrate, 2-methylbutyrate and isovalerate may be formed from the fermentation of amino acids that originate in proteolysis. The other end-products from bacterial metabolism of proteins include NH_3 , phenols, indoles and amines, some of which have toxic properties (Macfarlane & Macfarlane, 1995).

The amount of SCFA, which is usually in excess of 100 mmol/kg contents, and the molar ratios of the three principal acids produced by fermentation, vary substantially, depending on the substrate. This has been studied extensively *in vitro* using single-chamber chemostat models of the gut inoculated with intestinal micro-organisms. Yields vary from 40–60% (g SCFA/100 g substrate utilized), with molar ratios of acetate from 60–80, propionate 14–22 and butyrate 8–23 (Cummings, 1995). Whilst acetate is produced in all fermentation systems *in vitro*, it is the major product of pectin breakdown. Similarly, the highest molar ratios of propionate are seen characteristically with arabinogalactan and guar gum as substrate. Amounts of butyrate vary perhaps more than any other according to substrate but the polysaccharide that is associated with the highest relative amounts is starch. In animal studies, wheat bran seems to give rise to high concentrations of SCFA in the gut, despite the fact that it is relatively poorly fermented, especially in human subjects (Cheng *et al.* 1987; McIntyre *et al.* 1991). Studies in human subjects to determine amounts of SCFA in the gut are difficult, but evidence suggests that caecal concentrations of SCFA are approximately double those in the recto-sigmoid area (Cummings *et al.* 1987).

The amount of SCFA produced in human subjects is very difficult to determine. Studies of arterio-venous differences across the gut indicate that 300–500 mmol are produced each day, whilst in individual cases this may reach 1–2 mol. Few dynamic studies have been carried out in man because of problems accessing the portal vein and differential metabolism of SCFA by individual tissues. The situation is complicated by endogenous production of acetate by the liver. Future stable-isotope studies may give more information in this area.

SCFA production in the large intestine can be observed qualitatively by measuring levels in blood. However, only acetate appears in significant amounts in peripheral blood, although this responds in both time and amount to substrate fermentation in the large intestine (Pomare *et al.* 1985; Lifschitz *et al.* 1995).

2.2.1. Physiology and health. All SCFA are rapidly absorbed from the hindgut and stimulate salt and water absorption. They are then metabolized principally by the gut epithelium, liver and muscle, with virtually none appearing in urine and only small amounts in faeces.

One of the most important properties of SCFA is their trophic effect on the intestinal epithelium. All three major SCFA are trophic when infused into the large intestine, although butyrate seems to be the most effective and

propionate the least. What is perhaps more interesting is that infusion of SCFA into the hindgut leads to trophic effects in the small intestine (Sakata, 1987; Frankel *et al.* 1994) although the mechanisms for this are not fully determined. These trophic properties of SCFA have important implications, particularly for patients receiving enteral or parenteral nutrition, and in maintaining the mucosal defence barrier against invading organisms.

2.2.2. Acetate. Acetate is the principal SCFA in the gut. It is taken up by the epithelium, appears in portal blood and eventually passes through the liver to peripheral tissues where it is metabolized by muscle. In animal studies, the liver secretes free acetate when levels in portal blood fall below a critical level. Uptake and utilization of acetate by many tissues has been shown and is the principal route whereby the body obtains energy from carbohydrates not digested and absorbed in the small intestine. Current evidence suggests that the energy value of fermented carbohydrate is 6.3–8.4 kJ/g (1.5–2 kcal/g) (Livesey, 1990; Roberfroid *et al.* 1993).

2.2.3. Propionate. In ruminant species, propionate is a major glucose precursor but this is not an important role in hindgut fermenting species such as man. Propionate is largely cleared by the liver and has not been shown consistently to have significant effects on carbohydrate metabolism in human subjects. *In vitro*, propionate inhibits uptake of acetate into the cholesterol synthesis pathway, and in both rats and pigs propionate supplementation of the diet reduces cholesterol levels in blood. In human feeding studies of propionate only one out of three currently reported shows any change in blood cholesterol levels (Venter *et al.* 1989; Todesco *et al.* 1991; Stephen, 1994).

2.2.4. Butyrate. Butyrate is the most interesting of the SCFA, since in addition to its trophic effect on the mucosa it is an important energy source for the colonic epithelium and regulates cell growth and differentiation. Butyrate is almost entirely cleared by the colonic epithelium and is the principal energy source for the epithelial cells (Bugaut & Bentejac, 1993; Cummings, 1995). A defect in butyrate metabolism has been identified in ulcerative colitis patients and may be induced by S compounds generated in the large-bowel lumen (Roediger *et al.* 1993; Pitcher & Cummings, 1995).

The effect of butyrate on cell growth and differentiation is of great importance and has been the subject of a number of studies (Boffa *et al.* 1992; McIntyre *et al.* 1993). Butyrate brings about a concentration-dependent slowing of the rate of transformed cell growth and promotes expression of differentiation markers *in vitro*, thus leading to reversion of cells from a neoplastic to a non-neoplastic phenotype (Kim *et al.* 1980, 1994; Whitehead *et al.* 1986; Gibson *et al.* 1992). *In vitro* studies with colonocytes suggest an interaction between long-chain fatty acids, which result in decreased viability and differentiation of the cells, and butyrate, which has the opposite effect (Awad *et al.* 1991). In carcinogen-induced animal models of large-bowel cancer, however, butyrate, either from fermentable carbohydrate sources such as resistant starch or purified NSP such as pectin, leads to increased cell turnover and in some studies increased tumour formation (Sakamoto *et al.* 1996; Young *et al.* 1996). The proliferative effects of

butyrate are probably not of pathological significance. Butyrate increases the proliferative index at the bottom of the crypt and thereby has a trophic effect on the mucosa. It does not, however, increase the proliferative index of the surface of the crypt (type II abnormality), which is closely connected with risk of colorectal cancer. Moreover, the relevance of these models to human carcinogenesis is doubtful for a number of reasons (see section 6.6). The expression of several genes is affected by butyrate and butyrate response factors have been identified in the upstream element of certain genes (Kim *et al.* 1994).

2.3. Interactions between the intestinal microflora and epithelial cells

Although attachment to the epithelium is thought to be an important factor whereby bacteria colonize the gut, the mechanisms that allow certain species to maintain themselves in specific locations in the intestinal tract are largely unknown. An interesting new observation is that the intestinal microflora can influence expression of epithelial glycoconjugates, which may serve as receptors for

attachment of (pathogenic) micro-organisms. Recent papers by Bry *et al.* (1996) and Umesaki *et al.* (1995, 1997) report that host epithelial cells in the small intestine express fucosylated glycoconjugates in response to the presence of specific, strictly anaerobic bacteria (*B. thetaiotaomicron* and a segmented filamentous bacterium SIF13). Attachment of some pathogenic micro-organisms is decreased by the mutually beneficial crosstalk between the indigenous microflora and the host (Umesaki, 1989). The observation that one species can induce epithelial surface structures which influence attachment of other bacteria has significance for the use of CaCo-2 cell lines or gnotobiotic animals as model systems to study adherence or infectious diseases as well as for strategies to prevent and to treat gastrointestinal diseases. Thus, adhesion of bacteria to mucosal cell lines is important, but their mucus-adhering and degrading properties also need to be addressed.

2.4. The concept of healthy microflora

It is a long-held belief, originating probably with Metchnikoff at the turn of the century, that some gut bacteria are

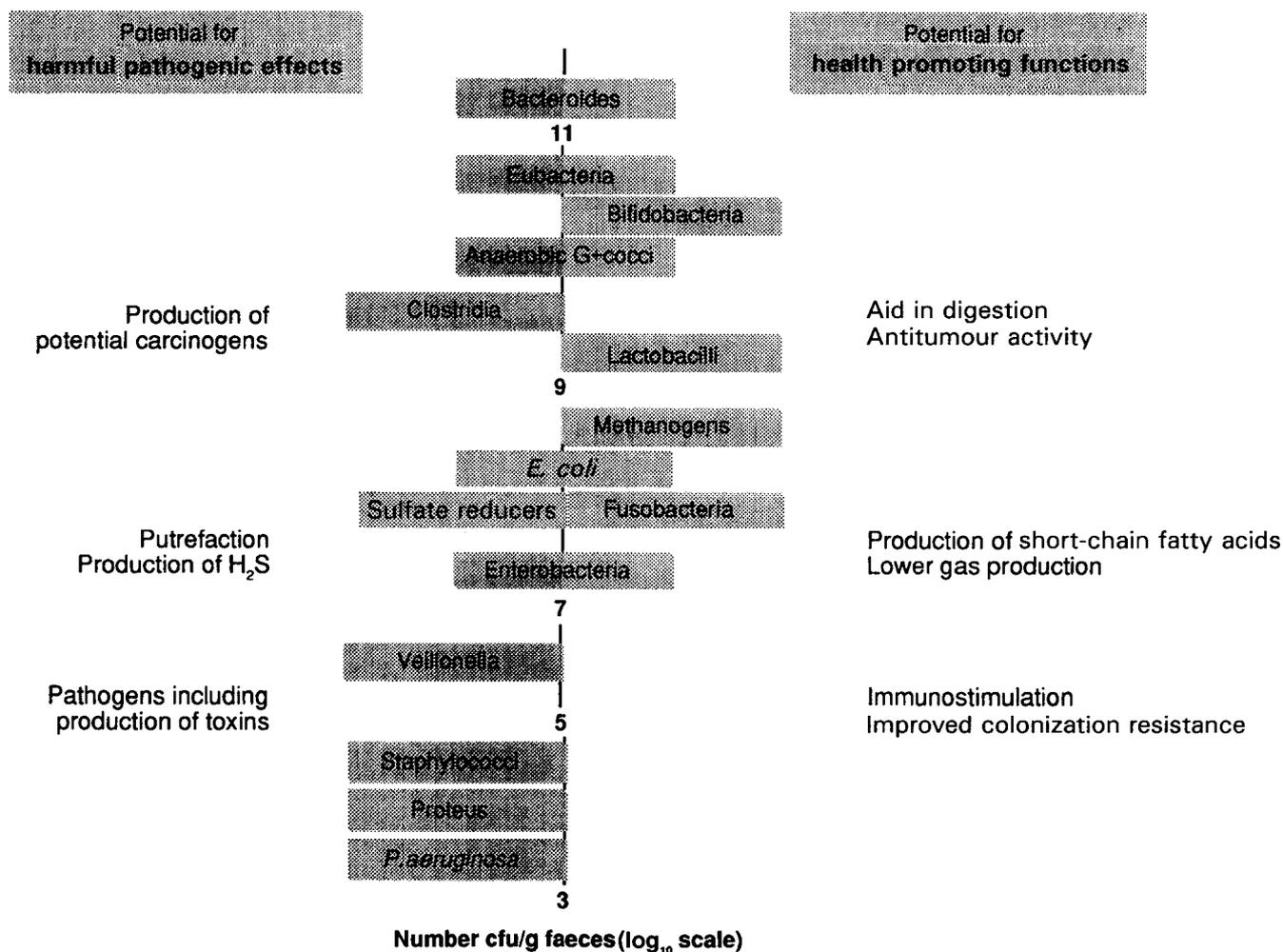


Fig. 1. Generalized scheme of predominant groups of colonic bacteria, indicating how the genera may exhibit potentially harmful and beneficial functions. G+, Gram positive; cfu, colony-forming units.

beneficial to health, whilst others may be harmful (Fig. 1). That bacteria in the gut can be harmful through production of toxins causing diarrhoea, mucosal invasion and activation of carcinogens is self-evident. Such bacteria are thought to include the Clostridia, sulfate reducers and amino acid-fermenting species.

Potentially health-giving bacteria are thought to include principally the bifidobacteria and lactobacilli. These two genera do not include any significant pathogenic species and their dominance in the faeces of breast-fed babies is thought to provide protection against infection. In adults they may be the principal species responsible for barrier function and for stimulating healthy immune function. In addition, bacteria act in symbiosis with the host through fermentation.

However, the colonic microflora is a complex interactive community of organisms and its functions are a consequence of the combined activities of the microbial components. Thus manipulation of the human intestinal flora offers the potential to improve health through a variety of mechanisms.

3. The gastrointestinal immune system

3.1. Gut-associated lymphoid tissue (GALT) (Brandtzaeg *et al.* 1989)

The first, and in normal individuals only, contact that ingested bacteria, including probiotics, have with the immune system is with the GALT. The human intestine represents the largest mass of lymphoid tissue in the body, containing over 10^6 lymphocytes/g tissue. In addition, about 60% of the total immunoglobulin (Ig; several grams) produced daily is secreted into the gastrointestinal tract. GALT is part of the mucosal immune system (i.e. gastrointestinal tract, respiratory tract, oral cavity, urogenital tract and mammary glands) and has unique cell types and mechanisms of immunity. The special nature of intestinal immunity has evolved under constant exposure to environmental antigens, whilst requiring an effective response to an invading pathogen despite the presence of dietary antigens. The difference between immune responses to dietary proteins and antigens of colonizing bacteria may play a role in the prevention of hypersensitivity reactions to food proteins.

3.2. The structure of GALT and cell distribution

Intestinal immune cells are organized in different compartments: aggregated in follicles and the Peyer's patches; distributed within the mucosa as diffuse lymphocyte populations; and in the epithelium (reviewed by McKay & Perdue, 1993). The GALT T-lymphocytes are not homogeneous. These are classified as CD4+ helper/inducer cells and CD8+ suppressor/cytotoxic cells, generating different cytokine profiles with distinct yet unproven functions (reviewed by Brandtzaeg *et al.* 1989; Brandtzaeg, 1995). The majority of the intra-epithelial T-cells have a suppressor/cytotoxic phenotype, contrasting with the lamina propria cells, which show mainly a helper/inducer phenotype. The lamina propria is also endowed with lymphocytes belonging to the B-cell lineage. These are mainly

memory cells and plasmocytes, where 70–90% of them are IgA-producing cells.

The epithelial layer of the small-intestinal mucosa is arranged in folds, consisting of villi and crypts, which increase the absorptive surface area. The epithelium consists of a single layer of absorptive columnar epithelial cells, goblet cells and intra-epithelial lymphocytes. The intra-epithelial lymphocytes are a heterogeneous population of cells. In the mouse, the primary intra-epithelial lymphocytes are CD3+, CD8+, T-cells with a γ/δ -T-cell receptor (T-cell receptor 1) and in man CD8 T-cells expressing an α/β -cell receptor (T-cell receptor 2). The proportion of T-cell receptor 1 cells in the epithelium is greater than in peripheral blood. The γ/δ -T-cell receptor cells are thought to mature in the epithelium rather than in the thymus, thus their development might be more susceptible to environmental exposures. Intra-epithelial lymphocytes are known to mediate both non-major-histocompatibility-complex-restricted and major-histocompatibility-complex-restricted cytotoxicity, and regulate neighbouring immune and epithelial cells by secreting cytokines.

The epithelium is surrounded by the lamina propria, which comprises lymphoid organs such as reticular tissue and which contains plasma cells, T-helper cells, granulocytes and mast cells. The lamina propria is surrounded by smooth-muscle tissue. Along the small intestine are Peyer's patches, which are organized lymphoid follicle aggregates. The Peyer's patches are more accessible to micro-organisms than other epithelial surfaces of the gut, because they have reduced numbers of the mucus-secreting goblet cells. In addition, the epithelial layer of the Peyer's patches contains specialized transport cells called M-cells, which lack microvilli and are able to phagocytose both soluble antigens and micro-organisms.

3.3. Immunophysiological regulation (Brandtzaeg, 1995)

Different components of the mucosal immune system act to focus a specific response against offending antigens. The first line in this defence, immune exclusion involving IgA antibodies, is non-inflammatory (Brandtzaeg, 1995). The best-characterized component of the mucosal immune defence is the secretory IgA system (Brandtzaeg, 1995). IgA antibody production is abundant at mucosal surfaces. IgG-, IgM- and IgE-secreting cells function also, but at a significantly lower frequency in GALT. In contrast to IgA in serum, secretory IgA is present in dimeric or polymeric form in the gut. The predominance of IgA in the mucosal immune system results from IgA-selective T-cell regulation in GALT, particularly in the Peyer's patches, where specific immune responses are generated (Biewenga *et al.* 1993). After being synthesized by IgA precursor cells, polymeric IgA is transported to the mucosal surface by epithelial transcytosis mediated by the polymeric immunoglobulin receptor, the secretory component. Secretory IgA is resistant to intraluminal proteolysis, and does not activate complement or inflammatory responses, which makes IgA ideal for protecting the mucosal surfaces. Hence, the main function of secretory antibodies is, in cooperation with non-immunological defence mechanisms (Sanderson & Walker, 1993), to mediate immune exclusion of foreign antigens by

preventing epithelial adherence and penetration of invasive pathogenic micro-organisms, neutralizing toxins and viral multiplication.

Although GALT is mainly involved in specific immune protection of the gut, there is evidence for a 'common mucosal immune system': an immune response initiated in GALT can affect immune responses at other mucosal surfaces (Brandtzaeg, 1995). The lymphocytes activated within Peyer's patches disseminate via mesenteric lymph nodes, thoracic duct and the bloodstream back to the lamina propria, and traffic between other secretory tissues, including the respiratory tract and the lachrymal, salivary and mammary glands.

There are differences between the upper and lower parts of the human GALT in isotype distribution of immunoglobulin-producing cells. Two IgA subclasses are available (IgA₁ and IgA₂). IgA₁ immunocytes predominate in the small-intestinal mucosa, while IgA₂ are most frequent in normal colonic mucosa. IgA₂ in the colon is resistant to most bacterial proteases that cleave IgA₁ (Brandtzaeg, 1995).

Immune elimination is directed towards removal of foreign antigens that have penetrated the mucosa. This second line of defence involves antibodies such as IgG and a large number of mediators such as inflammatory cytokines, which are considered to be responsible for the pathophysiology associated with local inflammation (Brandtzaeg, 1995). Immune regulation pertains to the state of specific hyporesponsiveness induced by prior oral administration of antigens, inducing oral tolerance. Consequently, hyporesponsiveness to ubiquitous antigens such as dietary antigens is a hallmark of the intestinal immune system (Weiner *et al.* 1994). This has been taken to be a combined effect of immune exclusion and suppression of the systemic immune response, but it is still a matter of debate.

3.4. Regulation of antigen transfer (Isolauri *et al.* 1993a, b)

Antigens are proteins foreign to the host. Factors that influence antigenicity include molecular complexity, solubility and concentration. Most antigens are macromolecules in the molecular mass range 10 000–70 000 Da.

Apart from the barrier function, the intestinal mucosa is efficient in assimilating antigens. For this purpose, there are specialized antigen transport mechanisms in the villous epithelium and particularly in the Peyer's patches (Heyman *et al.* 1982). The manner in which an antigen is transported across the mucosa determines the subsequent immune response (Heyman *et al.* 1982; Heyman & Desjeux, 1992; Isolauri *et al.* 1993a, b; Sanderson & Walker, 1993).

Most antigens are excluded by a well-functioning mucosal barrier but an immunologically important fraction of antigen does bypass it (Heyman *et al.* 1982; Isolauri *et al.* 1993a, b). Antigens are absorbed across the epithelial layer by transcytosis, and the main degradative pathway entails lysosomal processing of the antigen. A minor pathway allows the transport of unprocessed antigens. The Peyer's patches are covered by a unique epithelium and antigen transport across this is characterized by rapid uptake and

reduced degradation of antigens. In health, paracellular leakage of macromolecules is prevented because intact intercellular tight junctions maintain the barrier to macromolecules. Consequently, in healthy subjects antigen transfer is well controlled and aberrant antigen absorption does not occur.

There is evidence that during the process of absorption across the intestinal mucosa, dietary antigens are altered into a tolerogenic form (Weiner *et al.* 1994). By interfering with this process intestinal inflammation is an important risk factor for the development of hypersensitive disorders (Fargeas *et al.* 1995).

3.5. Interactions between the intestinal microflora and the GALT (Moreau & Coste, 1993)

After birth, the intestine is rapidly colonized by bacteria, which probably act as a source of antigens and non-specific immunomodulators. The dual role of the digestive flora on the immune system should be emphasized. Bacteria can be considered as antigens able to elicit specific systemic and local immune responses. Furthermore, they exert a considerable influence on the number and distribution of the GALT cell populations and play an important role in the regulation of immune responses. These data have emerged mainly from animal studies using germ-free and gnotobiotic animal models (see section 6.5). As direct evidence from human subjects is scarce, we can only extrapolate from experimental results obtained in mice. Such studies are important to determine the exact role played by different bacteria present in the digestive flora, with the aim of improving the bacterial equilibrium and allowing the best immune modulation by functional foods. The cellular and molecular events by which the digestive flora modulates the immune system are still poorly understood.

The digestive flora is the major antigenic stimulus responsible for the migratory pathway and maturation of precursor lymphoid cells present in the Peyer's patches. Consequently, it acts on the development and maturation of the IgA plasmocytes. In germ-free mice, IgA-plasmocyte number is decreased tenfold as compared with controls. It has been shown that the sequential establishment of the digestive flora from birth to weaning is responsible for the progressive increase in IgA plasmocyte numbers in the lamina propria of the small intestine in the growing normal mouse. In addition, Gram-negative bacteria such as *Escherichia coli* and *Bacteroides* play an important role in this immunologically non-specific effect.

The digestive flora also modulates the specific immune responses at local and systemic levels. It allows the persistence of the systemic unresponsiveness to an antigen, induced by a previous feeding with the same antigen (oral tolerance) (Moreau & Gaboriau-Routhiau, 1996) and shortens the abrogation of oral tolerance mediated by cholera toxin or *E. coli* toxin (Gaboriau-Routhiau & Moreau, 1996), which seems to be a property of Gram-negative bacteria (M. C. Moreau and V. Gaboriau, unpublished results). In another study the presence of the gut flora modulated the intestinal antibody IgA response to rotavirus. Recently, the development of an experimental model of adult germ-free mice infected with a heterologous strain of

rotavirus allowed investigation of the immunomodulating properties of a strain of *Bifidobacterium* on the enhancement of the intestinal anti-rotavirus IgA antibody response at cellular and faecal levels (Moreau *et al.* 1998). At the systemic level, in gnotobiotic mice harbouring a human strain of *Bifidobacterium* in the intestine or two bacterial strains from yoghurt, *Lactobacillus bulgaricus* and *Streptococcus thermophilus*, increases of the specific antibody response in serum and in the phagocytic activity of peritoneal phagocytes were observed respectively (Moreau *et al.* 1994).

4. Mucosal cell proliferation and differentiation (Wright & Alison, 1984)

The intestinal mucosa of rodents and other mammals is renewed every 2–3 d (Wright & Alison, 1984). Maintenance of the architecture of the colonic mucosa, in particular of mucosal crypts, is a consequence of the balance amongst a number of factors. Proliferation of stem cells occurs near the base of the crypt. As enterocytes migrate up the crypt they differentiate, mature and become functional in terms of absorption and mucin secretion.

4.1. Cell proliferation

This has been well described and a wide variety of methods are available for its estimation *in vitro* and *in vivo* in both animals and man (Goodlad & Wright, 1982; Goodlad, 1989).

4.2. Differentiation

Light and electron microscopic examination of human colonic tissue has revealed that stem cells differentiate into a number of cell types, including mucus-secreting cells, columnar cells (thought to have an absorptive and a secretory function) and intestinal endocrine cells. Histochemical studies have shown alterations in secreted glycoproteins between differentiated and undifferentiated regions of the small and large intestine, indicating that a modification of carbohydrate structures accompanies goblet cell differentiation in rat and man (Boland *et al.* 1992). Furthermore, the mucin of normal colonic mucosa differs markedly from that in cancerous tissue and 'transitional tissue' in the early stages of neoplastic development (Boland *et al.* 1992). A characteristic of tumours is the presence of poorly differentiated cells: consequently, a dietary treatment that encourages differentiation is potentially beneficial.

4.3. Apoptosis

Apoptosis (genetically programmed, autonomous cell death) associated with the removal of damaged cells is considered to be a protective event.

4.4. Mucosal enzymes (Szarka *et al.* 1995)

Phase I, cytochrome P450 enzymes and phase II drug-metabolizing enzymes such as glutathione *S*-transferase (*EC* 2.5.1.18) and UDP-glucuronosyl transferase (*EC*

2.4.1.17) are widely distributed in the intestinal mucosa. These enzymes are involved in the biotransformation of mutagens, procarcinogens, steroids and other compounds of exogenous and endogenous origin. Modulation by dietary compounds may result in protection against toxic and carcinogenic damage to tissues (Wattenberg, 1983). In terms of deactivation, the enzyme glutathione *S*-transferase is of particular importance. It is present in many tissues in a variety of forms (π , μ and α) and plays a critical role in protecting tissues from xenobiotics and carcinogens. Glutathione *S*-transferase activity has been found to be lower in individuals at high risk from colon cancer when compared with controls (Szarka *et al.* 1995).

5. Gastrointestinal function and disease

5.1. Gastrointestinal infections (Gracey, 1993; Savarino & Bourgeois, 1993)

Acute infections of the gut are usually self-limiting, characterized by diarrhoea and often vomiting. The principal pathogens are viruses and bacteria such as *Escherichia coli*, *Campylobacter* spp, *Vibrio cholerae*, *Staphylococcus aureus*, *Bacillus cereus*, *Clostridium perfringens*, *Salmonella* spp, *Shigella* spp, *Yersinia* spp and a number of protozoa, especially *Giardia lamblia*, *Entamoeba histolytica* and *Cryptosporidium parvum*.

Bacteria causing infection are usually classified according to whether they secrete an enterotoxin (toxigenic) or invade the bowel wall (invasive). Toxigenic diarrhoeas include cholera, and both enteropathogenic and enterotoxigenic *E. coli*, whilst the classic invasive organisms are *Shigella* (dysentery), *Salmonella* (typhoid) and enteroinvasive *E. coli*. Rotaviruses are most commonly found in diarrhoea of children and invade the small-intestinal epithelium. Acute diarrhoea is responsible for 3–4 million deaths annually worldwide, many of which are children, in which it accounts for 20–30% of all mortality.

Rotavirus is the most common cause of acute childhood diarrhoea. It is primarily seen in infants and young children, with a peak incidence between 6 months and 2 years of age. Rotaviruses invade the highly differentiated absorptive columnar cells of the small-intestinal epithelium, where they replicate. This results in partial disruption of the intestinal mucosa with loss of microvilli and decreased villus: crypt ratio. Rotavirus infection is associated with increased intestinal permeability. Jalonen *et al.* (1991) found increased lactulose:mannitol urinary recovery ratios in patients with acute diarrhoea compared with non-diarrhoeal patients. Concomitantly, the levels of immune complexes containing dietary β -lactoglobulin in sera were significantly higher in patients with rotavirus diarrhoea than in non-diarrhoeal patients. Enhanced macromolecular absorption in rotavirus gastroenteritis has been shown in several studies (Heyman *et al.* 1987; Isolauri *et al.* 1993a,b). A local immunoinflammatory reaction impairs the intestine's barrier function. Impaired barrier function and defective handling of intraluminal antigens in the epithelial cells may be an important pathogenic mechanism in acute and chronic gastrointestinal disorders. It may abrogate tolerance to ubiquitous antigens,

including bacteria residing in the intestine (Duchmann *et al.* 1995).

Chronic infection of the gut is much rarer and seen only in persons who have anatomical abnormalities of the gut such as blind loops, strictures or fistulas. Chronic infection with *Tropheryma whippelli* causes Whipple's disease and intestinal bacteria are responsible for tropical sprue. Tuberculosis affects the gut, especially the ileo-caecal region, and chronic carrier states occur with amoebas.

The main indigenous bacteria of the large intestine resist invasion by pathogenic species and this is part of the human host defence against diarrhoeal illness. This barrier function provided by the gut flora may be impaired during antibiotic use, where diarrhoea is common. Antibiotic-associated diarrhoea is usually due to invasion with toxin-producing species such as *Clostridium difficile* or *Clostridium septicum*.

5.2. Normal bowel habit (Cummings, 1993, 1994)

Bowel habit is defined by the amount of stool passed, frequency of defecation and consistency of stool. It varies very widely throughout the world with daily stool weights in the range 100–400 g/d and stool frequency of three times per day to three times per week. In European countries and North America, daily stool weight is of the order of 100–150 g/d (Cummings *et al.* 1992). Bowel habit is controlled principally by two factors, first diet, and second gut motor activity (transit time). The foods that affect bowel habit are those which reach the large intestine, i.e. are non-digestible. The dietary components falling into this category are lactose (in lactase-deficient individuals), sugar alcohols, non-digestible oligosaccharides, resistant starch and NSP. Dietary fat and protein have little effect on bowel habit unless they are rendered non-absorbable by some technique (e.g. sucrose polyester).

The mechanism by which non-digestible foods affect bowel habit depends on their fermentability. Foods that are not fermented appear in faeces and cause bulking depending on their inherent mass or water-holding capacity (i.e. bran and other intact cell-wall material). Most foods that reach the large intestine are fermented, yielding SCFA, which are absorbed and do not contribute to faecal bulk, and H₂ and CH₄, which can expand faecal bulk but not mass. Fermentation also stimulates bacterial growth to produce biomass, which is the principal mechanism of increasing stool mass. A final mechanism that needs to be borne in mind is the interrelationship between intestinal bulk and motor activity. As bulk in the large intestine increases, so motor activity is stimulated and, in general, the greater the bulk the more rapid the transit. Motor activity expressed as transit time may also modulate stool output independently of dietary bulk (Cummings, 1993, 1994).

5.3. Constipation

Constipation is a disorder of motor activity of the large bowel traditionally defined in terms of bowel frequency. The main symptom in constipation is straining at defecation, and discomfort, distension and incomplete rectal emptying are all considered part of the condition. Total gut transit time

is generally prolonged in constipated subjects. There are many causes of constipation, with diet one of the common reasons, particularly low-NSP diets, gluten-free diets, 'low-residue' diets and enteral feeds. Treatment of simple constipation is usually in the first instance by dietary means. The principle is to increase fermentable carbohydrates in the diet, especially NSP from whole-grain cereals. Thus, diet has a major role to play in controlling bowel habit.

5.4. Irritable bowel syndrome (IBS) (Thompson & Heaton, 1980; Thompson & Gomborone, 1993)

IBS is one of the commonest disorders seen in the hospital gastroenterology clinic, but it is poorly understood. IBS (or irritable colon, mucus colitis, spastic colon) is a disorder of motor activity of the whole bowel, although colonic symptoms usually predominate. It occurs very widely throughout the world and is commoner in women. IBS has two main presenting features, abdominal pain and altered bowel habit (Thompson & Heaton, 1980).

The cause of IBS is unknown but it occurs in many patients following dysentery or antibiotic use. In addition, patients often volunteer that specific foods upset them (food intolerance) and stress is clearly contributory. Wheat bran and other bulk laxatives are frequently given, but results have been very variable. They may aggravate symptoms through gas production, although in patients who are predominantly constipated they are of benefit. Because of a postulated disturbance in the colonic microflora in IBS a number of groups are currently trying the use of probiotics to 'normalize' the flora.

5.5. Inflammatory bowel disease (Podolsky, 1991; MacDonald, 1993; Tytgat *et al.* 1995)

Two major disorders, Crohn's disease and ulcerative colitis, are conventionally grouped together under the heading inflammatory bowel disease because both are characterized by chronic inflammation in the gut. However, it is best to consider them as separate conditions because they have characteristically different pathology, clinical courses, complications and management. The aetiology of neither is known.

5.5.1. Crohn's disease. Crohn's disease may affect any part of the gut from mouth to anus. Characteristically it occurs in the ileocaecal region and colon, and the inflammation is patchy or discontinuous. It frequently recurs after surgical resection of the affected areas of gut at or near the point of anastomosis of the bowel. The involved intestine is thickened, with ulceration of the mucosa, stricturing and fistula formation. Mouth ulcers and perianal abscesses are characteristic. Histologically there is transmural inflammation, with mononuclear cells, lymphoid aggregates and granulomata.

Crohn's disease occurs worldwide although it is uncommon in Central and South America, Africa and Asia. It is seen less frequently than ulcerative colitis, although rates have increased fivefold since 1950. It is predominantly a disease of the young with peak occurrence between the ages of 20 and 30 years with a second peak between 70 and 80 years. The cause is unknown but genetic factors are

important with 10% of patients having a close relative with the disease. Diet has been implicated, especially sugar, but a multicentre trial in which a diet excluding sugars and rich in NSP was given did not result in any conclusive benefit in patient management (Ritchie *et al.* 1987). More likely aetiological factors include bacteria and other micro-organisms.

Diet has an important role to play in the management of Crohn's disease. For patients who do not respond to conventional treatment in Crohn's disease, various enteral and parenteral regimens providing bowel rest have been used. The rationale for such treatments is that the absence of food antigens in the bowel lumen reduces inflammatory immune reactions, motor and digestive activity, and gives the mucosa a chance to heal itself.

Most patients relapse soon after introduction of normal food following bowel rest regimens. It has been suggested that dietary modification to exclude foods likely to cause symptoms (predominantly cereals, dairy products and meat) can lead to extended periods of remission.

5.5.2. Ulcerative colitis. Ulcerative colitis is a chronic inflammatory condition of the mucosa of the large bowel that causes bloody diarrhoea. It is one of the diseases of modern civilization, being first described with certainty in 1909 and predominantly affecting industrialized populations. It has an overall prevalence of 40–120 cases per 100 000 of the population in Western countries and is uncommon, although beginning to emerge, in Africa and India. It affects the sexes equally and usually presents between the ages of 20 and 40 years.

Its cause is unknown but there is probably a genetic component, with a 15-fold increased risk amongst close relatives of patients. Animal models point to an involvement of the immune system, but also to the necessary presence of bacteria in the colon to produce colitis. The observation that healthy colonocytes use butyrate for their metabolism and that this is defective in ulcerative colitis has raised the possibility of dietary factors in its aetiology. However, no convincing evidence for a dietary factor has emerged, although the inhibition of butyrate oxidation by S-compounds leaves the possibility of diet combining with the colonic microflora as a possible initiating factor of the inflammation (Pitcher & Cummings, 1996).

5.6. Food allergy (Isolauri, 1995)

Food allergy is defined as an immunologically mediated adverse reaction against dietary antigens. Food allergy can affect several organ systems, the symptoms commonly arising from the gut, skin and respiratory tract. Despite the wide spectrum of clinical manifestations, there are at least two prerequisites for the development of food allergy: dietary antigens must penetrate the intestine's mucosal barrier, and the absorbed antigens must cause harmful immune responses. The immaturity of the immune system and the gastrointestinal barrier may explain the peak prevalence of food allergies in infancy. In food allergy, intestinal inflammation and disturbances in intestinal permeability and antigen transfer occur when an allergen comes into contact with the intestinal mucosa. During dietary elimination of the antigen, the barrier and transfer

functions of the mucosa are normal. It has, therefore, been concluded that impairment of the intestine's function is secondary to an abnormal intestinal immune response to the offending antigens.

5.7. Colorectal cancer (Faivre *et al.* 1985)

Colorectal cancer is unequally spread throughout the world (Burkitt, 1971). It is amongst the three most common malignancies in most industrialized countries including Western Europe, and its survival rate has improved little during the past decades, being of the order of 40% at 5 years. The most common locations in high-risk countries are the left colon and the rectum, whereas right-colon cancers are proportionally more common in low-risk areas such as Japan. About 5% of colorectal cancers are truly genetic diseases (hereditary nonpolyposis colorectal cancer and familial polyposis coli), transmitted as autosomal dominant, but the majority of colorectal cancers are sporadic, and are mostly influenced by environmental factors, in particular diet, with a potential interaction between a genetic background and diet (Boutron *et al.* 1996). From 70 to 80% of left colon and rectal cancers in Western countries follow the so-called adenoma-carcinoma pathway, with possibly less in the case of right-colon cancers (Bedenne *et al.* 1992). This is of major importance as it provides the opportunity of studying precancerous lesions both in aetiological studies such as case-control and cohort studies, and for intervention studies, where studying adenoma recurrence or growth is easier and brings results more rapidly (Boutron & Faivre, 1993).

6. Methodology

6.1. Human intestinal microflora (Collins & Gibson, 1998)

The identification of factors controlling or influencing the composition of the human intestinal microflora, including prebiotics and probiotics, may be compromised by the precision of current methodologies for determining bacterial composition which are based almost entirely on phenotypic approaches. Whilst these have met with some success, when done properly, they are time-consuming, laborious and lack the resolving power necessary to analyse the complex microbiota at the species or subspecies level.

Traditional gut microbiological methodologies are usually based on morphological and biochemical properties of the organisms (Table 2). Whilst such an approach is cost-effective and allows the processing of replicate samples, the procedures used may be unreliable and may lack resolution. For example, the metabolic plasticity of organisms is problematic and the test used may not be reproducible. Phenotypic characterization does not allow a high degree of fidelity and is most useful for genus level identification. In some cases this situation is eased if the test organism exhibits a specific metabolic trait. For example, bifidobacteria may be detected, on a qualitative basis, by the production of fructose-6-phosphate phosphoketolase activity. An additional problem is that traditional cultivation-based methods may result in underestimation of microbial diversity, due to the presence of organisms that cannot be

Table 2. Methods for study of the human gut microbiota

Method	Advantages	Disadvantages
Morphological and biochemical characteristics	Straightforward to carry out. Can run in parallel a large number of replicates. Relatively inexpensive	Involves operator subjectively to recognize different colonial and cellular morphologies. Lack of discriminatory power and subject to metabolic plasticity of the organisms. Applicable only to culturable bacteria
Specific biomarkers, e.g. certain cell-wall antigens, cellular fatty acids, plasmid profiles, serotyping, resistance to antibiotics	Cultural procedures may not be required	Cannot assign the position of hitherto unknown species. Relies on all test organisms having unique biomarker. Stability of the biomarker may be questionable
Ribotyping (RNA polymorphisms)	Reliable. Very high discriminatory power	Applicable only to culturable forms. Cannot assign the taxonomic position of any unknown species
16 S ribosomal RNA typing	High fidelity. Reliable. Cumulative database allows placement of unknown species. Applicable to culturable and non-culturable forms. Allows probe development	Costly for both reagents and large-scale equipment, e.g. automated sequencers. Recommended for partial use only

cultivated, which therefore elude isolation. This has led to the development of alternative strategies for assessing microflora changes.

The detection of biomarkers that may be attributed to certain components of the flora offers some potential. However, to be wholly effective there would be a need for all the major bacterial components of interest to be separable by individual biomarkers, e.g. changes in cellular fatty acids. This may be feasible but would be difficult to prove in a reliable manner.

An attractive solution to the problem of determining microflora changes accurately, lies in the application of modern high-resolution molecular genetic techniques. Recent advances in the field of molecular biology are revolutionizing the characterization and identification of micro-organisms (Pace, 1996). For example, molecular sequence analysis, particularly of ribosomal RNA (rRNA), provides a powerful tool for determining the genetic inter-relationships of micro-organisms, and allows systematic monitoring of the gut flora response to dietary intervention. By utilizing diagnostic sequences within the rRNA, it is possible to design gene probes that facilitate precise identification. The use of polymerase chain reaction technologies may also allow access to non-culturable micro-organisms. Over the next few years, 16 S rRNA sequence analysis is expected to rapidly advance our knowledge of the true genetic diversity of the gut microbiota, including organisms that evade traditional identification, due either to a lack of taxonomic resolution and/or non-culturability.

Molecular approaches have already been used to determine changes in the composition of the microbial gut flora (Langendijk *et al.* 1995; Kok *et al.* 1996; McCartney *et al.* 1996; Wilson & Blitchington, 1996). Clearly, however, before such techniques are routinely used in gut microbiological applications, the fidelity and efficacy of such methods need to be rigorously evaluated. A comparative phylogenetic framework of gut micro-organisms, based on genetic material such as 16 S rRNA, would allow highly discriminatory and dependable diagnostic probes to be developed. Preferably, the probes should be validated using different procedures, such as *in situ* and dot blot hybridizations.

Another approach to analyse the genetic diversity of complex microbial populations is denaturing gradient gel electrophoresis or temperature gradient gel electrophoresis. The technique is based on the separation of polymerase chain reaction-amplified fragments of genes coding for 16 S rRNA, all of the same length (Muyzer *et al.* 1993). This results in unique separation patterns for different microbial populations, and will contribute to the description of changes or differences in microflora composition of uncharacterized microbial populations.

The potential benefits of such technologies in gut microbiology, especially dietary modulation for improved health, are large, particularly when used in conjunction with traditional phenotypic procedures. The use of molecular genetic approaches for qualitative and quantitative monitoring of the human intestinal microbiota will constitute an essential step forward for determining the validity of the functional food concept, when directed towards the role of the gut flora.

6.2. Functional analysis of the gut microflora (Rowland, 1995)

The complexity of the gut microflora, coupled with time-consuming procedures necessary to identify and enumerate the anaerobic components, makes their characterization by conventional methodology difficult and expensive. In particular, such methods are not suited to studies involving large numbers of subjects or treatment regimens. Less comprehensive studies (e.g. identification of major groups) are open to the criticism that any induced changes occurring in genera or species other than those being enumerated will be missed. Furthermore, although bacteriological investigations are useful in describing the basic ecology of the gut, they are of less value in studies of metabolism, nutrition and cancer.

An alternative approach is to use biochemical assays that measure the functional activity of the flora as a whole and thus permit deductions to be made regarding the role of the flora in the metabolism of dietary components. In addition, by selecting microbial enzyme activities or metabolic end-points resulting in compounds with potentially toxic or beneficial effects, probable health consequences for the host can be assessed.

6.2.1. Bacterial enzymes. The bacterial enzymes commonly assayed include β -glucuronidase (EC 3.2.1.31), β -glucosidase (EC 3.2.1.21), azoreductase, nitroreductase, nitrate reductase (EC 1.7.99.4), the conversion of pre-carcinogen 2-amino-3-methyl-7H-imidazo[4,5-f]quinoline (IQ) to 7-hydroxy-2-amino-3,6-dihydro-3-methyl-7H-imidazo[4,5-f]quinoline-7-one (7-OHIQ). The substrates of these enzymes and the functional and health implications of their products have been extensively reviewed (Rowland, 1995). For example, bacterial β -glucuronidase in the colon is able to release carcinogens from hepatically derived glucuronic acid conjugates and is a critical factor in the enterohepatic circulation of drugs and other foreign compounds. β -Glucosidase hydrolyses plant glycosides to release the aglycones, many of which are mutagenic, although some exert anti-carcinogenic activity (Rowland, 1995). Azo- and nitroreductases reduce their substrates to amines, which are usually more toxic than the parent compound, and nitrate reductase generates the highly reactive and toxic anion nitrite. Bacterial conversion to 7-OHIQ is one pathway of activation of the pre-carcinogen IQ (formed during cooking of meat and fish) to a genotoxic derivative.

6.2.2. Bacterial metabolites in faeces. Faecal metabolites that are indicators of bacterial activity relevant to colonic health include NH_3 , a toxic product of bacterial breakdown of protein and urea (Clinton, 1992), and phenols and cresols, which are derived from amino acid catabolism by gut bacteria (Macfarlane & Macfarlane, 1995). The production of NH_3 is closely related to bacterial activity and is associated with certain toxic events in the gastrointestinal tract. NH_3 is considered to be a potential tumour promoter in the colon, and has been postulated to enhance neoplastic transformation in the gut.

Other gut bacterial products with possible adverse effects on the colonic mucosa include *N*-nitroso compounds, which are potentially carcinogenic substances formed by bacterial catalysis of the reaction of nitrite and nitrogenous compounds in the colon (Rowland, 1995), diacylglycerol, a putative tumour promoter derived from lipid breakdown, and secondary bile acids, deoxycholic and lithocholic acids, also putative tumour promoters.

6.2.3. Assessment of cytotoxicity, genotoxicity and mutagenicity of faeces. An alternative approach to assaying enzymes or metabolites in faeces is to assess toxicological activity of fractions using short-term tests for toxicity, genotoxicity and mutagenicity. This provides a direct estimate of the potential of the faecal sample to damage the colonic mucosa and has been used to provide insights into possible processes involved in colon cancer. Usually, the aqueous phase of human faeces (faecal water) is used (Rafter *et al.* 1987). Cellular toxicity can be assessed using rapid colorimetric assays in multiwell plates. For example the 3-[4,5-dimethylthiazol-2-yl]-2,5-diphenyltetrazolium bromide (MTT) assay (Mosmann, 1983) involves the pH-dependent conversion of water-soluble MTT to water-insoluble formazan and provides a reliable estimate of viable cell number.

Recently, using the Comet assay, it has been shown that the genotoxicity of faecal water varies markedly between individuals, with at least some of the DNA

damage occurring via an oxidative mechanism (Venturi *et al.* 1997).

6.2.4. Susceptibility of functional markers to dietary change. In animal models, major changes in activities of bacterial enzymes and in levels of bacterial metabolic products have been seen after a wide range of dietary changes. These include the type and level of dietary fat and protein, supplementation with dietary fibre and resistant starch, and addition of oligosaccharides (Rowland, 1991). In many cases changes were seen in the absence of alteration in composition of the gut flora.

In contrast, modification by diet of bacterial metabolism in human subjects has proved more difficult. However, changes in enzyme activities and concentrations of NH_3 , phenol and cresol have been detected in volunteers consuming lactulose (Terada *et al.* 1992) and lactobacilli (Goldin & Gorbach, 1984).

A low level of NH_3 production in the gut is associated with low-protein, high-fibre diets, which appear to be protective against cancer of the colon. NH_3 levels have been shown to be elevated in rats consuming a diet containing high-risk factors for colon cancer (Hambly *et al.* 1997).

The cytotoxicity of faecal water strongly correlates with bile acid concentration in faeces and is increased in individuals on high-fat diets (Rafter *et al.* 1987) and decreased in subjects on high-resistant-starch diets (van Munster *et al.* 1994).

6.3. Digestibility and bioavailability of foods

The methodology for studying non-digestibility of foods is an important area for understanding the effects of food and food components on intestinal microflora and physiology. A compilation of current methodology is given in Table 3. It is important to identify the nature of molecules, their chemical bonds and molecular size to understand digestibility. *In vitro* digestion studies and markers for absorption and excretion are of value. Animal models offer a means of simulating different digestion extremes and human volunteer studies enhance the understanding of *in vivo* digestibility of foods and bioavailability of nutrients.

6.4. Large-bowel function

Study of large-bowel function is extremely difficult, mainly because of its inaccessibility. However, the large bowel has unique aspects of metabolism; the principal events in the lumen are anaerobic and end-products such as H_2 and SCFA are not produced by other biochemical reactions in the body. These products are absorbed and appear in blood and breath, and can be used to study intraluminal events. Many investigators have used faeces and their composition as a guide to intracolonic events. Such studies have often been criticized because of the lack of representativeness of faeces, but for some aspects of colonic metabolism, such as the gut microflora of the lumen, they are probably acceptable. The study of the large intestine has spawned a large number of *in vitro* models, particularly of fermentation. A short summary of methods is given in Table 4.

Table 3. Methods to study the digestibility and bioavailability of foods

Method	Advantages	Disadvantages
1. Chemistry	Identifies nature of molecules, chemical bonds, molecular size, etc. (e.g. most β -glucans are not digested)	Requires dedicated carbohydrate chemistry laboratory
2. <i>In vitro</i> model	Studies with pancreatic and other enzymes	Care needed to mimic conditions in gut and hence rate and extent of digestion
3. Blood appearance	Glucose tolerance, chylomicrons. Applicable to a large number of samples	Requires human subjects and can be affected by factors other than those determining digestion
4. Breath	H ₂ or CH ₄ , ¹³ CO ₂ . Simple non-invasive for human studies	Difficult to quantify. Prolonged studies (16–24 h) needed for fermentation. Large subject variability
5. Ileostomy model	Probably the gold standard for study of digestion in stomach and small bowel	Requires access to patient population. May underestimate losses to caecum because of microbial colonization of ileum and fermentation in bag
6. Intestinal intubation: jejunum, ileum, colon, perfusion/aspiration, single or multilumen tubes	Valuable for dynamic studies of flow rate, site of digestion in small bowel, nutrient concentrations and physical form, and identification of intermediate products of digestion	Very invasive human studies. Need X-ray control. Presence of tube may alter normal physiology. Difficult to make quantitative
7. Faecal analysis	Relatively straightforward and gold standard for overall digestion and fermentation	Requires accurate faecal collections with balance markers and good methods validated for faeces
8. <i>In vitro</i> fermentation	Various batch and continuous culture methods available. Good for modelling physiology	Needs well-founded microbiology laboratory
9. Animal models: germ-free/conventional, fistulated	Gives greater access to tissues. Germ-free, human associated and conventional flora	Animal facilities needed. Extrapolation to man

Table 4. Methods for studying large-bowel function

Focus of study	Method
Bowel habit	Diary record
Motor function	Motility recording Recto-anal manometry and pelvic floor neurophysiology Transit time: whole gut – non-absorbable markers Partial transit time: small bowel – isotope-labelled meal and gamma scanning – lactose breath H ₂ large bowel – X-ray following marker ingestion
Faecal analysis	Microflora, and enzymic activities Fat, N, biomass, carbohydrate, DM, bile acids, sterols, pH Faecal water: osmolality, pH electrolytes bile acids genotoxicity/cytotoxicity
<i>In vitro</i> models	Occult blood Transport physiology Batch cultures, single chemostats, multi-chamber chemostats
Blood (including portal blood)	Short-chain fatty acids, branched-chain fatty acids, bile acids
Breath	H ₂ , CH ₄ , ¹³ CO ₂
Mucosal biopsies	General histology and immuno-staining
Exfoliated cells	DNA adducts Mutational analysis (e.g. Kras) Proliferation markers Genotyping Apoptosis
Mucus	Composition and structure Thickness Breakdown
X-ray	Barium studies Plain film of abdomen
Endoscopy	Proctoscopy Sigmoidoscopy (including flexible) Colonoscopy
Angiography	Vasculature
Radiotelemetry	pH Motility

6.5. Gut-associated lymphoid tissue

The presence of the digestive flora has a considerable influence on the immune system of the host (see section 3.5) and the principal methodology to study this is the use of germ-free animals, also termed axenic animals. By comparing germ-free and conventional animals, it is possible to highlight the role of the digestive flora in immune function. Moreover, the role played by bacteria isolated from the digestive flora, or used as probiotics, can be analysed by inoculating the gut of germ-free animals with these bacteria. These are called gnotobiotic animals. Recently, germ-free mice associated with human flora have been developed allowing *in vivo* studies of functional properties of probiotics and prebiotics used in human nutrition.

The advantages of gnotobiotic animal studies are to determine which kind of immune response a given bacteria established in the gut is able to exert, e.g. non-specific and/or specific immune response; inductive or suppressive immune response. Many methodologies are available. They are summarized in Table 5 and marked with (H) when they are applicable to human studies. The disadvantages of gnotobiotic models are the expensive animal facilities they need and the limits of the animal species studied. Moreover, it is not certain that a modulating bacterial effect observed in gnotobiotic conditions will be expressed in conventional conditions. Thus, other studies using conventional animals are needed. Current methods utilize *in vitro* cultures of systemic or intestinal lymphoid cells (De Simone *et al.* 1993), cellular assays with colonic cell lines (Schiffrin *et al.* 1995) and *in vivo* assays with conventional animal models (Perdigon *et al.* 1996). Two types of specific immune response can be assessed at the intestinal level. These include the suppression of humoral and cellular immune responses to chronically administered antigens at the systemic level (immune regulation) and the induction of a protective IgA antibody response at the mucosal level

Table 5. Selected methods to study intestinal immune function

Immune response	Methodology	Advantages	Disadvantages
Proliferative assays	<i>In vitro</i> : measurement of the proliferation of systemic or intestinal lymphoid cells after stimulation with mitogens or cell components	Easy for systemic assays: from blood samples (H)	Not easy for intestinal assays in human subjects: need biopsy Technical difficulties Need correlation with <i>in vivo</i> assays to give a biological significance
Cytokine production	<i>In vitro</i> : after proliferative assays	Development of new methodologies (H) Biomarkers (H)	Need correlation with <i>in vivo</i> assays to give a biological significance
Phagocytic activity	<i>In vitro</i> : peritoneal cells, circulating cells	Easy for systemic assays: from blood samples (H)	Correlation with specific immune response poorly understood Technical difficulties for intestinal phagocytes
Modulation of molecular expression in intestinal cell lines	<i>In vitro</i> : HT-29, CaCo-2 cell line cultures Facs analysis, histochemical methods	Cell lines originated from human intestine Specialized line cells	Adenocarcinoma lines Absence of correlation with intestinal cellular environment
IgA antibody response	Measured by ELISA or ELISPOT at several levels: (1) In serum: soluble IgA antibodies (2) In blood: circulating IgA-producing cells (3) In faeces (4) In saliva (5) Whole gut lavage fluid	Biomarkers Easy in human subjects (H) Reflects intestinal response (H) Easy, allows kinetic studies (H) Easy (H) Invasive, but avoids intestinal biopsy (H)	Does not reflect the intestinal response Does not reflect transepithelial transport into gut lumen Individual and daily variations, proteolytic activity, reflects only colonic response Individual and daily variations Needs hospitalization
Oral tolerance to dietary antigens	<i>Ex vivo</i> : proliferative assays of lymphoid blood cells with specific antigen Inflammatory cytokine production by lymphoid blood cells <i>In vivo</i> : intestinal permeability <i>In vitro</i> : Ussing chamber	Direct measure of the unresponsiveness state (H) Biomarkers: TNF- α (H) Increase in food hypersensitivities (H) Direct correlation with antigen transfer	Cause or consequence of oral tolerance breakdown? Not specific to immunological changes Invasive and difficult technology

H, applicable to human studies; IgA, immunoglobulin A; TNF- α , tumour necrosis factor- α ; Facs, fluorescence-activated cell-sorting; ELISPOT, enzyme-linked immunospot assay.

(immune exclusion). The definition of biomarkers is still incomplete. Several methodologies have been developed for IgA response measurements. Sampling needs blood collection or intestinal biopsies or stool and saliva collection. Problems in faeces and saliva handling and daily variations in expression of IgA activities have not been solved. In the case of oral tolerance, the biomarkers available in human subjects are defined as the absence of proliferation of cultured blood cells with antigen or down-regulation of inflammatory cytokines involved with food allergies (Benlounes *et al.* 1996; Sütas *et al.* 1996a, b). Other methodologies must be developed according to further knowledge about oral tolerance mechanisms (Weiner *et al.* 1994).

6.6. Epithelial cell proliferation and colon carcinogenesis

6.6.1. Biological markers for colorectal carcinogenesis.

A relationship between colorectal carcinogenesis and abnormal cell proliferation has been demonstrated (Lipkin, 1988) in studies of the mucosa in patient groups at high risk of cancer, e.g. ulcerative colitis and familial adenomatous polyposis, and in animals exposed to carcinogens that target the colon. Two changes in colonic cell proliferation have

been described (Risio, 1992): an increase in the total number of proliferating cells, or hyperproliferation, which is not a specific marker of cancer risk, and a progressive shift of proliferating cells to the crypt surface (stage II abnormality) which is more specific for tumour risk.

6.6.2. Cell proliferation. Several techniques have been developed to measure cell proliferation in colonic mucosa and assess the influence of diet. Measurement of crypt-cell production rate in microdissected crypts is considered to provide the best assessment of proliferation with the fewest artifacts. However, since it requires *in vivo* treatment with vincristine, it is not suitable for human studies.

Change in the rate of cell proliferation in the normal mucosa may be less reliable as a biomarker for diet-related cancer risk (Wasan & Goodlad, 1996) and is just one of the processes contributing to colonic mucosal crypt architecture. Other events are differentiation, exfoliation and apoptosis. It is likely that the best predictor of cancer risk is an overall assessment of these events.

Markers of early epithelial events have been used in animal models. These include DNA damage, microadenomas and aberrant crypt foci in the mucosa. The microgel electrophoresis (Comet) assay has been used for assessing

DNA damage in the colonic mucosa. Induction of aberrant crypt foci has been particularly widely used, as it is easy to observe macroscopically. However, its reliability as a marker of colorectal tumour risk is a matter of debate. In man, aberrant crypts and microadenomas, similar to those described in animals, have been described (Roncucci, 1992) but their correlation with other well-known markers of risk has not been established. Other markers of cell proliferation include PCNA and Mib 1; both are proteins appearing at specific stages in the cell cycle.

6.6.3. Differentiation. Methods exist to measure the state of differentiation of the mucosal epithelial cells by histochemical staining of mucins using binding to specific lectins. The methods can be applied to tissue sections from human and animal biopsies after fixation. Such approaches have been used to study colonic epithelium in rats and human subjects at various stages of neoplasia. They have not achieved widespread use for investigating dietary modification of neoplastic processes.

6.6.4. Apoptosis. Identification of oligonucleotide fragments by *in situ* end labelling using immunoperoxidase techniques forms the basis for various methods that can be applied to sections of colonic tissue from human subjects and animals and to cell suspensions (Ansari *et al.* 1993). Apoptosis in cell cultures from transformed colon tissues has been determined by measuring cell loss from monolayers (Hague *et al.* 1993).

6.6.5. Products used in experimental carcinogenesis (Martin *et al.* 1981). Spontaneous colorectal cancer is exceptional in animal models, but tumours can be created easily in rats, mice or hamsters using chemicals such as *N*-methylnitrosourea and *N*-methyl-*N'*-nitro-*N*-nitrosoguanidine (Table 6). These products are direct carcinogens, which explains their local efficacy and their specificity when administered intra-rectally. Others such as 1,2-dimethylhydrazine or azoxymethane must be first metabolized by the liver, then at the level of the target cell, in order to be carcinogenic.

1,2-Dimethylhydrazine and derived azo and azoxy alkanes represent the carcinogens most commonly used to induce intestinal carcinomas in rats, mice and hamsters, by the oral or subcutaneous route. In rats, precancerous lesions such as adenomas are exceptional, cancers arising most commonly without any precursor adenoma, which is the opposite to what is observed in man. In mice, it is easier to induce adenomas than carcinomas (Maskens, 1976).

Secondary bile acids are cocarcinogens. They have been shown to promote colorectal carcinogenesis in animal models by increasing tumour formation rate induced by carcinogens, or increasing colonic cell hyperproliferation through the production of diacylglycerol and stimulation of protein kinase.

6.6.6. Types of lesion (Weisburger, 1973). The most studied lesion is adenocarcinoma induced either directly or, more often, indirectly. When azoxymethane is used by the subcutaneous or intramuscular route, intestinal carcinomas are formed in 100% of cases. Small doses induce tumours in the proximal colon and the caecum, whereas larger doses produce tumours mainly in the distal colon. Such tumours arise on flat mucosa and form plaques, thus mimicking human infiltrating tumours, which arise without any detectable adenomatous tissue and are named *de novo* cancers. However, this type of tumour is rare in human carcinogenesis, particularly in Western countries where tumours are mainly of the fungating type and arise in a pre-existing adenoma. Aberrant foci are interesting to study as they can be easily observed macroscopically. After a single injection of azoxymethane, aberrant foci have been described after early slaughter of the animals. These lesions are considered by some authors to be an early marker of tumour risk. They are more common in the rectum (90% of animals) than the caecum (10% of animals), but tend to migrate with time, with a decrease in rectal lesions and an increase in caecal lesions after 4 weeks.

Changes in colonic cell proliferation, which will be described in man elsewhere, are largely used to test the protective effect of products, e.g. Ca, against secondary bile acid-induced proliferation.

6.6.7. Transgenic mouse models for colon cancer studies. A number of inbred mouse models carrying germ-line mutations at the *Apc* gene (the murine homologue of *APC*, which is mutated in patients with familial adenomatous polyposis) have been developed. These animals exhibit spontaneous tumours throughout the intestinal tract, usually in the first few months of life. They are of use in studies of the interaction of diet and colon cancer and provide a model that dispenses with the need for chemical induction of carcinogenesis. The susceptibility of tumour incidence in these mice to dietary modulation is under investigation in a number of laboratories. Table 7 lists four such mouse models in current use.

Table 6. Current methods for studying colon carcinogenesis in animals

Products	Route	Lesions
MNNG (<i>N</i> -methyl- <i>N'</i> -nitro- <i>N</i> -nitrosoguanidine) and MNU (<i>N</i> -methylnitrosourea)	Intra-rectal instillations	Colon adenocarcinomas, squamous cell anal carcinomas, spleen and liver haemangiomas
DMH (1,2-dimethylhydrazine)	Oral or subcutaneous	Multiple colorectal adenocarcinomas and a few adenomas in rats
Azo and azoxy alkane derivatives	Subcutaneous or intramuscular	In mice adenomas, and a few carcinomas; adenocarcinomas in plaques (<i>de novo</i>)
Secondary bile acids	Intra-rectal or oral	Alone: colonic cell hyperproliferation After DMH: increased number of carcinomas

Table 7. Transgenic mouse models currently in use in colon cancer studies

Model	Mutation	Tumour yield per animal
Min (multiple intestinal neoplasia), usually used in the heterozygous cross with C57B16/J mice	Germline nonsense mutation at codon 850 of <i>Apc</i>	About 100 adenomas with the majority in the small intestine
Apc1638N	<i>Apc</i> disrupted at codon 1638, probably leading to a null allele for <i>Apc</i>	About five intestinal tumours
Apc 1638T	<i>Apc</i> disrupted at codon 1638 leading to a truncated polypeptide	No intestinal tumours at 5 months
Apc Δ716	<i>Apc</i> disrupted at codon 716, leading to a truncated protein	200–500 intestinal adenomas

6.6.8. *Limits of experimental models (Maskens & Dujardin Loits, 1981).* Experimental carcinogenesis in animals creates mainly infiltrating *de novo* carcinomas, and is an indirect process in most cases, i.e. multiple metabolic transformations are needed, the last occurring in the colon itself and involving bacterial enzymes such as β -glucuronidase, azoreductase or nitroreductase. In man, in particular in high-risk countries such as Western Europe, North America or Australia, most colorectal tumours are polypoid, fungating, and arise on a pre-existing adenoma. It has been estimated that, in the distal colon and rectum, where most tumours arise, over 80% of cancers arise through the adenoma–carcinoma pathway. Indirect evidence, such as the type of mutation of the p53 protein, points to a major role played by secondary bile acids, i.e. endogenous carcinogenesis, whereas carcinogenesis in animals is called exogenous carcinogenesis. The role of the latter may be more important in low-risk countries such as Japan, but is likely to be of little importance in high-risk countries. Therefore, it is difficult to extrapolate from animal models to man, in particular regarding the importance of bacterial enzymes, apart from the major role played by the 7 α -dehydroxylase, which converts primary into secondary bile acids.

7. Human studies on the effects of food and food components

Among the components likely to be used in functional foods, prebiotics and probiotics are already used as food ingredients.

Probiotics have been variously defined (Fuller, 1991, 1992; Havenaar & Huis in't Veld, 1992) largely on the basis of their initial use in animal feeds. For the purposes of human nutrition we suggest that a probiotic is best defined as 'a live microbial food ingredient that is beneficial to health'.

A prebiotic is a 'nondigestible food ingredient that beneficially affects the host by selectively stimulating the growth and/or activity of one or a limited number of bacteria in the colon, that have the potential to improve host health' (Gibson & Roberfroid, 1995).

Another approach is the use of synbiotics. A synbiotic has been defined as 'a mixture of probiotics and prebiotics that beneficially affects the host by improving the survival and implantation of live microbial dietary supplements in the gastrointestinal tract, by selectively stimulating the growth and/or activating the metabolism of one or a limited number of health-promoting bacteria, and thus improving host welfare' (Gibson & Roberfroid, 1995).

7.1. Prebiotics (Gibson & Roberfroid, 1995)

The key criterion for a food ingredient to be classified as a prebiotic is selective stimulation in human subjects of the growth of potentially beneficial bacteria in the gut. A prebiotic may repress pathogen growth or virulence and induce systemic effects that can be beneficial to health.

The prebiotics identified today and which have served to introduce the concept (Gibson & Roberfroid, 1995) are non-digestible oligosaccharides that are fermented in the colon. They are obtained either by hot-water extraction from plants, eventually followed by enzymic hydrolysis of the extracted molecules, or synthesis from a disaccharide using -osyl transferases. The prebiotics that are available in Europe at present or that are being developed, belong to two groups, characterized by the major -osyl monomer they are composed of; namely, the fructosyl prebiotics (fructo-oligosaccharides) and the galactosyl prebiotics (galacto-oligosaccharides). Daily intake of fructosyl prebiotics for a few weeks leads to a selective stimulation of the growth of bifidobacteria. Many studies using different dose regimens and different methods of microbial analysis have shown selective stimulation of bifidobacteria (Roberfroid *et al.* 1997). Ito *et al.* (1993) have reported the same effect for galactosyl prebiotics. In addition, the experiments reported by Gibson *et al.* (1995) demonstrate that the growth of bifidobacteria is accompanied by a reduction in the number of other populations, e.g. bacteroides, clostridia and fusobacteria, thus leading to a major modification in the composition of the colonic microflora. Lactitol (4-*O*- β -D galactopyranosyl-D-glucitol) has been shown to have prebiotic properties (Ballongue *et al.* 1997).

The nutritional properties of prebiotics have been summarized in the report of an International Life Sciences Institute (ILSI) Europe workshop entitled *Colonic Microflora: Nutrition and Health* (Roberfroid *et al.* 1995) and in the proceedings of the 1st International Conference on East–West Perspectives in Functional Foods organized by ILSI (Roberfroid, 1996). In a recent monograph, Cummings (1997) also reviewed the nutritional properties of prebiotics.

The health-promoting consequences of prebiotic fermentation include increased faecal biomass and consequently stool weight and/or stool frequency (Roberfroid *et al.* 1993; Gibson *et al.* 1995). Colonic fermentation of prebiotics produces SCFA mainly acetate, propionate and butyrate (Roland *et al.* 1995). Through fermentation and absorption of SCFA prebiotics have part of their energy salvaged. Their

digestible energy is of the order of 4–9 kJ/g (1–2 kcal/g) (Roberfroid *et al.* 1993; Molis *et al.* 1996). This property has led to their use as bulking ingredients, sugar substitutes and, for inulin, as a fat replacer (Coussement, 1996).

7.2. Probiotics (Salminen *et al.* 1996a,b,c)

The bacterial genera most often used as probiotics are lactobacilli and bifidobacteria. They can be given with fermented foods such as yoghurt, fermented vegetables or meats and they may briefly establish in the gut. A number of health-related effects are documented. Such established effects are listed in Table 8 and each effect has been shown in at least two human clinical studies by different research groups.

Colonic fermentation has been shown to be altered following probiotic intake either as fermented milks or freeze-dried cultures. Japanese studies have shown that oral administration of certain lactic-acid bacteria increases the numbers of endogenous lactobacilli in faeces. Similarly, an increase in faecal bifidobacteria has been observed, and the numbers of clostridia have decreased (Hosoda *et al.* 1994; Benno *et al.* 1996). Recent studies by Alander *et al.* (1996) have indicated that the potential to colonize human colonic mucosa differs from that of faecal samples. Thus, more human studies are clearly needed to understand changes in both colonic microflora and colonic mucosal microflora during probiotic intake.

7.2.1. Alleviation of lactose intolerance symptoms. The majority of the world's population have low levels of β -galactosidase in their small-bowel mucosa. Most lactase-deficient people are symptom-free if they consume only limited amounts of milk, except those subjects who are severely lactose intolerant. A beneficial effect of probiotics on lactose digestion has been demonstrated (Sanders, 1993). In particular, in studies comparing yoghurt and milk consumption it was shown that yoghurt consumption enhances lactose digestion in lactase-deficient subjects (Kolars *et al.* 1984; Marteau *et al.* 1990) and slows oro-caecal transit.

7.2.2. Immune enhancement. An enhancement of the circulating IgA antibody secreting cell response was observed in infants supplemented with a strain of *Lactobacillus casei*, and was correlated with shortened duration of diarrhoea in the study group when compared with a placebo group (Kaila *et al.* 1992). Other studies reported an enhancement in the non-specific immune phagocytic activity of granulocyte populations in the blood of human volunteers after consumption of *Lactobacillus acidophilus* and *Bifidobacterium bifidum* (Schriffin *et al.* 1995; Marteau

et al. 1997a,b). Since phagocytic activity is involved with natural immunity and phagocytes are implicated in antibody immune responses as antigen-presenting cells, it is possible that stimulation of intestinal IgA antibody responses induced by lactic-acid bacteria may be explained partly by an effect on phagocyte cell functions. Ingestion of yoghurt has been reported to stimulate cytokine production, including interferon- γ in human blood mononuclear cells (Solis-Pereyra & Lemonnier, 1996).

7.2.3. Acute gastroenteritis. Several studies, by different groups and in different conditions, have shown that some probiotic lactobacilli significantly shorten the duration of rotavirus diarrhoea in developed (Guarino *et al.* 1977) and developing countries (Raza *et al.* 1995; Pant *et al.* 1996). For one strain, a similar clinical effect on the duration of rotavirus diarrhoea without an increase in immune response has been reported (Shornikova *et al.* 1997). *Bifidobacterium bifidum* (now reclassified as *Bifidobacterium animalis*) has been reported to prevent rotavirus diarrhoea (Saavedra *et al.* 1994).

7.2.4. Faecal mutagenicity and enzymes. Lactic acid bacteria influence the mutagenicity of intestinal contents and the levels of faecal microbial enzymes, such as β -glucuronidase, β -glucosidase, nitroreductase and urease (EC 3.5.1.5). *Lactobacillus acidophilus* has been shown to decrease faecal and urinary mutagenicity in healthy volunteers consuming fried minced beef. The same strain decreased faecal *E. coli* levels in colon-cancer patients and reduced faecal β -glucuronidase levels. Similar results have been reported for many probiotic lactobacilli strains (Goldin & Gorbach, 1984; Goldin *et al.* 1992; Ling *et al.* 1994; Morotomi, 1996).

The prophylactic effects of oral administration of a lactobacillus strain on the recurrence of superficial bladder cancer have been reported in two Japanese studies (Aso & Akazan, 1992; Aso *et al.* 1995).

7.3. Diet and colon cancer

The principal risk factors for sporadic colorectal cancer are high intakes of animal fat and meat, particularly red meat, excess energy intake, high intakes of refined cereals and of alcohol, particularly beer for colorectal carcinogenesis, and lack of physical exercise. A number of dietary factors have been proposed as potentially protective (Potter *et al.* 1993).

7.3.1. Dietary protective factors. A high intake of vegetables and fruit is associated with a decreased risk of colorectal cancer and adenomas throughout the world (Trock *et al.* 1990). Although vegetables such as the cruciferae may have a specific protective affect via antioxidant substances such as indoles, all fresh vegetables seem to be protective, in particular against cancer of the distal bowel. Components of fruit and vegetables that have been implicated as protective against colorectal cancer include NSP (dietary fibre), micronutrients (vitamins C, D and E, β -carotene, Ca and Se), glucosinolates, phenols, lignans, flavonoids and isoflavonoids.

One of the most important protective components of vegetables and unrefined cereals is dietary fibre. The mechanism may be through a diluting effect of carcinogens, a reduction in transit time, fermentation and the production

Table 8. Health-related effects of currently available probiotics (for detailed references, see Marteau & Rambaud, 1993; Lee & Salminen, 1995; Salminen *et al.* 1996a, b, c)

Alleviation of symptoms of lactose intolerance
Immune enhancement
Shortening the duration of rotavirus diarrhoea
Decreasing faecal mutagenicity
Decreasing faecal bacterial enzyme activity
Prevention of recurrence of superficial bladder cancer

of SCFA and alterations in N and bile acid metabolism. Although results of individual case-control studies have been disappointing, a meta-analysis of thirteen of these studies (Howe *et al.* 1992) found a significant inverse association between fibre intake and risk of colorectal cancer with a relative risk close to 0.5 for consumers of over 27 g/d. When extrapolating these data to the American population, the authors estimated it to be possible to reduce the risk of colorectal cancer by 31% i.e. 50 000 cases per year by increasing the daily consumption by 13 g. However, the multiplicity of methods for assessing dietary fibre intake in these studies makes any quantitative recommendations difficult.

Ca and vitamin D are other potentially protective factors that could easily be used as preventive agents. A recent American cohort study summarized the relations between Ca, vitamin D and dairy products (Bostick *et al.* 1993). Of thirteen studies (nine case-control and four intervention studies), eight suggested an inverse relationship, which was statistically significant in five. Most studies that observed a protective effect of Ca came from Nordic or Anglo-Saxon communities, whereas no study demonstrated a protective effect of Ca or dairy products in Latin communities. It can, therefore, be suggested that the way Ca is consumed, and perhaps the general level of Ca consumption, is of importance. It has been suggested that fermented dairy products could be protective. In a French study (Boutron *et al.* 1996), a reduced risk of large adenomas was associated with a moderate intake of yoghurt.

Other dietary protective factors which have been investigated in intervention studies of recurrence of adenomas are the so-called antioxidant vitamins. The results of these studies have so far been disappointing (Greenberg *et al.* 1994; MacLennan *et al.* 1995).

8. Safety issues

(Donohue & Salminen, 1996; Roberfroid *et al.* 1997)

Safety issues with new prebiotics and probiotics, including genetically modified bacteria, should be assessed according to European Union Novel Food Regulations on a case-by-case basis. Safety aspects of currently used prebiotics and probiotics are discussed in relation to their physiological properties.

8.1. Prebiotics (Van Loo *et al.* 1995)

Although the European Union regulation on novel foods and novel food ingredients leaves room for interpretation, it is very likely that most prebiotics placed into the European market after this regulation comes into force will fall within its scope. They will, therefore, be subject to safety and nutritional evaluation on a case-by-case basis.

Fructosyl-type ingredients, for which prebiotic properties are claimed, are already in the market. Fructosyl-type ingredients are natural components of a variety of fruits, vegetables and cereals, and consequently are consumed regularly (a few g/d) as part of the current diet (Van Loo *et al.* 1995). They are classified as natural food ingredients and cleared as novel food ingredients. Similarly galactosyl prebiotics have been cleared as novel food ingredients by

the Health Department of The Netherlands (Staatscourant, 1996).

8.2. Probiotics

(Adams & Marteau, 1995; Donohue & Salminen, 1996)

The use of lactic acid-producing bacteria in foods has a long history and most strains are considered commensal micro-organisms with little or no pathogenic potential. Their ubiquitous presence in intestinal epithelium and the human gastrointestinal tract, and their traditional use in fermented foods and dairy products attest to their safety. Members of the genus *Lactobacillus* are most commonly given safe or generally recognized as safe (GRAS) status, whereas members of the genera *Streptococcus* and *Enterococcus* contain many opportunistic pathogens.

Case reports from the literature of lactic acid-producing bacteria causing clinical infection in human subjects have recently been analysed in reviews by Gasser (1994) and by Aguirre & Collins (1993). Both reviews conclude that, considering their widespread consumption, lactic-acid bacteria appear to have a very low pathogenic potential. Two recent Finnish studies confirm that the number of infections associated with lactic-acid bacteria is extremely small and no case could be linked to commercial probiotics (Saxelin *et al.* 1996a,b).

9. Critical evaluation of present knowledge

Each of the foregoing sections has examined in depth one aspect of the science of gastrointestinal physiology and function in relation to probiotics and prebiotics. The following conclusions are derived from the discussions within each section.

9.1. Intestinal microflora

The intestinal microflora has been studied using traditional methods. A current problem is the presence of non-culturable species, which require new methodologies to be developed for their detection and measurement.

The establishment of the normal human intestinal microflora, its components and metabolic activities, requires further study. Similarly, knowledge is needed on the composition and activities of the flora in different ethnic groups, at different ages and in different countries.

Prebiotics and a few probiotics have already been shown to have the potential to modify significantly the composition of the intestinal microflora. In particular, the stimulatory effect of fructosyl prebiotics on the growth of bifidobacteria is well established. Moreover, prebiotics and probiotics are interesting tools with which to study the physiological consequences of changes in metabolic activities following such modification.

9.2. Mucosal function

There is evidence for a strong interaction between the intestinal microflora, gut mucosa and GALT and the functions and dysfunctions of the gastrointestinal tract. It has been shown that probiotics may change the gut mucosal

barrier by stabilizing the intestinal mucosa, normalizing intestinal permeability and improving gut immunology. However, there are differences in the function and activity of different probiotics. Another consequence of intake of prebiotics and probiotics is the prevention of overgrowth of pathogenic bacteria and viruses. Taken together, these modifications have been shown to influence the gut barrier system.

Future human studies should consider intestinal immunity and its modulation by resident probiotic bacteria or prebiotic components in more detail. Local release of cytokines induced by inflammatory reactions may amplify adverse reactions to food components within the intestinal tract and other parts of the body.

9.3. Gastrointestinal physiology

Gut bacteria play a role in bowel functions like faecal mass, stool frequency, regulation of colonic pH, production of SCFA and salvage of energy from non-digestible food components. Any modification of the microflora is likely to influence these functions, but most of the results of human studies with pro- and prebiotics are preliminary and yet to be confirmed. In particular, the interaction of probiotics and prebiotics with the endocrine activity of the gut requires investigation.

9.4. Methodology

There is clearly a need for new methodology to measure and characterize the composition of faecal microflora, in particular in large human nutrition studies.

New biomarkers specifically involved with immune responses need to be defined and validated for nutritional studies. Germ-free animals (gnotobiotic mice) offer a model to clarify the specific effect of a bacterial strain or a food component on a given immune response.

Development of early markers of carcinogenesis is urgently required for human intervention studies.

9.5. Human studies on health benefits

9.5.1. Prebiotics (Table 9). There are currently few reports of well-designed and well-documented human

Table 9. Established and postulated functional effects of currently available prebiotics in human subjects

Established functional effects	Postulated areas for future research
Non-digestibility and low energy value (<9 kJ/g)	Prevention of intestinal disorders (IBS, ulcerative colitis) and infections, including diarrhoea
Stool bulking effect	Modulation of immune response
Modulation of the gut flora, promoting bifidobacteria and repressing clostridia	Prevention of colon carcinogenesis
	Reduction in serum levels of triacylglycerols and cholesterol
	Improved bioavailability of minerals (Ca, Mg)

IBS, irritable bowel syndrome.

Table 10. Postulated clinical effects of probiotics

Areas for future research	Regulation of intestinal motility. Modulation of intestinal and systemic immune responses. Reduction and protection of radiotherapy-associated intestinal dysfunction. Prevention of intestinal cancers
Research on mechanisms	Intestinal microflora effects, immunomodulation effects, competitive exclusion, cholesterol lowering

intervention studies with prebiotics. Future studies may indicate differences between the effects of prebiotic components. Thus, it is important that each compound is tested separately or in products designed for a particular function.

9.5.2. Probiotics (Table 10). Alleviation of lactose maldigestion symptoms is well characterized for some probiotics. The ability of certain probiotic strains to shorten the duration of rotavirus diarrhoea has been established in several studies. Immunomodulation has been demonstrated for some probiotic strains and one strain has been documented to reduce the recurrence of superficial bladder cancer in human subjects. Novel methodologies are required to provide further data on the mechanisms of competitive exclusion and microflora modification.

For other areas of interest there are still few well-designed and well-conducted human intervention studies with probiotics. There are indications of significant differences between probiotic bacterial genera and species, and between strains of the same species. Thus, it is important that each strain is tested on its own or in products designed for a particular function. Further research is needed on the immune response, intestinal cancer and the mechanisms that underlie probiotic effects.

9.5.3. Diet and colon cancer. The major challenge at present is to demonstrate within intervention studies which specific dietary factors are able to decrease the risk of colon cancer. Recurrence of large adenomas, the step prior to cancer, is a promising tool for use in human studies and could be applied to prebiotics, probiotics and synbiotics using functional food products as test products. Colon cancer-related variables need to be carefully characterized in the diet and among the microflora.

9.6. Safety

The human consumption of prebiotics and probiotics, at least lactic-acid bacteria, appears to be safe.

10. Recommendations for future research priorities

Evidence has accumulated to support an important role for gastrointestinal function and the intestinal microflora in maintaining health and preventing diseases. Disturbances of the intestinal microflora may lead to other disturbances and dysfunctions of the gut. Thus, understanding the normal microflora with regard to its metabolic activity and

influence on the immune and endocrine systems remains a key area for future research.

10.1. Intestinal microflora

- (1) Develop and validate robust methods that are applicable to large-scale human studies of the intestinal microflora.
- (2) Characterize the normal microflora and its activities in healthy persons of all ages.
- (3) Identify changes in microflora composition and activity associated with major dysfunctions of the gut.
- (4) Identify dietary factors that lead to changes in the microflora and the mechanisms that bring about improvement in health.

10.2. Short-chain fatty acids and intestinal microflora

- (1) The mechanism by which mixed populations of anaerobic gut bacteria produce different amounts and patterns of SCFA needs to be further investigated.
- (2) The role of butyrate in cell differentiation and growth requires further study, particularly of the butyrate response elements in genes and identification of the genes involved.
- (3) The role of acetate in metabolism and its regulation, particularly in fasting or starving subjects, requires further study.
- (4) *In vivo* production rates of SCFA and their relation to H₂ metabolism and microbial growth need to be determined.

10.3. Diet and cancer

- (1) Determine the role of the intestinal microflora with respect to composition and activities in carcinogenesis, in particular of the colo-rectum.
- (2) Modulation of these aspects by probiotics and prebiotics requires further study.
- (3) Novel biomarkers of colorectal carcinogenesis need to be developed and validated.
- (4) The influence of diet and the intestinal microflora on DNA damage and repair in normal mucosa requires further study.

10.4. Immune system

- (1) Improve understanding of GALT function and regulation, and interaction between GALT and the digestive epithelium.
- (2) Understand the role of the intestinal microflora and its modification by dietary factors in regulating immune function in health and disease.
- (3) Develop and validate biomarkers for immune function and their long-term effects.

10.5. Gut mucosa

- (1) Develop novel methodologies to study the function and changes in the intestinal mucosa in human subjects.
- (2) Characterize the microflora associated with a healthy mucosa and mucus metabolism.

- (3) Determine the effects of changes in phase I and phase II mucosal enzymes on xenobiotic metabolism and health.

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