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Prebiotic Effects: Metabolic and Health Benefits

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Table of contents

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Abbreviations : AAD, antibiotic-associated diarrhea, ACF, aberrant crypt foci, BMC, bone mineral content, BMD, bone mineral density, CD, Crohn's disease, CFU, colony forming unit, DGGE, Denaturing Gradient Gel Electrophoresis, DP, degree of polymerisation, FISH, fluorescence in situ hybridization, GALT, gut-associated lymphoid tissue, GI, gastro-intestinal, GLP, glucagon-like peptide, GOS, galacto-oligosaccharides, GSH, glutathione transferase, IBS, Irritable Bowel Syndrome, IBD, Inflammatory bowel disease, ITF, inulin-type fructans, ITT, Intention To Treat, LAB, lactic Acid Bacteria, LPS, lipolysaccharide, NK, Natural Killer, NNT, number needed to treat, OTUs, operational taxonomic units, PBMC, Peripheral Blood Mononuclear Cell, PCR, polymerase chain reaction, PP, per protocol, RCT, randomized controlled trials, SCFA, short chain fatty acids, TER, Trans-Epithelial Resistance, TGGE, Temperature Gradient Gel Electrophoresis, TLR, Toll-Like Receptor, UC, Ulcerative Colitis

Running Title: Prebiotic concept and health

Keywords: Prebiotic, Gut microbiota, Infant nutrition, Immune functions, Irritable bowel syndrome, Inflammatory bowel disease, Metabolic syndrome, Mineral absorption, Metabolic endotoxemia, Osteoporosis, Colonization resistance.

Abstract:

The different compartments of the gastrointestinal tract are inhabited by populations of microorganisms. By far the most important predominant populations are in the colon where a true symbiosis with the host exists that is key for well-being and health. For such a microbiota, 'normobiosis' characterizes a composition of the gut "ecosystem" in which microorganisms with potential health benefits predominate in number over potentially harmful ones, in contrast to 'dysbiosis', in which one or a few potentially harmful microorganisms are dominant, thus creating a disease-prone situation.

The present document has been written by a group of both academic and industry experts (in the ILSI Europe Prebiotic Expert Group and Prebiotic Task force respectively). It does not aim to propose a new definition of a prebiotic nor to identify which food products are classified as prebiotic but rather to validate and expand the original idea of the prebiotic concept (that can be translated in 'prebiotic effects'), defined as:

"The selective stimulation of growth and/or activity(ies) of one or a limited number of microbial genus(era)/species in the gut microbiota that confer(s) health benefits to the host".

Thanks to the methodological and fundamental research) of microbiologists, immense progress has very recently been made in our understanding of the gut microbiota. A large number of human intervention studies have been performed that have demonstrated that dietary consumption of certain food products can result in statistically significant changes in the composition of the gut microbiota in line with the prebiotic concept. Thus the prebiotic effect is now a well established scientific fact. The more data are accumulating, the more it will be recognized that such changes in the microbiota's composition, especially increase in bifidobacteria, can be regarded as a marker of intestinal health.

The review is divided in chapters that cover the major areas of nutrition research where a prebiotic effect has tentatively been investigated for potential health benefits.

The prebiotic effect has been shown to associate with modulation of biomarkers and activity(ies) of the immune system. Confirming the studies in adults, it has been demonstrated that, in infant nutrition, the prebiotic effect includes a significant change of gut microbiota composition, especially an increase of faecal concentrations of bifidobacteria. This concomitantly, improves stool quality (pH, short chain fatty acids, frequency and consistency), reduces the risk of gastroenteritis and infections, improves general well-being, and reduces the incidence of allergic symptoms such as atopic eczema.

Changes in the gut microbiota composition are classically considered as one of the many factors involved in the pathogenesis of either inflammatory bowel disease or irritable bowel syndrome. The use of particular food products with a prebiotic effect has thus been tested in clinical trials with the

objective to improve the clinical activity and well-being of patients with such disorders. Promising beneficial effects have been demonstrated in some preliminary studies, including changes in gut microbiota composition (especially increase in bifidobacteria concentration). Often associated with toxic load and/or miscellaneous risk factors, colon cancer is another pathology for which a possible role of gut microbiota composition has been hypothesized. Numerous experimental studies have reported reduction in incidence of tumors and cancers after feeding specific food products with a prebiotic effect. Some of these studies (including one human trial) have also reported that, in such conditions, gut microbiota composition was modified (especially due to increased concentration of bifidobacteria). Dietary intake of particular food products with a prebiotic effect has been shown, especially in adolescents, but also tentatively in postmenopausal women, to increase calcium absorption as well as bone calcium accretion and bone mineral density. Recent data, both from experimental models and human studies, support the beneficial effects of particular food products with prebiotic properties on energy homeostasis, satiety regulation and body weight gain. Together with data in obese animals and patients, these studies support the hypothesis that gut microbiota composition (especially the number of bifidobacteria) may contribute to modulate metabolic processes associated with syndrome X, especially obesity and diabetes type II. It is plausible, even though not exclusive, that these effects are linked to the microbiota-induced changes and it is feasible to conclude that their mechanisms fit into the prebiotic effect. However, the role of such changes in these health benefits remains to be definitively proven.

As a result of the research activity that followed the publication of the prebiotic concept 15 years ago, it has become clear that products that cause a selective modification in the gut microbiota's composition and/or activity(ies) and thus strengthens normobiosis, could either induce beneficial physiological effects in the colon and also in extra-intestinal compartments and/or contribute towards reducing the risk of dysbiosis and associated intestinal and systemic pathologies.

Introduction[1]

In the 1980s, Japanese researchers ([1; 2]) had already demonstrated that specific non-digestible oligosaccharides (especially fructo-oligosaccharides) were selectively fermented by bifidobacteria and had the capacity, upon feeding, stimulating their growth in human faeces. These observartions were confirmed and further expanded by Gibson & Roberfroid who introduced the concept of prebiotics in 1995 ([3]) and have recently published a review of the research which includes the most recent development ([4]) (Table 1). During the last fifteen years, this concept has attracted the interest of many academic as well as industrial scientists and it has become a popular research topic in nutrition and, more recently, in the biomedical fields.

Early research in the mid 1990s on prebiotics has contributed towards the development and validation of new molecular biology-based methods resulting in of easy-to-handle, sensitive, and highly specific methods to identify and quantify the large variety of microorganisms composing the gut microbiota ([5-16]). The application of such methods has improved our knowledge of the gut microbiota composition in terms of variety, classification, identity and relative concentrations of genera or species of microorganisms, as well as in terms of their properties and interactions/cooperations with each other and with intestinal epithelial cells. This has led the International Scientific Association for Probiotics and Prebiotics (ISAPP) (6th meeting in Ontario, USA, November 2008) to propose the concept of 'normobiosis' to characterize a normal gut microbiota in which genera/species of microorganisms with potential health benefits predominate in number over potentially harmful ones as opposed to 'dysbiosis' which characterizes a gut microbiota in which one or a few potentially harmful genus(era)/species of microorganisms are dominant, thus creating a disease-prone situation.

A large part of the research activity has concentrated, and still does focus on the *in vitro* and *in vivo* ability of selective modification in the composition of the complex gut microbiota, in particular research has focused on the selective stimulation of growth of mainly bifidobacteria, but also lactobacilli. In the future, it is likely this may be expanded towards other genera eg *Eubacterium, Faecalibacterium* and *Roseburia*. It has become clear that products, causing such a selective modification in gut microbiota's composition and/or activity(ies), could, in addition, either induce beneficial physiological effects not only in the colon but also within the whole body and/or contribute towards reducing the risk of miscellaneous intestinal and systemic pathologies. These effects are summarised in Table 2 and

[1] The main author of this section is Prof. Marcel B. Roberfroid.

have been discussed, on a regular basis, at international conferences ([17-19]) and were, more recently, reviewed in a handbook ([20]). They are also topics for the present document.

The intensiver research of the past 15 years has contributed towards an improved understanding of the complexity of the gut microbiota. This includes the discovery of new phyla/genera, their relative concentration in the gut microbiota, the key role of diet in modulating its composition, the changes associated with ageing or chronic diseases and the individual character of gut microbiota composition. In addition, past research has given us insights into its roles in human physiology and miscellaneous pathophysiological conditions. The gut microbiota is thus now perceived as a key player in health and well-being with, as a principal condition, a composition in which potentially health promoting dominant microorganisms (especially the saccharolytic genera/species e.g. bifidobacteria) are elevated and/or more active than the potentially harmful ones (especially the proteolytic/putrefactive genera/species) ([3; 21]) a situation known as 'normobiotic' or 'eubiotic'. It is now well recognized that, within such a potentially health beneficial dominant microbiota, the genus *Bifidobacterium*, plays an important role although future research may show different genera/species to also be important. Indeed, it has been hypothesized that increasing bifidobacteria in gut microbiota, might improve health status and reduce disease risk.

As a result of discussions with both academic and industry experts (in the ILSI Europe Prebiotic Expert Group and Prebiotic Task force respectively), the present document does not aim at proposing a new definition of a prebiotic nor at identifying which food components/ingredients/supplements classify as prebiotic but rather to validate and expand the original idea of the prebiotic concept, as:

"**The selective stimulation of growth and/or activity(ies) of one or a limited number of microbial genus(era)/species in the gut microbiota that confer(s) health benefits to the host**",

with

"**selectivity**" being the key condition that needs to be demonstrated, *in vivo*, in the complex human (animal) gut microbiota by applying the most relevant and validated methodology(ies) to quantify a wide variety of genera/species composing the gut microbiota;

"**activity(ies)**" meaning a metabolic profile(s), molecular signalling, prokaryote-eucaryote cell-cell interaction linked to one specific microbial genus/species or resulting from the coordinated activity of a limited number of microbial genus(era);

"**confer(s)**" referring to one or a limited number of selectively stimulated genus(era)/species in the gut microbiota.

In this concept, the use of "**gut microbiota**" is limited to the application to food/feed components.

Moreover it is implicit that "**health benefit(s)**" must be linked/correlated, directly or indirectly, to the presence in relatively high concentrations and/or activity(ies) of one or a limited number of selectively stimulated microorganisms in the gut microbiota. Indeed, such a conceptual approach emphasizes the link between "selective stimulation of growth and/or activity(ies) of one or a limited number of specific bacteria genus/species" and "health benefit(s)". Consequently, only food components/ingredients/supplements for which both such a selective stimulation has been scientifically substantiated and health benefits have been evaluated are included in the review process. The expression 'prebiotic effect(s)' will be used to identify or refer to selective changes in gut microbiota composition as well as specific (patho-) physiological effects both in experimental and human intervention studies. But it must be kept in mind that, to substantiate a 'prebiotic' effect, will require the demonstration that such an effect is likely to be 'causally' linked to or at least correlated with selective change(s) in gut microbiota composition.

Currently and mostly for historical reasons, the majority of the scientific data (both experimental and human) on prebiotic effects have been obtained using food ingredients/supplements belonging to two chemical groups namely inulin-type fructans (ITF) and the galacto-oligosaccharides (GOS) (for more details on the chemistry, nomenclature and abbreviations used in the present review see Table 3). These have repeatedly demonstrated the capacity to selectively stimulate the growth of bifidobacteria and, in some cases, lactobacilli leading to a significant change in gut microbiota composition. Concurrently, most of the health benefits possibly associated with the prebiotic effects were discovered and demonstrated using the same food ingredients/supplements. This, by no means, precludes other products of demonstrating such prebiotic effects with the same or other health benefits. However, since the aim of the present review is, primarily, to expand and validate the prebiotic concept, it will neither emphasize nor identify which specific products can be classified as 'prebiotic'. A precise list of potential

candidates for such a classification would require a detailed review of all published studies using each potential candidate as well as the evaluation of their validity and their relevance. This was not the mandate given to the group of experts who collectively wrote the manuscript. For such a discussion the reader should consult the different chapters in the recently published Handbook of Prebiotics ([20]). It is important to emphasize the fact that the prebiotic effect and the dietary fibre effect have two different attributes. Being resistant (partly or totally) to digestion and being fermented (at least the so-called soluble dietary fibres) both may concern gut microbiota composition and activity. What makes them different is the selectivity of the prebiotic effect as described above.

In the concluding chapter, tentative answers to the above questions will be presented and discussed with the main objective to prospectively prioritise topics for further research in the field.

1 Prebiotic effects in the gut[2]

1.1 Microbiota of the gastro-intestinal tract

The microbiota of the human gastro-intestinal (GI) tract inhabits a complex ecosystem ([22]). Factors such as pH, peristalsis, nutrient availability, oxidation–reduction potential within the tissue, age of host, host health, bacterial adhesion, bacterial co-operation, mucin secretions containing immunoglobulins, bacterial antagonism and transit time influence the numbers and diversity of bacteria present in the different regions of the GI tract ([23]). Until 20 years ago, our knowledge of the GI microbiota relied upon cultivation-based methods and recovery of bacteria from faecal samples. However, with the advent of molecular techniques and their application to biopsy and faecal samples, our knowledge of the GI microbiota has increased dramatically ([5-16]). An understanding of the bacteria making up the GI microbiota is important due to its involvement in the development of the GI mucosal immune system, maintenance of a normal physiological environment and for providing essential nutrients ([24]).

[2] The main authors of this section are Prof. Gibson, Dr. Hoyles and Dr. McCartney and specifically Prof. Robert Rastall for the *in vitro* subsection.

1.1.1 The stomach

Although the bacterial load in the stomach is low in healthy adults [~10^2 Colony Forming Unit (CFU) (ml contents)$^{-1}$ ([25])], the walls of the stomach are colonized with bacteria. In the healthy adult stomach, the predominant organisms isolated include lactobacilli, enterococci, 'catenabacteria' and bacilli ([26]). Of the bacteria that inhabit the stomach, *Helicobacter* species have been studied most intensively due to their association with various gastric complaints. *Helicobacter pylori* is present in the stomach of a subset of the population (10 % of those between 18 and 30 years of age; 50 % of those age 60 and over), where it resides in the mucous layer next to the gastric epithelium ([23]). Infection with *Helicobacter pylori* can be asymptomatic, but the organism is known to cause symptoms such as acute gastritis (i.e. pain, bloating, nausea and vomiting) and/or chronic gastritis; it has also been associated with peptic ulcers and gastric carcinomas ([23]).

1.1.2 The small intestine (duodenum, jejunum and ileum)

The environment of the duodenum is acidic (pH 4–5) with lactobacilli and streptococci predominating, and numbers of bacteria are higher than those found in the stomach [10^2–10^4 CFU (ml contents)$^{-1}$; ([27])].

Cultivation studies have shown lactobacilli, streptococci, veillonellae, staphylococci, actinobacilli and yeasts to be most prominent in the duodenum and jejunum ([23]). However, due to limitations in cultivation techniques and the ethical issues surrounding the obtention of biopsy samples from humans, our knowledge of the microbiota of the small intestine was poor until recently. Table 4 gives details of the results of recent molecular studies that have provided additional understanding of the microbiota of the small intestine. But these studies are only informative, because only one or a few donors have been used in each study, and their ages have not been representative of the general population. However, the results of the molecular studies appear to confirm those of cultivation-based work.

The microbiota changes markedly from the duodenum to the ileum, as the velocity of the intraluminal content decreases, pH increases and oxidation–reduction potentials lower, with bacterial loads increasing to 10^6–10^8 CFU (ml contents)$^{-1}$ ([23]). As transit time in the small intestine is rather rapid (2-4h) and the bacterial density relatively low, its impact in terms of overall fermentation is low

comparedto the large intestine (see below). The small intestine is also the site of many bacterial infections, such as salmonella and some *E. coli*. For this reason, the small intestine is also a target for probiotics known to compete with pathogens.

1.1.3 The large intestine

The combination of increased transit time of the large intestine, increased nutrient availability (i.e. undigested food material from the upper GI tract, sloughed-off bacterial cells, microbial cell debris and by-products of microbial metabolism) and a more-neutral pH ensure that the large intestine is a highly favourable environment for microbial colonisation. As the environment is strictly anaerobic (>100mV), in particular obligate anaerobes prevail. Table 5 gives details of some bacteria that have been isolated from the GI microbiota. Table 6 gives details of molecular studies on biopsies from different regions of the large intestine. In addition to characterizing the mucosa-associated microbiota, Zoetendal *et al.* ([11]) demonstrated that the faecal microbiota differs from that inhabiting the GI mucosa.

Even today, due to the difficulty of obtaining samples from the different regions of the intestine, much of the work done in relation to the ecology and activity of bacteria within the GI tract is carried out using faecal samples. However, the faecal microbiota is not representative of that of the GI tract as a whole ([11; 14]), and inferences made from *in vitro* studies in relation to specific GI diseases, particularly those involving the more-proximal regions of the intestine, should always be made with this in mind. However, a study examining the GI microbiota of sudden-death victims has shown that the faecal microbiota reflects that of the luminal contents of the descending colon in terms of the culturable component ([28]). Molecular based methods have been used to examine the faecal microbiota in recent years. Identification of specific strains isolated from faecal samples has become more accurate due to the use of 16S rRNA gene sequence analysis, and has improved taxonomic schemes and our understanding of the bacteria involved in specific metabolic processes (e.g. the role of *Roseburia* spp. in butyrate production ([29]), and the identification of the mucin-degrading bacterium *Akkermansia muciniphila* ([30])). This improved characterization of viable bacteria has also aided in the design of probes for use in fluorescence *in situ* hybridization (FISH) analysis (e.g. Rrec584 for *Roseburia* spp. ([31])).

Early cloning studies examined relatively small numbers of clones to generate a phylogenetic inventory of the faecal microbiota of healthy adults. Wilson & Blitchington ([22]) generated two clone libraries [one from a 9-cycle polymerase chain reaction (PCR) (50 clones, 27 operational taxonomic units (OTUs)), the other from a 35-cycle PCR (39 clones, 13 OTUs)] from a faecal sample from a healthy 40-year-old male. Of the clones they analysed, 35 % were related to the *Bacteroides* group, 10 % to the *Clostridium coccoides* group (*Clostridium* cluster XIVa) and 50 % to the *Clostridium leptum* group (*Clostridium* cluster IV). Less than a quarter of the sequences analysed were derived from known bacteria. Suau *et al.* ([5]) found that, of the 284 clones they generated from a faecal sample from a 40-year-old male, the majority of the sequences fell into three phylogenetic groups: *Bacteroides* (31 %), *Clostridium coccoides* (44 %) and *Clostridium leptum* (20 %). The remaining clones were derived from *Streptococcus salivarius* and *Streptococcus parasanguinis* and bacteria related to *Mycoplasma* spp., clostridia, the *Atopobium* group, *Verrucomicrobium spinosum* and the *Phascolarctobacterium faecium* subgroup. Seventy-six per cent of the clones analysed were derived from previously unknown bacteria. Blaut *et al.* ([32]) used a cloning approach to demonstrate that microbial diversity in faeces increases with age ([32]). It was found that the number of OTUs corresponding to known molecular species was highest in infants and lowest in the elderly, with 92 % of sequences from the elderly subjects corresponding to previously unknown bacteria.

As molecular methods have become more widely available and less time-consuming and their relative costs have decreased, more-ambitious cloning studies in which thousands of sequences have been examined have been carried out ([14; 33]). The results of these studies in terms of the groups of bacteria represented by the largest number of clones and the identification of previously unknown bacteria are in accordance with those of Wilson & Blitchington ([22]) and Suau *et al.* ([5]), but are notable for the characterization of several actinobacterial and proteobacterial sequences from human faecal samples.

Techniques such as Temperature Gradient Gel Electrophoresis (TGGE) and Denaturing Gradient Gel Electrophoresis (DGGE) allow higher numbers of samples from more donors to be examined than traditional cloning studies. TGGE was used by Zoetendal *et al.* ([9]) to examine the total bacterial communities of faecal samples from 16 adults. Host-specific fingerprints were generated, demonstrating interindividual variation in the composition of the faecal microbiota and confirming the results of cultivation studies. Some bands were seen in fingerprints from multiple donors, suggesting

that species of the predominant microbiota were common across individuals. In addition, by obtaining samples from two donors over a 6-month period, the authors showed that the profiles of these donors did not differ significantly over time, demonstrating that predominant microbial species were relatively stable without dietary intervention. Excision and sequencing of bands of interest allowed the authors to perform a phylogenetic analysis on their samples, the results of which demonstrated that the majority of bacteria represented in their fingerprints did not correspond to known bacterial species. Of the prominent bands identified in almost all samples, most belonged to different *Clostridium* clusters, with the remainder identified as *Ruminococcus obeum*, *Eubacterium hallii* and *Faecalibacterium prausnitzii*. Zoetendal *et al.* ([10]), using DGGE, demonstrated that host genotype affects the composition of the faecal microbiota. In that study, the authors examined faecal samples from 50 donors of varying relatedness. A higher similarity was seen between fingerprints from monozygotic twins living apart than between those of married couples or pairs of twins. There was a significant difference between the fingerprints of unrelated people grouped by either gender or living arrangements, and no relationship between the fingerprints generated and the age difference of siblings. Temporal TGGE and DGGE studies examining the faecal microbiota of children and infants have confirmed the impact of host genotype on the composition of the faecal microbiota ([34]). Other studies employing DGGE have used primer sets that allow examination of the composition and dynamics of specific groups of bacteria (Table 7). The detection limit seems to be the main barrier to overcome in these studies, particularly when examining populations such as bifidobacteria and lactobacilli – the commonest prebiotic targets.

With respect to the prebiotic concept it is important to understand that, apart from knowledge on the complexity of the gut microflora, it is also known that certain bacteria are associated with toxin formation and even pathogenicity when they become dominant. Others are associated with carcinogen generation and the metabolism of other xenobiotics. These potentially harmful bacteria belong to species within groups such as clostridia and bacteroides. Whereas knowledge on overt or latent pathogens has advanced markedly, due to the symptoms they can cause, there is less consensus on what characterises potentially harmful bacteria (without direct pathogenicity) and potentially healthy bacteria. Still potentially healthy bacterial groups are characterized by a beneficial metabolism to the host through their short chain fatty acids (SCFA) formation, absence of toxin production, formation of defensins or even vitamin synthesis. They may also inhibit pathogens

through a multiplicity of mechanisms. Their cell wall is devoid of lipoplysaccharides or other inflammatory mediators (i.e. mainly Gram positive). Some may also compete with receptor sites on the gut wall and inhibit pathogen persistence and thus reduce the potential risk of infection. They may also compete effectively for nutrients with pathogens. One subject of intensive research is their stimulation of immunological defence systems, as discussed in the section *Prebiotic effects and immune system* of this paper. Acknowledged examples are bifidobacteria and lactobacilli – known as useful probiotics. Intermediate genera like streptococci, enterococci, eubacteria and bacteroides can be classified as potentially beneficial to health or potentially harmful, depending on the species. With regard to some of the most recently identified genera in the major phylla (Firmicutes, Actinobacteria and Bacteroidetes), classification as potentially beneficial to health or potentially harmful still remains to be made. A scheme describing the hypothesis of a balanced microbiota has been proposed by Gibson and Roberfroid ([3]) and recently endorsed by ISAPP (2008) even though it is stillsubject of ongoing discussion. A revised version of that scheme including the most recent knowledge on gut microbiota composition is presented in Figure 1.

The prebiotic concept is based on the selective stimulation of the host's own beneficial microflora by providing specific substrate for their growth and metabolism. Today, the effect is measured by using bifidobacteria or lactobacilli as markers, but may include others in the future, if their positive nature can be confirmed.

It has been shown by several studies (see the section *Human studies showing prebiotic effects in healthy persons* of this paper) that dietary intervention can selectively modulate the indigenous composition of the gut microbiota. This is the basis of a prebiotic effect and this has been assessed through reliable molecular based analyses.

1.2 Prebiotic effects and fermentation and physiology

1.2.1 Bacterial fermentation in the large gut

It is clear that a complex, resident gut microflora is present in humans. Whilst the transit of residual foodstuffs through the stomach and small intestine is probably too rapid for the microbiota to exert a significant impact, this slows markedly in the colon. Colonic microorganisms have ample opportunity

to degrade available substrates ([35; 36]). These may be derived from either the diet or by endogenous secretions ([37]).

Due to the high residence time of colonic contents, as well as a diverse and profuse flora, the colonic microbiota plays a more important role in host health and well-being than is the case in the small intestine. Beneficial effects can be related to their metabolism (i.e. fermentation profiles and end products), capacity for producing vitamins, antioxidants (reduction equivalents), defensins against potentially harmful competitors, exchange of molecular signals between the different genera/species but also with the eukaryotic epithelial cells. Potentially beneficial bacteria are further characterized by the absence of secondary metabolic pathways leading to toxic metabolites of,for example xenobiotics, bile acids or phytochemicals.

The prebiotic concept emphasizes the specific stimulation of such a microbiota leading to a reduction of the metabolic activity of potentially harmful bacterial. This section focusses essentially on primary metabolism whereas the following ones deal with adverse effects and their prevention.

1.2.2 Substrate utilisation in the colon

The colonic microflora derive substrates for growth from the human diet (e.g. non-digestible oligosaccharides, dietary fibre and un-digested proteins reaching the colon) as well as from endogenous sources such as mucins, the main glycoprotein constituents of the mucus which lines the walls of the GI tract ([38]). The vast majority of the bacteria in the colon are strict anaerobes and thus derive energy from fermentation. The two main fermentative substrates of dietary origin are non-digestible carbohydrates (resistant starch, non-starch polysaccharides, dietary fibres, non-digestible oligosaccharides of plant origin) and proteins which escape digestion in the small intestine ([39; 40]). Of these, carbohydrate fermentation is more energetically favourable, leading to a gradient of substrate utilization spatially through the colon ([41]). The proximal colon is a saccharolytic environment with the majority of carbohydrate entering the colon being fermented in this region. As digesta moves through to the distal colon, carbohydrate availability decreases, proteins and amino acids become increasingly important energy sources for bacteria ([41]).

The main substrates for bacterial growth are dietary non-digestible carbohydrates ([42]) that evade upper intestinal hydrolysis and absorption. Non-digestible carbohydrates comprise resistant starch

and resistant dextrins, non-starch polysaccharides (e.g. pectins, arabinogalactans, gum Arabic, guar gum and hemicellulose), non-digestible oligosaccharides (e.g. raffinose, stachyose, ITF, galactans and mannans) as well as undigested portions of disaccharides(eg lactose) and sugar alcohols (e.g. lactitol and isomalt) ([37; 43; 44]). Resistant starch, non starch polysaccharides, most dietary fibres but also some non-digestible oligosaccharides (e.g. lactose) are fermented by a wide range of the colonic bacterial although the degree of their breaking down might vary ([45]). However, some non-digestible oligosaccharides entering the colon are rapidly and quantitatively but selectively fermented (e.g. raffinose, ITF and galactans) by a small number of bacteria (e.g. bifidobacteria and lactobacilli) ([46]).

The overall intake of non-digestible carbohydrate in a Western diet is estimated between 20-30 g/day ([47]). Endogenous carbohydrates, chiefly from mucins and chondroitin sulphate, contribute about 2-3 g/day of fermentable substrate ([48]). The main saccharolytic species in the colonic microflora belong to the genera *Bacteroides, Bifidobacterium, Ruminococcus, Eubacterium, Lactobacillus* and *Clostridium*.

The second important group of substances for bacterial growth are proteins, peptides and amino acids: Approximately 25 g of protein enters the colon daily ([49]). Other sources of proteinsin the colon include non-digestible food components, bacterial secretions, sloughed off epithelial cells, bacterial lysis products and mucins. The main proteolytic species belong to the genera *Bacteroides* and *Clostridium*.

1.2.3 Products of microbial fermentation in the colon and their effects on the host

Carbohydrates in the colon are fermented to SCFAs, mainly, acetate, propionate and butyrate ([50-52]) and a number of other metabolites such as the electron sink products lactate, pyruvate, ethanol, succinate as well as the gases H_2, CO_2, CH_4 and H_2S ([53]). As a whole, SCFAs acidify the luminal pH which suppresses the growth of pathogens ([54]). They are rapidly absorbed by the colonic mucosa and contribute towards energy requirements of the host ([50; 55; 56]). Acetate is mainly metabolised in human muscle, kidney, heart and brain Propionate, that is cleared up by the liver, is a possible gluceogenic substrate and it might contribute to inhibition of cholesterol synthesis. It might also play a role in the regulation of adipose tissue deposition ([57; 58]).

Butyrate on the other hand is largely metabolised by the colonic epithelium where it serves as the major energy substrate as well as a regulator of cell growth and differentiation ([51; 59]). It is also

acknowledged that it may reduce the risk of colon cancer through stimulating apoptosis. Evidence for the role of butyrate in relation to the administration of ingredient showing a prebiotic effect is described later in this review. Rectally administered butyrate was also shown to relieve subjects from inflammatory bowel disease symptoms ([60]).

Proteins reaching and/or produced in the colon are fermented to branched chain fatty acids such as isobutyrate, isovalerate and a range of nitrogenous and sulphur-containing compounds. Unlike carbohydrate fermentation products which are recognized as beneficial to health, some of the end products of amino acids metabolism may be toxic to the host e.g. ammonia, amines and phenolic compounds ([49]). Consequently, excessive fermentation of proteins, especially in the distal colon, has been linked with disease states such as colon cancer and inflammatory bowel diseases, which generally start in this region of the large intestine before affecting more proximal areas. Thus, it is favourable to shift the gut fermentation towards saccharolytic fermentation over a prolonged period of time into the distal parts.

Conclusions

- Overall, saccharolytic fermentation leads to the formation of end products (SCFAs) that are recognized as being beneficial to the host.
- Protein degradation on the other hand is likely to give rise to toxic substances such as ammonia, and amines
- Non-digestible carbohydrates with prebiotic effects selectively stimulate the growth of bacterial genera/species characterized exclusively, or preferably, by saccharolytic fermentation. .Such a selective effect on gut microflora composition is likely to be more beneficial to host health than one which would favour the metabolism of both carbobohydrates and proteins. This is well established today for prebiotic effects favouring the growth of bifidobacteria and lactobacilli. Emerging genera are *Eubacterium, Faecalibacterium* and *Roseburia* –although more evidence is needed on their physiological properties

1.3 In vitro tests for prebiotic effect

In vitro models aim at studying prebiotic effects independently from their passage through the upper parts of the gastro-intestinal tracteven if digestion is sometimes partly simulated. These models are thus only indicative of a potential prebiotic effect however, they do not prove the prebiotic attribute of a particular product as *in vivo* studies need to be performed to definitively demonstrate that the compound under investigation selectively stimulates the growth and/or activity(ies) of one or a limited number of microbial genus(era)/species in the gut microbiota that confers health benefits to the host. Since, as discussed above (see the *Introduction* section), the aim of the present paper is not to provide a list of food ingredients/supplements that classify as prebiotics, the following sections will only refer to a few examples to illustrate the potentials and the limits of *in vitro* tests as well as the advantages and disadvantages of the different experimental models.

Batch culture (pH or non-pH controlled) studies where different substrates are incubated with either pure culture of selected bacteria or faecal slurries subsequently analysed for microbial composition can be used:

- to study the selectivity of fermentation (including possible mechanism of selectivity) by, for example, bifidobacteria, lactobacilli of different substrates (e.g. main oligosaccharides contained in soybeans are raffinose and stachyose which have been found to be good growth promoters of *Bifidobacterium infantis* but not *Escherichia coli*, *Streptococcus faecalis* or *Lactobcaillus Lactobacillus acidophilus* ([61]) or similar substrates differing in molecular weights (e.g. wheat arabinoxylans) showing e.g. that molecular weight can be an important factor in selectivity ([62])..

- to show changes in faecal microbiota (e.g. increase in bifidobacteria) but also to compare the efficacy of different substrates (e.g. ITF, starch, polydextrose, fructose and pectin, galactans, xylo-oligosaccharides, soybean oligosaccharides ([63-65])
- to measure and to compare the evolution of gas and SCFAs production as a result of the fermentation of different substrates ([64]).

Single stage chemostat studies with ITF were used to compare differing techniques to analyze microbiota composition, demonstrating that discrepancies might exist between classical microbiological techniques and molecular approaches. Agar plate counts showed an increase in the

combined populations of bifidobacteria and lactobacilli reaching 98.7% of the total bacterial flora by steady state. However, 16S rRNA genus-specific probes indicated an initial increase in the bifidobacteria population which decreased after 6 days, whilst lactobacilli thrived in the low pH fermenter (pH 5.2-5.4) maintaining a high population at steady state. Changes observed in the SCFAs profile corresponded well with the population data obtained through probe methods ([66]).

Continuous culture systems inoculated with faecal slurries can be used to investigate fermentation profiles showing for example that, in accordance with earlier studies, bifidobacteria, and to a lesser extent lactobacilli preferred ITF to glucose, whereas bacteroides could not grow on these substrates ([67; 68]). By varying parameters in the chemostat, the conditions for growth of bifidobacteria and inhibition of bacteroides, clostridia and coliforms can be further analyzed showing that low pH (pH 5.5), high culture dilution rate (0.3h-1) and 1% (w/v) concentration of carbohydrate, (i.e. similar to the physicochemical environment of the proximal colon) are optimum.

The three-stage gut model reproduces the three segments of the colon (proximal/ascending, transverse, distal/descending). It is used to confirm the effects observed in the previous models. Studies using this model show enhanced proliferation of bifidobacteria and/or lactobacilli by ITF and galactans in conditions resembling the proximal/ascending colon ([67; 69; 70]). Whereas studies using models of vessels two and three (modeling transverse and descending colon respectively) displayed very little change in microbiota when fermenting galactans ([70]). In the same model changes in enzyme activities (β-glycosidase, β-glucuronidase, azoreductase and arylsulphatase) can also be monitored showing their suppression after fermentation of galactans ([70]) or soybean-oligosaccharides ([71]). Investigating the effect of pH and substrate concentration on the fermentation selectivity of galactans alongside other products, Palframan et al ([72]) reported a strong bifidogenic effect at pH 6 and at 2% (w/v) and suggested that they may be well-fermented in the distal colon. In another study galactans of rather low molecular weight (1% w/v) had a strong bifidogenic effect which showed good persistence through the first two vessels, with a weaker response in the third ([73]).

The Simulator of the Human Intestinal Microbial Ecosystem (SHIME) model consists of a series of five temperature and pH-controlled vessels that simulate the stomach, small intestine, ascending,

transverse and descending colons respectively. It can be fed with a complex growth medium containing selected substrates (e.g. ITF) to study their fermentation including the monitoring of metabolites and to analyze their effect on enzyme activities and composition of the microbiota by using a multiphase approach consisting of plate counting, quantitative PCR and DGGE ([74]). Results have shown a significant increase in lactobacilli in the transverse and descending colon vessels. Low levels of bifidobacteria were recorded in the colon vessels. DGGE analysis revealed that bacteria in the ascending colon vessel grouped together as did bacteria in the other colon vessels. Bifidobacteria clustered according to time point rather than vessel. Quantitative PCR, however, revealed a significant increase in bifidobacteria population in all three colon vessels. ITF feeding also resulted in an increase in the production of SCFAs, particularly propionate and butyrate, indicating a shift towards a more saccharolytic fermentation. The same model system and metabolic analysis can also be used to investigate the effect of different composition of the same substrates (e.g. of ITF with different molecular weight) on fermentation properties ([75]).

A more sophisticated in vitro model of fermentation in the proximal large intestine is the TIM-2 model ([76; 77]). This consists of a series of linked glass vessels containing flexible walls. This arrangement allows simulation of peristalsis together with temperature regulation by means of pumping water through the space between the glass and flexible walls. The flow is controlled by computer to more accurately simulate peristalitc mixing. The vessels are further equipped with a hollow fibre membrane in the lumen to simulate absorption of water and short chain fatty acids. TIM-2 has been used to investigate the population changes on the fermentation of lactulose using culture-based methods coupled with DGGE ([77]). Increases in lactobacilli and enterococci were seen.

Conclusions

- *In vitro* models allow comparative studies on fermentation by and/or effects of ingredients showing a potential prebiotic effect on isolated or mixture of bacterial strains, including faecal flora, as well as identification and eventually quantification of the resulting fermentation products especially the SCFAs. They also allow comparative analysis of the different analytical methods available to identify and quantify the various genera/species.
- They further allow the analysis of the potential/absence of toxin formation or change in enzyme activities potentially associated with beneficial or harmful effects.

The multi-stage models that are designed to mimic the different segments of the intestine, especially the proximal/ascending, transverse and distal/descending colon are useful in localizing the site of the selective stimulation of bacterial growth

- The results can be used to select potential candidate showing prebiotic effect(s) for *in vivo* studies especially in human volunteers, which remain the obligatory steps to definitively prove the prebiotic effect attribute.

1.4 Human studies showing prebiotics effect in healthy persons

By reference to the prebiotic concept as defined in the introduction, criteria for classification as a prebiotic are ([4]):

- resistance to gastric acidity, hydrolysis by mammalian digestive enzymes and GI absorption
- fermentation by intestinal microflora
- selective stimulation of the growth and/or activity(ies) of of one or a limited number of intestinal bacteria beneficially associated with health and well-being.

Any dietary component that reaches the colon intact (or partly so) is a potential candidate for prebiotic attribute, however it is the latter of the 3 above criteria which is crucial but still the most difficult to fulfil (and which is often ignored when citing ingredients as “prebiotics”). Even if in addition to ITF and GOS, several dietary carbohydrates (e.g polydextrose, soybean oligosaccharides, lactosucrose, isomalto-oligosaccharides, gluco-oligosaccharides, xylylo-oligosaccharides, gentio-oligosaccharides, mannan-oligosaccharides, lactose, hemicellulose, resistant starch, resistant dextrins, oat bran, oligosaccharides from melibiose, β-glucans, N-acetylchito-oligosaccharides, sugar alcohols such as lactitol, sorbitol and maltitol), show some fermentation selectivity when tested in laboratory systems (see section *In vitro tests for prebiotic effect* in this paper). However, the ultimate test for prebiotic activity (i.e. human volunteer trials) is lacking for the majority of these compounds. As for today ITF and GOS are the compounds the most extensively tested in human trials that have confirmed their prebiotic effects as evidence by their ability to change the gut flora composition after a short feeding period at reasonably low doses ([20]) (Table 8). ITF, the most extensively tested forms in the literature, occur naturally in several foods such as leek, asparagus, chicory, Jerusalem artichoke, garlic, artichoke, onion, wheat, banana and oats, as well as soybean. However, these foods contain only trace levels of ITF, so developments have taken the approach of removing the active ingredient from such sources (especially chicory roots) and adding them to more frequently consumed products in

order to attain levels whereby a prebiotic effect may occur, e.g. cereals, confectionery, biscuits, infant feeds, yoghurts, table spreads, bread, sauces, drinks, etc ([4]). Other food ingredients/additives with potential prebiotic effects are already under investigations and will certainly be further developed in the future from dietary fibres and other non-digestible food ingredients. Very preliminary data already exist for some but many more replicate human studies including the quantitative analysis of a wide variety of bacterial genera in faecal microbiota using the more recent methodologies (as described in the section *Microbiota of the gastro-intestinal tract – The large intestine* of this paper) are needed before this can be the case. Human trials may be carried out on volunteers who are on controlled diets, or are free living. To ensure consistency and exclude incidental findings, more than one human trial is needed and the totality of several human studies for a candidate prebiotic should be considered.

When evaluating a potential prebiotic effect it must be kept in mind that a dose-effect relationship and consequently a minimum effective dose is difficult to establish. Indeed, the major determinant that quantitatively controls the prebiotic effect is the number of targeted bacteria genus/species per gram of feces the volunteers have before the supplementation with the compound presumed to show a prebiotic effect. This issue has been extensively discussed previously ([78]).

1.5 Conclusion

Apart from protein fermentation, harmful substances may arise from bacterial secondary metabolism.

A prebiotic effect should not lead to stimulate the proteolytic microbiota and thereby reduce overall formation of bacterial metabolism.

2 Prebiotic effects and immune system[3]

2.1 Outline of benefit area

To provide optimal resistance against a large variety of pathogenic encounters, the immune system has evolved to comprise multiple, functionally differing cell types enabling the development of an

[3] The main authors of this section are Prof. Watzl and Dr. Wolvers.

immune response that is specifically tailored to clear the pathogen involved. Consequently, a large spectrum of immune parameters involved in various types of responses, exist, of which comprehensive descriptions can be found in many textbooks (e.g. Janeway's Immunobiology by Murphy *et al*. ([79])). Some of these may be measurable in humans, and can be divided into innate vs adaptive, mucosal vs systemic, pro-inflammatory vs anti-inflammatory, etc. Modulating aspects of the immune system may, in theory, serve several clinical purposes. First, boosting or restoring the very purpose of immune function, i.e. the resistance against infections, may serve as a clinical tool to prevent or treat infectious diseases. Second, preventing or treating consequences of an aberrant or undesired immune response, such as those occurring with an allergic response or during chronic inflammatory diseases, are other targets with high clinical relevance.

Although there is no single immune marker that accurately reflects or predicts an individual's resistance to infection, parameters can be identified that play a more prominent role in certain types of infections or conditions than others. For instance, if resistance against the common cold, i.e. a viral upper respiratory tract infection, is the topic of interest, it seems appropriate to investigate natural killer cell and CD8+ lymphocyte activity, whereas in case of inflammatory bowel disease the balance between pro-inflammatory and immuno-regulatory cytokines will be of interest (see section *Prebiotic effects and IBD* of this paper). Moreover, in a previous ILSI Europe activity, the suitability of immune markers to measure immuno-modulation by dietary intervention in humans was assessed, leading to the identification of four high-suitability markers that are the result of an integrated immune reaction (vaccine-specific serum antibody production, delayed-type hypersensitivity response, vaccine-specific or total secretory IgA in saliva, the response to attenuated pathogens). In addition, a range of medium and low-suitability markers, such as functional activity of cells of the innate immune system (NK cell activity, phagocytosis, T cell proliferation and various cytokines) were identified ([80]). Although the combined measurement of high- and medium-suitability markers may be a way to address aspects of immune status, the ultimate proof of accurate or even improved immune function in practice is a change in the incidence, severity or duration of infectious episodes or conditions with a prominent immune component such as allergies and chronic inflammation.

That modulation of certain aspects of the immune system may result from prebiotic effects and is based on the pivotal interaction between the intestinal microbiota and the host immune system. From

several studies in germ-free and gnotobiotic animals, it is clear that the microbiota is essential for an optimal structural and functional development of the immune system ([81-84]). The interactive co-existence of the immune system and the microbiota is especially apparent in the intestinal tract where the gut-associated lymphoid tissue (GALT) has evolved to provide optimal defense against intestinal pathogens, while at the same time tolerating dietary and self-antigens, as well as large populations of commensal non-pathogenic microbes.

Although specialized cells such as the M-cells and, as discovered more recently, also dendritic cells sample material directly from the intestinal lumen ([85]), enterocytes are key intermediates that convey signals from the intestinal lumen to the mucosal immune system ([86; 87]) and are thus a target for a prebiotic effect on the immune system.

Prebiotic effects may influence the immune system directly or indirectly as a result of intestinal fermentation and promotion of growth of certain members of the gut microbiota. Firstly, the mere presence of increased numbers of a particular microbial genus or species, or a related decrease of other microbes, may change the collective immuno-interactive profile of the microbiota. Through pattern-recognition receptors such as the toll-like receptors, both immune cells and enterocytes interact with so-called pathogen-associated molecular patterns (PAMPs), such as lipopolysaccharides (LPS, a membrane component of Gram negative bacteria), lipoteichoic acids and unmethylated CpG DNA that are in fact present on all microorganisms surface regardless of pathogenicity. These interactions, possibly in combination with contextual cues of pathogenicity, result in a variety of downstream events eventually leading to cytokine production steering towards an appropriate immune response for the microbial event ([88-90]).

Secondly, microbial products such as SCFAs may interact with immune cells and enterocytes and modify their activity. G-protein coupled receptors (GPR) 41 and GPR 43 are identified as receptors for SCFA and are expressed on leukocytes, especially polymorphonuclear cells, ([91; 92]) as well as on enterocytes and enteroendocrine cells in the human colon ([93; 94]). SCFAs modulate chemokine expression in intestinal epithelial cells ([86]), differentially affect pro-inflammatory IL-2 and IFNγ and immuno-regulatory IL-10 production by rat lymphocytes *in vitro* ([95]) and a recent publication shows the importance of ligation to GPR43 in mice to maintain intestinal homeostasis ([96]).

Thirdly, the potential direct ligation of pattern recognition receptors on immune cells by prebiotic carbohydrate structures may result in immunomodulation, although there is currently very little evidence for the presence of, for example, a fructose-receptor on immune cells.

In summary, there are plausible mechanisms by which prebiotic effects can modulate immune function parameters. The inaccessibility of the human GI immune system complicates the investigation in this area and most human studies rely on the measurement of *ex vivo* systemic immune markers, of which the predictive value for overall resistance to infections or outcome of immune-related disorders is limited.

2.2 Summary of key studies

Several comprehensive reviews have summarized the current knowledge of the immunomodulatory potential of prebiotic effects (especially ITF) ([97-101]). A limited number of human studies have been performed but most have limitations as they investigated prebiotic effects in combination with the administration of other ingredients or did not included an appropriate control group.

The prebiotic effects on immune markers that represent a more or less integrated immune response, such as response to vaccination, was investigated in only a few studies (see Table 9). Bunout *et al*. ([102]) supplemented healthy elderly with an oligofructose/inulin mix (6 g per day) in combination with a nutrient supplement, while the control group received maltodextrin with the nutrient supplement. No significant differences were observed in antibody titers after vaccination or on secretory IgA levels ([102]). In a second study the same authors investigated the effect of a supplement with oligofructose on various immune markers including delayed type hypersensitivity (DTH) and vaccination. Elderly subjects attending a clinic received oligofructose as part of a complex nutritional supplement including *Lactobacillus paracasei*. Elderly subjects attending another clinic not receiving this supplement served as controls. DTH response and antibody titers after vaccination did not differ between groups ([103]).

In infants aged 6-12 months (87 % breast-fed) the intake of oligofructose as part of an infant cereal had no effect on diarrhea prevalence (see section *Use of prebiotic effects for pediatric disorders – Diarrheal diseases* of this paper) and on vaccination-induced antibody titers to *H. influenza* when

compared to the infant cereal alone ([104]). Besides the fact that a rather low dose of oligofructose was supplemented, breast-feeding may already have provided adequate amounts of human milk oligosaccharides in this study. Also in infants at high risk for allergies, supplementation witha GOS/FOS mixtures did not change antibody levels after a standard vaccination ([105]). In contrast, early life exposure of non-breast fed infants to oligosaccharides had an effect on natural immunoglobulin production, as a mixture of GOS/FOS was shown to result in significantly higher faecal SIgA concentrations as a consequence of the prebiotic effect ([106; 107]). Overall, there are currently no data that support beneficial prebiotic effects on the response to vaccination, but data on faecal secretory IgA in infants are promising when supplemented with a specific combination of compounds showing prebiotic effects.

In addition to effects on integrated immune responses, the prebiotic effect on specific immune markers has been tested in a few studies of varying quality with differential outcomes (see Table 9). In healthy elderly people receiving ITF-$_{\text{DPav 3-4}}$ (6g/d) a decrease in phagocytosis and IL-6 mRNA expression in peripheral blood mononuclear cell was found ([108]). This study was a one-arm study using baseline for comparison. Whether the tested ingredient induced the observed immunological changes cannot be answered from this study. Increased NK cell activity and IL-2 production by PBMC (Lymphokine production by mononuclear cells) was found in a synbiotic study in elderly ([103]). As this was a synbiotic intervention, a causal conclusion about an immunomodulation of the prebiotic intervention cannot be drawn. No effect was observed on secretion of IL-4, IFNg, and lymphocyte proliferation in cultured PBMC ([102]).

A study investigating the application of ingredients showing a prebiotic effect in pregnant women showed no effect on the composition of lymphocyte subsets or cytokine secretion patterns in circulating lymphocytes of the off-spring as assessed in cord-blood ([109]).

A well-designed and controlled human intervention study investigated the effect of a mixture of galactans on the immune system of healthy elderly volunteers. This study reported that intake of such galacto-oligosaccharides (galactans) (5.5 g/d) for 10 weeks significantly increased phagocytosis, NK cell activity and the production of the anti-inflammatory cytokine IL-10, while the production of pro-inflammatory cytokines IL-1β, IL-6, TNFα was reduced ([110]). A clear positive correlation between numbers of bifidobacteria in faecal samples and both, NK cell activity and phagocytosis, was

observed. This study suggests that a mixture of galactans beneficially affects the immune system and that the achieved effects may be indirect and mediated via a prebiotic effect i.e. a change in microbiota composition. A few of the trials described above also show changes in immune markers alongside changes in the fecal microbiota, mainly increase in bifidobacteria. These studies thus provide data for the suggested link between a change in the flora and immunomodulation, but more studies showing correlative findings are required for convincing evidence.

Only a few studies that investigated the prebiotic effect on immune-related clinical endpoints such as resistance to infections, allergies and inflammatory bowel disease, have also included measurements on immune markers. Combining clinical endpoints with such functional markers may provide a possible mechanistic explanation for the observed effects. In a small number of patients with moderately active Crohn's disease, consumption of 15 g ITF per day reported positive clinical outcomes (see section *Prebiotic effects in Crohn's disease* of this paper), while IL-10 production by mucosal dendritic cells isolated from biopsies was increased as did expression of TLR-2 and TLR-4 ([111]). Although some of the findings correlate with those found in animals studies ([112]), the open label character of the study needs to be considered.

In infants at high risk of allergies, a mixture of GOS/FOS supplemented for 6 months reduced plasma level of total IgE, IgG1, IgG2 and IgG3, whereas no effect on IgG4 was observed. In addition, cow's milk protein-specific IgG1 was significantly decreased ([105]). This may be beneficial change in infants at risk of allergies, and although no direct correlations were reported, the same study found a significant reduction in the incidence of atopic dermatitis in a subpopulation of the GOS/FOS group ([113]).

Experimental data from animal studies indicate that, besides the systemic immune system, the gut-associated lymphoid tissue (GALT) may be the primary target of immunomodulatory prebiotic effects. Biomarkers to assess functional changes in the GALT include SIgA, cytokine production, and lymphocyte numbers. Prebiotic effects have been shown to increase SIgA concentration in the intestinal lumen, to increase B cell numbers in Peyer's patches, and, in intestinal tissues, to enhance IL-10 protein secretion, and to decrease mRNA expression and protein concentrations of pro-inflammatory cytokines ([98-101]). Genes related to intestinal immune responses seem to be a primary

target of the prebiotic effects ([114]). Further, functional activities of NK cells and phagocytes isolated from various immune tissues were significantly increased but depending on the source of immune cells (Peyer's patches, mesenteric lymph nodes, intraepithelial lymphocytes) the prebiotic effects may differ ([115-117]). This illustrates the need to differentially study the prebiotic effects of on various immune compartments. The lack of sufficient tools to investigate prebiotic effects in the human GALT hampers insights into the possible differential impact on the mucosal vs the systemic immune system.

2.3 Key points

- Plausible hypotheses exist that ingredients showing a prebiotic effect may potentially affect the immune system as a direct or indirect result of the change in the composition and/or fermentation profile of the microbiota
- There is currently limited, yet promising evidence that such ingredients modulate immune markers in humans. Well designed human intervention studies are few.
- Data that showing increased fecal sIgA levels in infants are promising and need to be confirmed
- While several studies report changes in the fecal microbial composition alongside with changes in immune markers, only one study so far has correlated these findings. More studies addressing such correlation are needed to establish a firm link between changes in the microbiota and immune markers
- Despite the wealth of evidence that compounds with prebiotic effects affect the intestinal microbiota, and modulate immune parameters, it is of importance to know whether these immunomodulatory effects result in a clinically relevant outcome, i.e. improved resistance against infections, or impairment of allergies and inflammation. Preliminary yet promising clinical endpoint studies exist that integrate the measurement of immune markers as possible explanation of prebiotic efficacy.
- Animal studies indicate that immunological effects may vary depending upon the anatomical site of origin of the immune cell (e.g., Peyer's patches vs. intraepithelial lymphocytes). However, as the human GALT as primary target of the prebiotic effects cannot be easily addressed in human intervention studies, insights are difficult to obtain and thus still limited.

2.4 Recommendations

Data from well-designed, controlled human intervention studies with healthy subjects do not allow a final conclusion about the effects of ingredients showing a prebiotic effect on the immune system. Data so far are available for ITF and GOS, but few studies have been published sofar. Therefore, further studies with adequate methodology, investigating immune parameters such as laid out by the ILSI Task Force on Nutrition and Immunity in Man ([80]) are warranted to obtain further insights on how prebiotic effects may modify immune function markers. Furthermore, tools should be developed to measure the impact of prebiotic effects on the GALT in humans, so an understanding of the tissue-specific effects can be achieved. Findings of such immuno-modulation should lead to hypotheses on the potential use of compounds with prebiotic effects in relevant health-related conditions, which could then be tested in well designed clinical endpoint studies. In addition, effects of different prebiotic chemical structures of prebiotics, dosing and timing of supplementation have to be studied.

3 Prebiotic effects in paediatrics [4]

3.1 Oligosaccharides and prebiotic effects in infant formulae

The use of nondigestible carbohydrates in infant formulae and follow-on formulae has been commented on by the Committee on Nutrition of the European Society for Paediatric Gastroenterology, Hepatology and Nutrition (ESPGHAN) ([118]). Based on the evidence obtained in a search up to January 2004, the Committee concluded that only a limited number of studies have evaluated the effects of the addition of substances with prebiotic effects to dietetic products for infants. Only one type of oligosaccharide mixture of galactans and ITF consisting of galacto-oligosaccharides and a high molecular weight fraction of inulin in a ratio of 9:1 (GOS/FOS) was evaluated. The Committee stated that although the administration of oligosaccharides with prebiotic effects has the potential to increase the total number of bifidobacteria in feces and may also soften stools, there is no published evidence of any clinical benefits after addition of oligosaccharides with prebiotic effects to dietetic products for infants. No general recommendation on the use of oligosaccharide supplementation in infancy for preventive or therapeutic purposes can be made. **T**he

[4] The main authors for this section are Prof. Szajewska and Dr. Stahl.

available data on the oligosaccharide mixtures in infant formulae do not demonstrate adverse effects. Validated clinical outcome measures of prebiotic effects in infants should be characterized in further well-designed and carefully conducted randomized controlled trials (RCTs), with relevant inclusion/exclusion criteria and adequate sample sizes. Such trials should also define the optimal quantities, types and intake durations.

A number of studies have been published thereafter on the addition of ingredients showing a prebiotic effect to dietetic products for infants and recently reviewed ([119-121; 121]). These ingredients have been used either as one compound or as a mixture of different neutral and acidic oligosaccharides ([122-124]). Collectively, these studies confirm that the administration of oligosaccharides with prebiotic effects in dietetic products have the potential to increase dose-dependently the total number of bifidobacteria in feces, although at present, it is not possible to define the number of bifidobacteria that would constitute normal/optimal microbiota, and to soften stools. Furthermore, prebiotic effects modulate stool pH, SCFAs pattern similar to those of breast fed infants. Whether any of these effects per se is of benefit is currently not well established. Clinical outcomes related to the use of dietetic products for infants supplemented with prebiotic effects are discussed in the sections below (e.g. effect on allergic diseases, infections).

Currently, the Directive 2006/141/EC on infant formulae and follow-on formulae specifically allows the addition of GOS-FOS in a ratio of 9/1 and in a quantity of 0.8g/ 100 ml prepared product ([125]). This Directive also states that other combinations and maximum levels of FOS and GOS may be used if they satisfy the nutritional requirements of infants in good health as established by generally accepted scientific data.

3.2 Use of prebiotic effects in complementary foods for children

One controlled trial (RCT) ([126]) conducted in 56 healthy, term infants aged 4-12 months evaluated the tolerance and GI effects of an infant cereal supplemented with either ITF or placebo for 28 days. Compared with the control group, stool consistency was less often described as 'hard' and more likely to be described as 'soft' or 'loose' in the ITF-supplemented group. There was no difference between

the groups in crying, spitting-up or colic. No difference in stool pH between the groups was found. There was also no significant difference in growth between the two groups. Clinical outcomes were not reported. The limitations of this study include the use of non-validated tool for parental assessment of stool consistency, a small sample size, and a short follow-up period.

Another double blind RCT ([127]) involving 35 infants aged 4 to 6 months studied the effect of adding GOS/FOS to solid foods results in an increase in the fecal proportion of bifidobacteria in the intestinal microbiota. Intention-to-treat analysis revealed no significant difference between the 2 study groups. Only per-protocol analysis involving 20 children who complied with the protocol showed that the fecal percentage of bifidobacteria increased from 43% to 57% (p=0.03) from week 0 to week 6 but did not significantly change in the control group (36% and 32%, respectively, p=0.4). There were no statistically significant differences in stool frequency and consistency.

More recently the prebiotic effect of IFT in chidren aged 7-8 years has also been reported ([128]).

3.3 Use of prebiotic effects for pediatric disorders

3.3.1 Diarrheal diseases

It can be hypothesized that the continuous use of products with prebiotic effects might, by providing an immunologic stimulus (see section *Prebiotic effects and immune system* of this paper), be useful in preventing infectious diseases commonly encountered by young children.

In a large well-designed RCT performed in infants aged 6 to 12 months (n=282), Duggan *et al*. ([104]) compared an infant cereal supplemented with oligofructose with a non-supplemented cereal. There was no difference in the number of diarrheal episodes, episodes of severe diarrhea, or episodes of dysentery. No significant difference was found in the mean duration of diarrhea. During a second part of the same trial involving 349 subjects, zinc was added to both oligofructose-supplemented and control cereals ([104]). Again, no significant difference was found in any of the outcomes studied between the groups. In both trials, post immunization titers of the antibody to *Haemophilus influenzae* type B were similar in all groups, as were gains in height (no data on weight), number of visits to the clinic, hospitalizations, and use of antibiotics.

More recently, Bruzesse *et al*. ([129]) evaluated the effect of an infant formula containing the prebiotic mixture GOS/FOS) compared with a standard infant formula in an open placebo-controlled involving 342 healthy infants with 12 months follow-up. Compared with controls, the use of prebiotic supplemented formula was associated with a significant reduction in the incidence of gastroenteritis (0.12±0.04 vs. 0.29±0.05 episodes/child/12 months; p=0.015), and in the rate of children with ≥1 episode of acute diarrhea (10/96 vs 26/109, RR 0.44 (95% CI 0.22 to 0.86)). The findings regarding the prevention of GI infections are promising for efficacy. However, there are some methodological limitations to the study, including no allocation concealment, and no blind control, and no Intention-To-Treat analysis (ITT analysis aims to test for effectiveness under field conditions); this may result in selection, performance, and/or attrition biases. The impact on respiratory tract infections is discussed under 'Respiratory tract infections'.

One RCT ([130]) found similar number of episodes of diarrhea in the group of infants fed extensively hydrolyzed whey formula supplemented either with 0.8g GOS/FOS or maltodextrin as placebo.

3.3.2 Acute infectious gastroenteritis

The efficacy and safety of administering a mixture of nondigestible carbohydrates, including soy polysaccharide 25%, α-cellulose 9%, gum arabic 19%, oligofructose 18.5%, inulin 21.5%, and resistant starch 7%, as an adjunct to oral rehydration therapy in the treatment of acute infectious diarrhea was assessed in one RCT involving 144 boys with mild to moderate dehydration. It was hypothesized that with the incorporation of nondigestible carbohydrates, some of them (e.g. galactans and ITF) with prebiotic effects might promote fermentation in the colon, and thus, decrease fecal volume and the duration of the diarrheal illness. Intention-to-treat analysis (relevant for effectiveness) did not show a significant difference in the mean 48-hour stool volume, the duration of the diarrhea after randomization, the duration of hospital stay, and unscheduled intravenous rehydration. No significant adverse effects were noted ([131]). An explanation for the negative results could originate from the type and the amount of nondigestible carbohydrates added to the ORS. An average dose of 10 to 15g per episode in relatively mild diarrhea may be simply insufficient to achieve a shorter duration of diarrhea. Furthermore, it is possible that the timing of the intervention was inappropriate,

making the addition of nondigestible carbohydrates to exclusive oral rehydration therapy an insufficient measure.

3.3.3 Antibiotic-associated diarrhea

The rationale for the use of ingredients showing a prebiotic effect for the prevention of antibiotic-associated diarrhea (AAD) is based on the assumption that the use of antibiotics leads to intestinal dysbiosis and that this is a key factor in the pathogenesis of AAD ([132]). In contrast to probiotics, ([133-137]) there is a paucity of data on the prebiotic effects in preventing AAD. One pediatric double-blind RCT ([138]) involved 140 children (1 to 2 years of age) who were treated with amoxicillin for acute bronchitis. This study revealed no significant difference in the incidence of diarrhea in children receiving ITF administered in a milk formula (4.5g/L) for 21 days after completion of antibiotic treatment compared with placebo (10% vs. 6%, RR 0.6, 95% CI 0.2-1.8). However, ingredients showing a prebiotic effect in a milk formula increased fecal bifidobacteria early after amoxicillin treatment.

3.3.4 Respiratory tract infections

In the most recent RCT by Bruzesse *et al*. ([129]) described above, it was found that compared with controls, the use of an infant formula with GOS/FOS was associated with a similar number of episodes of upper respiratory tract infections (p=0.4), similar number of children with >3 episodes upper respiratory tract infections (17/60 vs. 29/65; p=0.06), although the number of children with multiple antibiotic courses per year was lower in children receiving ingredients showing a prebiotic effect (24/60 vs. 43/65; p=0.004).

One RCT ([130]) found that infants fed extensively hydrolyzed whey formula supplemented with 0.8g GOS/FOS compared with the placebo group had fewer episodes of physician-diagnosed overall and upper respiratory tract infections ($P<0.01$), fever episodes ($P<0.00001$), and fewer antibiotic prescriptions ($P < 0.05$).

3.4 Prebiotic effects and atopy

Atopic eczema is an itchy inflammatory skin condition with associated epidermal barrier dysfunction. Therapeutic options (emollients and topical steroids for mild-to-moderate eczema; topical or systemic calcineurin inhibitors, ultraviolet phototherapy, or systemic azathioprine for moderate-to-severe eczema) are relatively limited and often unsatisfactory, prompting interest in alternative treatment methods.

The rationale for using prebiotic effects in preventing atopic disorders is based on the concept that prebiotic effects modify the intestinal flora of formula-fed infants towards that of breast-fed infants. The intestinal flora of atopic children has been found to differ from that of controls with atopic subjects having more clostridia and tending to have fewer bifidobacteria than non-atopic subjects ([139]). Thus, there is indirect evidence that differences in the neonatal gut microbiota may precede or coincide with the early development of atopy. This further suggests a crucial role for a balanced commensal gut microbiota in the maturation of the early immune system.

The Cochrane Review published in 2007 ([140]), aimed at determining the effect of different ingredients showing a prebiotic effect (GOS/FOS, only FOS, GOS together with polydextrose and lactulose) on the prevention of allergic disease or food hypersensitivity in infants. Only 2 RCTs of reasonable methodological quality according to the reviewers and involving 432 infants reported outcomes related to allergic disease. The reviewers concluded that there is insufficient evidence to determine the role of prebiotic supplementation of infant formula for prevention of allergic disease and food hypersensitivity.

One of the included RCT ([140]) investigated the effect of the prebiotic mixture (GOS/FOS; dosage: 0.8g/dl) on the intestinal flora and the cumulative incidence of atopic dermatitis during the first 6 months of life in infants at risk for allergy (with at least one parent with documented allergic disease confirmed by physician). Two hundred six of 259 (79.5%) infants who were randomly assigned to receive extensively hydrolyzed whey formula supplemented either with 0.8g GOS/FOS (experimental group, n=102) or maltodextrin as placebo (control group, n=104) were included in the per-protocol analysis. The frequency of atopic eczema in the experimental group was significantly reduced compared with the placebo group (9.8% vs. 23.1%, RR 0.42 (95% CI 0.2-0.8)), number needed to

treat (NNT) 8 (95% CI 5-31). In a subgroup of 98 infants, the parents provided fresh stool samples for microbiological analysis using plating techniques; the fecal counts of bifidobacteria were significantly higher in the group fed the GOS/FOS formula compared to the placebo group. No significant difference was found for the lactobaccilli count between groups. Follow-up of this study, showed that at 2 years the cumulative incidences of atopic dermatitis, recurrent wheezing, and allergic urticaria were higher in the placebo group (27.9, 20.6, and 10.3%, respectively) than in the intervention group (13.6, 7.6, and 1.5%) ($P<0.05$). This is the first observation that prebiotic effects are able to reduce the incidence of atopic diseases, and that this effect persists beyond the intervention period. This assessment is based on a Per Protocol (PP) evaluation which aims at testing efficacy; due to the high drop-out rate (20% at 6 months and 48% at 2 years of age) and lacking ITT analysis, effectiveness for field practice needs to be confirmed ([141]). (See section *Prebiotic effects and mineral absorption* of this paper)

4.5 Conclusions

- Only two dietary nondigestible oligosaccharides fulfill the criteria for prebiotic classification. These are galactans and ITF. Only a limited number of randomized controlled trials evaluating the efficacy and safety of in pediatric population are available. Some of these studies had methodological limitations.
- Typically, the studies could show efficacy, i. e. statistical effects based on PP analysis. However, they may need to be confirmed by effectiveness using ITT analysis.
- Supplementation with such ingredients has the potential to increase the total number of bifidobacteria in feces and reduce some pathogens. It also can reduce stool pH, increase the concentrations of fecal short-chain fatty acids like observed in breast fed infants. The clinical meaning of these findings is still under debate.
- There is evidence from controlled trials that effects are able to reduce the incidence of atopic diseases, and that this effect persists beyond the intervention period. Confirmation of these data for effectiveness is needed.
- A reduction in the risk of some infectious diseases is likely, but needs to be confirmed for effectiveness.

- The available data on prebiotic effects do not demonstrate adverse effects.

4 Prebiotic effects and Gastro-intestinal disorders[5]

4.1 Prebiotic effects and Gastro-intestinal infections

In adults, the use of ingredients showing a prebiotic effect in the fight against infections has hardly been studied. A few studies, dealing with different infectious problems, have been reported.

One study dealing with traveller's diarrhea reports that consumption of 10g ITF per day for a 2-week pre-travel period continued during a 2-week travel period to high-and medium risk destinations, had no effect on the prevention of traveller's diarrhea, although the sense of 'well-being' was improved ([142]). Furthermore, a study of patients consuming 12g ITF /day while taking broad-spectrum antibiotics for 7 days, followed by another 7 days of the same treatment reported no difference from the placebo group regarding diarrhea incidence, *Clostridium difficile* infection and hospital stay, while the number of fecal Bifidobacteria increased significantly ([143]). In contrast, continued consumption of 12g ITF /day for 30 days after the cessation of *Clostridium difficile*-associated diarrhea, reduced the relapse rate, while increasing bifidobacteria levels ([144]).

Overall, the number of studies on the efficacy of ingredients showing a prebiotic effect in the prevention of infectious diseases is limited. Some positive outcomes exist alongside studies reporting no-effects. Clearly, a rationale is present for the use of such ingredients. However, any direct effect of the studied ingredients on the immune system can not be excluded and the measurement of the putative associated effect on the microbiota is not always included in these studies, hindering the formation of any conclusions on possible underlying mechanisms.

4.2 Prebiotic effects and IBS

The Irritable Bowel Syndrome (IBS) is a functional bowel disorder manifested by chronic, recurring abdominal pain or discomfort associated with disturbed bowel habit, in the absence of structural

[5] The main authors for this section are Prof. Guarner and Dr. Respondek (IBS), Dr. Whelan (IBD) and Prof. Rowland (colon cancer and bacterial activities).

abnormalities likely to account for these symptoms ([145]). The symptomatic array may include abdominal pain, discomfort, distension, cramping, distress, bloating, excess flatulence, and variable changes in frequency and form of stools. Such symptomatic episodes may be experienced by almost every individual, and in order to separate IBS from transient gut symptoms, experts have emphasized the chronic and relapsing nature of IBS and have proposed diagnostic criteria based in the recurrence rate of such symptoms ([146]). IBS is one of the most common intestinal disorders both in industrialized and developing countries and it is known to generate significant health care costs ([145]).

A precise aetiology for IBS is not recognized. However, epidemiological studies have identified a series of pathogenetic factors, including genetic and early environmental conditioning, cognitive /emotional adaptation, altered response to stress and inflammatory post-infectious processes of the gut mucosa, etc. ([145]). It has been shown that IBS patients have abnormal reflexes and perception in response to gut stimuli ([147]). In subsets of patients the underlying defects appear to be altered GI motility, visceral hypersensitivity, small bowel bacterial overgrowth, excess gas production, abnormalities in the composition of the gut microbiota (Table 10) or combinations of them ([148]).

Among the modifications of the gut microbiota, a decrease of Bifidobacteria and more specifically *Bifidobacterium catenulatum*, has been observed in IBS patients in comparison to healthy subjects ([149-151; 151; 152; 153; 154; 155]).

Hypothetically, some of these disturbances may be corrected or counteracted by prebiotic effects. Indeed compounds showing such effects are known to modulate the digestive microbiota and particularly to stimulate the growth of Bifidobacteria especially when the initial level is low ([156]). Furthermore human studies with ITF or lactulose have shown that such prebiotics modulate gut transit ([148; 157]), decrease putrefactive activity within the gut lumen ([158]), prevent GI infections ([142; 144]), and mitigate inflammatory responses ([111; 159; 160]).

Indirect evidence for beneficial effects of ingredients showing a prebiotic effect on abdominal well-being was initially obtained in human trials addressing other primary endpoints. For instance, Cummings *et al* ([142]) tested the effectiveness of ITF in preventing diarrhoea in 244 healthy subjects,

travelling to high and medium risk destinations for travellers' diarrhoea (see the section *Prebiotic effects and gastro-intestinal infections* of this paper for discussion of the effects on risk of intestinal infections). This randomized, double-blind, placebo-controlled study showed that consumption of 10g ITF daily gave a significantly better sense of `well-being' during the holiday, as recorded in post-study questionnaires. Likewise, Casellas *et al* ([160]) performed a prospective, randomized, double-blind, placebo controlled trial to test the effect of ITF (12g/day) in patients with active ulcerative colitis. Interestingly, the study observed a significant decrease in abdominal symptoms with treatment but not with placebo, as assessed with the validated questionnaire of dyspepsia-related health scale ([161]).

Few studies have investigated the effect of ingredients showing a prebiotic effect in patients with IBS. The study by Olesen *et al* ([162]) tested a large dose of finally 20g ITF during 12 weeks. The authors hypothesized that IBS symptoms may be provoked by large quantities of fermentable carbohydrates in the colon. After 4-6 weeks on treatment, IBS symptoms worsened, as expected, in patients on 20g ITF per day and improved in patients on placebo. However, continuous treatment for 12 weeks resulted in adaptation and there were no differences between groups: symptoms improved in 58% of the ITF group and in 65% of the placebo group, and symptoms worsened in 8% of the ITF group and in 13% of the placebo group. Large doses of any fermentable carbohydrates should not be recommended to IBS patients.

Hunter and co-workers ([163]) found no effect of 2g ITF (three times daily) against placebo in a reduced group of IBS patients studied in a double blind crossover trial. The Rome team of experts on functional bowel disorders do not recommend the use of a crossover design for IBS treatment trials as they have the potential disadvantages of carryover effects and unmasking the study product by differences in taste and palatability ([164]). Dughera *et al* ([165]) reported a positive effect of a synbiotic (including short chain ITF at 2.5g per day) on clinical manifestations and intestinal function in patients with IBS. However, this was an open-label and uncontrolled study and IBS studies with subjective outcomes are prone to study bias ([148]).

To date, there are two published studies of adequate study design reporting the effects of an ingredient showing a prebiotic effect in IBS. The first study screened 2235 subjects and recruited and

randomized 105 patients with IBS fulfilling Rome II criteria with minor intensity of symptoms as assessed by an initial questionnaire. Treatment with short chain ITF at 5g per day for 6 weeks reduced incidence and intensity of symptoms as compared to the placebo product. Prebiotic treatment also improved functional digestive disorders related quality of life ([166]).

The second study randomized 44 subjects according to Rome II criteria into 3 groups either receiving 7g/d placebo, 3.5g/d of ingredient showing a prebiotic effect and 3.5g/placebo and 7g/d of the tested ingredient for 6 weeks. The prebiotic treatment significantly improved flatulence, bloating, and composite score of symptoms as well subjective global assessment. It also increased the proportion of Bifidobacteria in faecal samples ([167]).

In summary, the two available studies with up to date standard, both provided positive outcomes for the ITF and GOS tested up to 7g. Results with less positive outcomes either used higher or lower doses.

4.2.1 Recommendations:

Ingredients showing a prebiotic effect are likely to play a role in the symptomatic control of IBS. Evidence accumulated so far in well-designed clinical studies is limited, but suggests possible benefits at moderate doses. Further studies with adequate methodology are warranted.

4.2.2 Key Points:

- The Irritable Bowel Syndrome (IBS) is a functional bowel disorder manifested by chronic, recurring abdominal pain or discomfort in the absence of structural abnormalities.
- The symptomatic array includes abdominal distension, cramping, distress, bloating, excess flatulence, and variable changes in frequency and form of stools. Such symptomatic episodes may be experienced by almost every individual.
- The underlying defects appear to be altered GI motility, visceral hypersensitivity, small bowel bacterial overgrowth, excess gas production and abnormalities in the composition of the gut microbiota or combinations of these.

- Ingredient showing a prebiotic effect may counteract these disturbances as they were shown to modulate gut transit, decrease putrefactive activity within the gut lumen, prevent GI infections, and mitigate inflammatory responses.
- To date, there are only two published studies of adequate study design testing such ingredient in IBS. Both studies improved the subjects' symptoms.

4.3 Prebiotic effects and IBD

4.3.1 Introduction

Inflammatory bowel disease (IBD) is a chronic relapsing and remitting disorder characterised by inflammation, ulceration and stricturing of the GI tract. Ulcerative colitis (UC) and Crohn's disease (CD) are the two main types of IBD. In Europe, the incidence ranges from 1.5 to 20.3 cases per 100,000 person-years for UC and from 0.7 to 9.8 cases per 100,000 person-years for CD, meaning that up to 2.2 million people in Europe currently live with IBD ([168]).

Ulcerative colitis causes continuous mucosal inflammation that is restricted to the colon whereas CD causes discontinuous transmural inflammation anywhere throughout the GI tract, although it most frequently affects the terminal ileum ([169]). Symptoms common to both UC and CD include diarrhoea, faecal urgency and incontinence. Severe abdominal pain and rectal bleeding are common and complications such as fissuring and abscesses may occur. These symptoms can have a profound impact on patients, with evidence of impaired nutritional status ([170]) and quality of life ([171]).

The primary treatment approach in IBD is usually drug therapy. Patients can be treated with a variety of drugs, including 5-ASAs (e.g. mesalazine), steroids (e.g. prednisolone) and immunosuppressants (e.g. azathioprine). In addition, patients with CD may also receive new biological drugs such as monoclonal antibodies (e.g. the anti-TNF-α antibody infliximab) when standard drug treatment fails ([172]). Despite their general efficacy, such drugs can carry a significant burden. They are not only expensive, but side effects are common, with an incidence of 28% for immunosuppressants, rising to 50% for steroids ([173]). In addition, approximately 30% of patients with UC and 50% of patients with CD

will require surgery at some point in their life ([173]). In the case of UC, a colectomy and formation of an ileo-anal pouch may be curative. However, following this procedure, a minority of patients will experience relapsing, remitting pouch inflammation, described as pouchitis.

Nutritional approaches to treating IBD have been investigated. In clinical trials, enteral nutrition has been shown to induce remission in 60-85% of patients with CD, however it remains less effective than steroids ([174]) and patients report problems with palatability and abstinence from food ([175]). In view of these findings, safe and effective interventions that induce and maintain remission in IBD with a low incidence of side effects are urgently needed.

In order to identify potential therapeutic targets for IBD, examination of its pathogenesis is required. Although the precise mechanisms are not yet known, it appears that IBD results from a heightened mucosal immune response to the GI microbiota in genetically susceptible individuals.

The immunological processes underlying IBD involve alterations in the balance of proinflammatory and immuno-regulatory cytokines within the mucosal immune system. Much of the inflammation is mediated via cytokines released by activated Th1/Th17 lymphocytes. In addition, tumour necrosis factor (TNF)-α has been shown to play a key role, exerting its effects via stimulation of other proinflammatory cytokines such as interleukin (IL)-1, IL-6 and interferon (IFN)-γ. Each of these proinflammatory cytokines have been shown to be elevated during active IBD ([176]), and biological therapies such as anti-TNF-α-antibodies directly target this immunological cascade. Other proinflammatory cytokines include IL-12 and IL-18, both of which are involved in IFN-γ production. In contrast, the immuno-regulatory response is mediated by cytokines such as IL-10, which downregulates IFN-γ production ([177]). Furthermore, some animal studies have indicated immuno-regulatory roles for IL-4 and transforming growth factor (TGF)-β in IBD ([178]).

There is convincing evidence that the inflammation observed in IBD is driven by the GI microbiota. For example, it has been shown that animal models of IBD do not develop inflammation when reared in germ-free conditions, whereas they subsequently develop inflammation once transferred to non-sterile conditions or are artificially colonised with bacteria ([179]). Similar observations have been described in humans with IBD. In patients with colonic CD, formation of an ileostomy, which diverts the faecal stream away from the site of inflammation, results in disease remission in 65% of patients,

whilst reversal of this procedure results in disease relapse in 60%, implying that the content of the faecal stream is in part responsible for driving inflammation ([180]). Patients with active IBD also have elevated GI permeability, thereby increasing the exposure of the mucosal immune system to the resident microbiota ([181]). An underlying pathogenic mechanism linking CD and the GI microbiota was realised when it was found that mutations in the caspase activating recruitment domain 15 (CARD15) gene, involved in bacterial recognition, were found to result in a 38 fold increase in risk for CD ([182]). Interestingly, this mutation does not result in a higher risk of UC and further genome wide association studies have identified numerous other mutations associated with increased risk of either UC or CD but that are unrelated to bacterial recognition or sensing ([183]). Therefore, there are clearly genetic and environmental triggers related to the onset of IBD other than those involving the GI microbiota.

Despite the evidence that the GI microbiota is necessary to drive the inflammation in IBD, some bacteria may indeed protect the mucosa from such inflammation. Studies in both animals models and patients with IBD have shown that some bacteria decrease abnormal GI permeability ([184; 185]), thereby reducing exposure of the mucosal immune system to the GI microbiota. Meanwhile, some probiotics, in particular bifidobacteria, upregulate immuno-regulatory IL-10 production by dendritic cells ([186; 187]), the production of which is therapeutic in animal models of IBD ([177]). In view of this, studies have shown some success of both antibiotics and probiotics in the management of IBD and these have been extensively reviewed elsewhere ([188; 189]).

Components of the GI microbiota therefore drive proinflammatory and/or immuno-regulatory cytokine production during IBD. Interestingly, numerous studies demonstrate alterations in the GI microbiota of patients. Such studies are varied, utilising a wide variety of microbiological techniques (e.g. traditional culture; molecular microbiology) in different samples (i.e. faeces, inflamed mucosa, non-inflamed mucosa). Comparisons have been made between UC and/or CD and/or healthy controls, and these vary as to whether patients were in relapse or remission. Consequently, studies of the GI microbiota in IBD are too varied to review in detail here. However, some conclusions can be drawn regarding the alterations in GI microbiota in IBD that suggest that ingredients showing a prebiotic effect may be of potential benefit in its treatment or maintenance.

In general studies adopt two different approaches to investigating the microbiota in IBD. Some investigate differences in concentration, proportion or diversity of microbial communities (i.e. dysbiosis

theory), whereas others investigate the presence or absence of selected species (i.e. single strain theory). For example, patients with inactive CD have been shown to have lower proportions of faecal bifidobacteria ([190; 191]), whereas both patients with active UC or active CD have lower faecal bifidobacteria, Clostridium coccoides and Clostridium leptum compared with healthy controls ([191]). Lower concentrations of bifidobacteria ([192; 193]) and higher concentrations of bacteroides ([194]) have also been found in the mucosa of both patients with UC or CD. Meanwhile, another study has shown that some patients with CD or UC have lower numbers of mucosal Firmicutes and Bacteroidetes ([195]). Increased presence of Escherichia coli has been demonstrated in patients with UC or CD ([196; 197]) and more recently, lower concentrations of Faecalibacterium prausnitzii were found in the faeces of patients with CD or UC compared with controls ([191]). This is important as Faecalibacterium prausnitzii is immuno-regulatory and higher mucosal concentrations are associated with longer maintenance following surgically-induced remission of CD ([198]).

In view of the role of the certain components of the GI microbiota in driving intestinal inflammation, combined with the apparent dysbiosis in IBD, the use of ingredients showing a prebiotic effect as an approach to modifying the microbiota in order to induce or maintain remission in IBD has been investigated.

The prebiotic concept is defined as the selective stimulation of growth and/or activity of one or a limited number of microbial genera, species or strains in the gut microbiota that confers health benefits to the host. Ingredients showing a prebiotic effect have been shown to increase faecal and mucosal bifidobacteria in healthy subjects ([199; 200]). This is relevant because bifidobacteria are present in lower concentrations in the faeces and mucosa of patients with IBD ([191; 193]), whilst *in vitro* experiments have shown that some species of bifidobacteria stimulate IL-10 production, potentially via interaction with toll-like receptors (TLR) on lamina propria dendritic cells ([186]). In addition, prebiotic ITF have recently been shown to increase concentrations of Faecalibacterium prausnitzii in healthy subjects ([201]), although this has not yet been confirmed in patients with IBD. Furthermore, SCFAs, produced through the fermentation of such ingredients, modulate inflammation, with cell culture studies showing that butyrate inhibits pro-inflammatory IL-2 and IFN-γ production and acetate and propionate increases immuno-regulatory IL-10 production ([95]).

Numerous experiments have been conducted to investigate the impact of these ingredients on chronic intestinal inflammation in animal models of inflammatory bowel disease, and these have been reviewed elsewhere ([202]). However at the current time, their use amongst patients with IBD remains relatively low ([203]). However, over the last decade there has been an increase in the number of clinical trials investigating their use in inducing or maintaining remission in IBD (Table 11).

4.3.2 Prebiotic effects in pouchitis

Two studies have been identified that investigate the use of ingredients showing a prebiotic effect in patients with pouchitis. The first, published in abstract form only, involved 10 patients with active pouchitis who were treated with a synbiotic combination of *Lactobacillus rhamnosus* GG and ITF in an open label study in whom 'all patients experienced complete clinical and endoscopic remission' ([204]). Unfortunately, further details of the outcomes are limited and the cause of any benefit, be it a placebo effect, the probiotic, a prebiotic effect or a combination, is unclear. In a larger, controlled study, 20 patients with inactive pouchitis were randomised to consume 24 g/d ITF or placebo for 3 weeks in a cross-over study ([205]). There was a significant reduction in pouchitis disease activity index during the ITF intervention, despite nobody having active disease. In addition, there was a reduction in faecal *Bacteroides fragilis* and an increase in butyrate. Interestingly, bifidobacteria remained unchanged, perhaps due to the absence of a colon preventing the complete fermentation and prebiotic effects of the ITF to be realised. Clearly, larger parallel controlled trials in both active and inactive pouchitis are warranted.

4.3.3 Prebiotic effects in ulcerative colitis

Two trials have used ingredients showing a prebiotic effect to investigate their efficacy in the management of UC. The first was a pilot study of 18 patients with active UC, who were randomised to receive either a synbiotic (6g/d of ITFand *B. longum*) or a placebo. Only 14 completed the study (8 intervention, 6 control) and there was no difference in clinical scores between the intervention and control group, but there was a lower degree of inflammation ([159]). In addition, there was an increase in

mucosal bifidobacteria, decrease in TNF-α, IL-1α and antimicrobial human β-defensin peptides in the synbiotic group. Although this data suggests promising effects, the use of a synbiotic combination makes it difficult to ascertain the specific effects of the prebiotic on clinical outcome.

In another pilot study in active UC, 19 patients were randomised to receive either an ingredient showing a prebiotic effect (12 g/d of ITF) or placebo, in conjunction with 3 g/d mesalazine for two weeks ([160]). Only 15 patients completed the study (7 intervention, 8 control) and although there was a reduction in disease activity, this occurred in both groups, potentially due to them both starting concomitant drug therapy. However, compared with placebo, the intervention group had significantly lower concentrations of the inflammatory marker faecal calprotectin. This trial provides the first indicator that a prebiotic alone may be of benefit in treating active UC. Its major limitations include low numbers in each group, that increase the chance of type II errors, and a short treatment duration that may be insufficient to allow a prebiotic effect to translate into a clinical effect ([160]).

In addition to these, a number of studies in UC have investigated the use of compounds that although described as prebiotic, are not generally considered to be so. Trials of these fibre compounds have therefore not been included in Table 11. For example, a series of studies have shown that germinated barley foodstuff increases remission rates when used to treat active UC ([206]) and results in longer remission when used in maintenance of UC ([207]). More recently a trial of psyllium or the probiotic *Bifidobacterium longum* did not result in a significant improvement in quality of life or reduction in serum C-reactive protein, whereas when used together they did ([208]).

There remains little data on the clinical, microbiological and immunological effects of prebiotics specifically in maintaining remission in UC.

4.3.4 Prebiotic effects in Crohn's disease

In a small, open-label study a semi-elemental enteral formula containing ingredients showing a prebiotic effect (4 g/L of ITF) was fed via nasogastric tube as a sole source of nutrition for six weeks to 10 children with active CD ([209]). There was a reduction in disease activity alongside improvements in markers of inflammation including reduced erythrocyte sedimentation rate and improved white cell

scans. In light of the evidence for the efficacy of enteral nutrition in inducing remission in active CD ([174]), this study design does not allow the clinical consequences of the prebiotic effect to be separated from those of the enteral nutrition.

A small open label study of ingredients ITF (15g/d) in patients with active CD, demonstrated a significant reduction in disease activity after three weeks, with 4 out of 10 patients entering disease remission ([111]). In addition, faecal, but not mucosal, bifidobacteria increased and there was an increase in dendritic cell IL-10 production together with TLR-2 and TLR-4 expression. Clearly caution is required in interpreting and applying the results of this small uncontrolled trial.

The same group have recently presented the clinical data from a large double-blind, randomised, placebo-controlled trial of ITF (15g/d) in 103 patients with active CD ([210]). Analysed on an intention-to-treat basis there were no significant differences in disease activity or the numbers entering disease remission between groups. However, as the data has only been presented as a conference abstract there is currently limited clinical data and no microbiological and immunological data published.

Finally, one study has investigated the effect of ingredients showing a prebiotic effect on preventing relapse in 30 patients following surgically induced remission of CD. This study supplemented a synbiotic *(Pediacoccus pentoseceus, Lactobacillus raffinolactis, Lactobacillus paracasei susp paracasei 19, Lactobacillus. plantarum*, 2.5 g β-glucans, 2.5 g ITF, 2.5 g pectin, 2.5 g resistant starch) or placebo for 24 months ([211]). In view of the long follow-up period, only nine patients completed the study (7 intervention, 2 control) and there were no differences in relapse rates between groups. It is noteworthy that the amount of the used ingredient contained within the synbiotic was relatively low.

4.3.5 Limitations of existing studies on prebiotic effects in IBD

Of the identified clinical trials of ingredients showing a prebiotic effect in IBD, numerous limitations in their reporting and trial design have been highlighted. Firstly, a number have only been published as conference abstracts ([204; 209; 210]), therefore impeding detailed data extraction. Many of the studies used different compounds, some with unconfirmed prebiotic properties, and in different doses. In addition, many of the studies use a synbiotic combination, making it unclear whether the probiotic, the

prebiotic or the combination is effective. The majority of the studies have poor study design, with numerous small pilot studies, some of which do not have control groups. Where control groups are used they do not always receive a placebo, making subjective outcomes such as patient reports of disease activity or quality of life difficult to interpret. This is important in view of the high placebo rates reported in clinical trials of IBD ([212; 213]). Furthermore, of the trials in CD none have analysed the influence of disease location, which may be important as ingredients showing a prebiotic effect may have different efficacy in colonic and ileal disease, due to the site of fermentation and augmentation of bacterial growth.

4.3.6 Key points

Inflammatory bowel disease results from a heightened mucosal immune response to the GI microbiota in genetically susceptible individuals.

Patients with IBD have a GI dysbiosis characterised by, amongst other things, lower concentrations of luminal and mucosal bifidobacteria, suggesting potential for prebiotic intervention Prebiotic effects have potential for benefit in IBD by increasing luminal and mucosal bifidobacteria and SCFAs concentrations and stimulating immuno-regulatory cytokine production.

Numerous small pilot studies have been conducted in pouchitis, UC and CD indicating potential benefit in treating active disease.

Although some larger trials have been conducted, they are generally limited in study design, interpretation and analysis, therefore definitive conclusions regarding the clinical efficacy of the prebiotic effect in IBD are not yet possible. One large RCT has demonstrated no clinical benefit of treating active CD with ingredients showing a prebiotic effect.

So far, results are substance- and study-specific, but do not warrant a conclusion for prebiotic effects in general.

None of the trials conducted thus far have reported concerns regarding the safety of ingredients showing a prebiotic effect in patients with IBD, and so their use at the doses used would appear safe.

4.3.7 Recommendations

Further large, multi-centre randomised, double-blind, placebo-controlled trials of ingredients showing a prebiotic effect in IBD are required. There is a particular lack of research on maintenance of remission of IBD and for the treatment colonic IBD (either UC or colonic CD).

Inter-disciplinary research is required that addresses clinical, as well as mechanistic, outcomes that are validated and relevant to this patient population.

In vivo and *in vitro* research is also required to further understand the mechanisms by which ingredients showing a prebiotic effect may achieve their potential benefit.

Healthcare professionals should keep informed of the latest evidence relating prebiotic effect in IBD. Not only is this an emerging area of research, with clinical trials currently underway, but it is also an area of interest to patients.

4.4 Prebiotic effects and colon cancer

4.4.1 Colon carcinogenesis- the role of diet and gut microbiota

Evidence suggests that diet plays an important role in the aetiology of colorectal cancer, However, identifying conclusively which constituents (e.g. vegetables, meat, fibre, fat, and micronutrients) exert an effect on risk has been more problematic due to inconsistent data. The 2007 World Cancer Research Fund report ([214]) concluded that the epidemiological evidence was convincing or probable for associations between overweight and obesity (in particular waist circumference), processed meat, alcohol and increased risk of colorectal cancer. Fibre, garlic, milk and calcium are associated with decreased risk. There are no published epidemiological studies on ingredients showing a prebiotic effect and cancer risk.

Evidence from a wide range of sources supports the view that the colonic microbiota is involved in the aetiology of cancer ([215]) and that bacterial metabolism of unabsorbed dietary residues and endogenous secretions is the origin of many of the genotoxic, and tumour promoting agents found in faeces ([216]).

4.4.2 Prebiotic effects and CCR (colorectal cancer)

It follows from the above, that modification of the gut microbiota may interfere with the process of carcinogenesis and this opens up the possibility for dietary modification of colon cancer risk. Prebiotic modulation of the microbiota by increasing numbers of lactobacilli and/or bifidobacteria in the colon, has been a particular focus of attention in this regard. Evidence that such an effect can influence carcinogenesis is derived from a variety of sources:

1- Effects on bacterial enzyme activities.

2- Antigenotoxic effects in vivo.

3- Effects on pre-cancerous lesions in laboratory animals.

4- Effects on tumour incidence in laboratory animals

5- Epidemiological and experimental studies in humans

4.5 Prebiotic protective effects and bacterial activities

4.5.1 Prebiotic effects and secondary bacterial enzyme activities.

The ability of the colonic microbiota to generate a wide variety of mutagens, carcinogens and tumour promoters including N-nitrosocompounds, secondary bile acids, ammonia, phenols and cresols from dietary and endogenously-produced precursors is well documented ([215; 217]). In addition, the bacterial enzyme ß-glucuronidase is involved in the release in the colon from their conjugated form of a number of dietary carcinogens, including polycyclic aromatic hydrocarbons.

Ingredients showing a prebiotic effect should not stimulate bacteria capable for such metabolism. During *in vivo* experiments this should result in an overall decrease in toxic substances.

In general, species of Bifidobacterium and Lactobacillus, have low activities of enzymes involved in carcinogen formation and metabolism by comparison to other major anaerobes in the gut such as bacteroides, eubacteria and clostridia ([218]). This suggests that increasing the proportion of these two lactic acid bacteria (LAB) in the gut could modify, beneficially, the levels of xenobiotic metabolising enzymes. It may lead to decreases in certain bacterial enzymes purported to be involved in the synthesis or activitation of carcinogens, genotoxins and tumour promoters. Such manipulations have been suggested to be responsible for decreased levels or preneoplastic lesions or tumours in animal models ([219; 220]) and suggests a reduction in the damaging load.

Studies in laboratory animals have in general shown that ITF and galactans decrease caecal enzyme activities ([221; 221; 222]). However, human studies have yielded inconsistent or negative results on such enzyme activities or on production of toxic bacterial metabolites such as ammonia and phenols ([65; 223; 224]).

4.5.2 Prebiotic and synbiotic effects on pre-cancerous lesions in laboratory animals

Aberrant crypts (AC) are putative pre-neoplastic lesions seen in the colon of carcinogen treated rodents. In many cases a focus of two or more crypts is seen and is termed an aberrant crypt focus (ACF). Aberrant crypts are induced in colonic mucosa of rats and mice by treatment with various colon carcinogens such as azoxymethane (AOM), DMH and IQ ([225]).

Ingredients showing a prebiotic effect alone appear to give inconsistent results on carcinogen induced ACFs which may be partly a consequence of differences in carcinogen and treatment regimes used. For example Rao *et al* ([226]) reported that ITF (10% in diet) had no significant effect on total ACF in colon, or their multiplicity, in F344 rats, although curiously a significant decrease in ACF/cm2 of colon was reported. A study by Gallaher *et al* ([227]) on Bifidobacterium spp and FOS (2% in diet) gave inconsistent results with only 1 out of 3 experiments showing a decrease in DMH-induced ACF. In contrast Verghese *et al* ([228]), reported a dose-dependent decrease the incidence of ACF and total crypts ($P<0.01$) after ITF supplementation (0, 2.5, 5 and 10 g /100 g diets) in AOM challenged rats.

The effects of prebiotics on ACF may be dependent on the chain length of the ITF, since a number of studies report more potent inhibition by longer than by shorter chains ([229-231]). For example,

Buddington *et al* ([230]) reported that inulin (10% in diet), but not oligofructose fed mice had significantly lower ACF numbers than controls

Some studies have found that ITF have differential effects on ACF and tumours. For example Jacobson *et al* ([232]), reported that oligofructose or long chain inulin (15% in diet) increased the number of ACF but significantly reduced the tumour incidence. A study by Caderni *et al* ([233]) showed similar results when rats were fed the synbiotic containing ITF alongside *Lactobacillus GG*, *L. delbrueckii* subsp. Rhamnosus and *Bifidobacterium lactis* Bb12. Supplementation caused increased ACF multiplicity after 16 weeks, however significantly reduced tumour incidence following 32 weeks in AOM challenged rats.

There are limited studies on ingredients showing a prebiotic effect other than ITF in this area. Challa *et al* ([234]) demonstrated a small reduction (22%) in total ACF in AOM treated F344 rats when the synthetic, non-digestible disaccharide lactulose was incorporated in the diet at 2%. Hsu CK *et al* ([235]) compared the influence ITF (60 g/kg) and xylo-oligosaccharides supplementation on DMH induced aberrant crypts in ratsreporting a decrease in the mean number of multicrypt clusters of aberrant crypts by 56 and 81%, respectively ($P<0.05$). Wijnands et al ([236]) compared AOM-induced ACF in F344 rats fed diets containing low or high GOS (5% vs 20% w/w of a GOS syrup comprising 38% GOS). There were no significant differences between the dietary groups in total ACF after 7 or 13 weeks of treatment although there was a significant decrease in ACF multiplicity in the high GOS fed group (4.4 vs 3.07 $P<0.5$).

Both Challa *et al* ([234]) and Rowland *et al* ([220]) studied the effect of combined treatment of probiotic and prebiotic on ACF numbers. The combination of *Bifidobacterium longum* and lactulose resulted in a 48% inhibition of colonic ACF, which was significantly greater than that achieved by either *Bifidobacterium longum* or lactulose alone ([234]). Similarly Rowland *et al* reported a decrease in total ACF of 74% in rats given *Bifidobacterium longum* + ITF (by comparison to 29% and 21% reduction achieved by *Bifidobacterium longum* or ITF alone). Importantly, the combined administration of probiotic and prebiotic reduced large ACF by 59% whereas the individual treatments had no effect ([220]). Nakanishi *et al* ([237]) showed that supplementation with *Clostridium butyricum* (CB) in AOM

challenged rats had no significant effect on ACF occurrence. However, CB supplemented alongside high amylose maize starch (a poorly digestible carbohydrate) decreased the number of ACF significantly (P<0.05) indicating a degree of synbiotic activity.

4.5.3 Prebiotic effects and colon tumour incidence in laboratory animals

There are fewer reports on prebiotic and synbiotics than on probiotics in terms of tumour incidence but overall the studies indicate protective effects. Jacobsen *et al* ([232]) compared the incidence of tumours in AOM challenged rats following consumption of ITF (15 % diet w/w). Significantly less rats developed colon tumours in the treated group (P<0.05) compared to the control diet. The total number of tumours developed per rat was significantly reduced following both oligofructose (P<0.01) and Inulin (P<0.05) supplementation. However supplementation had no effect on the malignancy of the tumours. Wijnands et al ([238]) compared the effect of cellulose and GOS syrup on induction of DMH-induced colorectal tumours in Wistar rats consuming basal diets containing low, medium or high fat content. The cellulose diets contained 4.5 - 5.2% w/w (low cellulose) or 22.6 - 24.5% (high cellulose) and the GOS syrup diets 8.3 – 9.5% (low GOS) or 26.3 – 28.7% (high GOS). The GOS syrup used comprised 38% GOS with additional lactose, glucose and galactose, thus the high GOS diets contained about 10.5% dry weight GOS. The cellulose content of the diet had no effect on total tumours, but high cellulose increased adenomas and significantly decreased carcinomas. There were no significant effects of high GOS diets on tumour incidence. Multiplicity of tumours (i.e. number per tumour-bearing animal), both adenoma and carcinoma was significantly decreased in the hig GOS fed group.

Femia *et al* ([239]) investigated the protective effects of prebiotic (ITF), probiotic (Bifidobacterium lactis Bb12 and Lactobacillus rhamnosus GG, (5x108 CFU/g diet) or synbiotic combination of the two, against AOM-induced colon tumours in rats. Prebiotic fed groups (prebiotic and synbiotic groups) resulted in lower adenoma (P < 0.001) and adenocarcinoma (P<0.05) incidence than in the rats not given prebiotic (probiotic & control). Interestingly, in the groups treated with probiotics (probiotic and synbiotic groups) the proportion of cancers relative to the total number of tumours was significantly lower (P=0.04) (9 cancers out of 84 tumours [11%]) than in the control and prebiotic groups (19

cancers out of 83 tumours [23%]), suggesting a protective effect of probiotics, but not ingredients showing a prebiotic effect, on development of malignant tumours.

In the transgenic Min mice model, the mice develop spontaneous adenomas throughout the small intestine and colon within a few weeks. Results from studies on ITF in this model have been conflicting, with both inhibitory and stimulatory effects on tumours reported. In one study Min mice were fed various diets containing wheat bran, resistant starch or oligofructose (5.8% in diet) for 6 weeks. Tumour numbers remained unchanged from the control (low [2%] fibre diet) in the mice fed either wheat bran or resistant starch, but a significant reduction in colon tumours was observed in rats receiving the diet supplemented with oligofructose. Furthermore 4 out of the 10 oligofructose fed animals were totally free of colon tumours ([240]). These results contrast with those of Mutanen and co-workers using the same model. In the first of their studies, Min mice fed a purified high fat (40% energy) diet with 2.5% ITF showed non-significant increases in adenomas in the small and large intestins compared with the control animals fed the high fat, fibre -free di*et al*one ([241]). A subsequent study ([242]) using a higher ITF dose (10%) confirmed these results with increases, again non-significant, being seen in the number of adenomas in the small intestine and colon and significant increases in tumours in the distal small intestine after 9 weeks of treatment. Interestingly, although the adenoma size in the small intestine was significantly increased in the inulin-fed mice, in the colon the size was reduced from 3.72mm to 2.54mm (non significant). It has been suggested that the reasons for the discrepancies in the Min mouse studies are due to major differences in the basal diet fed: high fat, high glucose diet in the Mutanen studies and high starch diet in the studies of Pierre *et al* ([78; 243]).

Taper & Roberfroid ([244]) investigated the effects in mice of inulin–type fructans or pectin (15% in the diet) on the growth of intramuscularly transplanted mouse tumours, belonging to two tumour lines - TLT (a mammary tumour) and EMT6 (a liver tumour). The growth of both tumour lines was significantly inhibited by supplementing the diet with non-digestible carbohydrates. In subsequent studies, the same authors demonstrated that ITF (15% in diet) reduced the incidence of mammary tumours induced in Sprague-Dawley rats by methylnitrosourea; and decreased the incidence of lung metastases of a malignant tumour implanted intramuscularily in mice ([245]).

4.5.4 Prebiotic effects in human intervention studies

For human intervention trials, cancer is an impractical endpoint in terms of numbers of subjects, cost, study duration and ethical considerations. An alternative strategy employed in recent studies is to use early or intermediate biomarkers of cancer such as DNA damage and cell proliferation in colonic mucosa and genotoxic activity of faecal extracts ('faecal water') ([246]).

In a larger scale, randomized, double blind, placebo-controlled trial, patients with resected polyps (n=37) or colon cancer (n=43) were given a synbiotic food supplement composed of ITF and the probiotics Lactobacillus rhamnosus GG and Bifidobacterium lactis Bb12 for 12 weeks ([247]). The effect of synbiotic consumption on a battery of intermediate biomarkers for colon cancer was examined. The intervention significantly reduced colorectal proliferation as assessed by *in vitro* [3H]thymidine incorporation and autoradiography in colorectal biopsy samples. Given the correlation between colorectal proliferativeactivity and colon cancer risk, these results suggest that synbiotics might be beneficial for patients with an increased risk of colon cancer. In addition in the polyp patients, the synbiotic intervention was associated with a significant improvement in barrier function as assessed by trans-epithelial resistance (TER) of Caco-2 cell monolayers after exposure to fecal water samples. This anti-promotion effect may reflect changes to the balance of SCFAs and secondary bile acids (deoxycholic acid and lithocholic acid) in the samples because these gut microbial metabolites have been shown to influence TER, beneficially and adversely respectively, in this system. Genotoxicity assays of colonic biopsies and faecal water indicated a decreased exposure to genotoxins in the polyp patients at the end of the intervention period.

Thus several colorectal cancer biomarkers were altered favorably by the intervention and the results show consistency with animal studies conducted in parallel ([239]).

Also of interest was the observation that the polyp patients and cancer patients appeared to respond differently to the synbiotic, as evidenced by the different effects observed on each biomarker.This may have been due to the fact that the intestinal microbiota was more refractory to changes induced by the synbiotic in the cancer patients than in the polyp patients.

4.5.5 Mechanisms of anticarcinogenicity and antigenotoxicity

4.5.5.1 Prebiotic effects and in vivo prevention of genotoxicity

More direct evidence for protective properties of probiotics and ingredients showing a prebiotic effect has been obtained by assessing the ability to prevent DNA damage and mutations (which are considered to be early events in the process of carcinogenesis) in cell cultures or in animals.

Using the technique of single cell microgel electrophoresis (Comet assay), the prebiotic effect of lactulose on DNA damage in the colonic mucosa has been evaluated. Rats that were fed a diet containing 3% lactulose and given dimethylhydrazine (DMH), exhibited less DNA damage in colon cells than similarly treated animals fed a sucrose diet. In the latter animals, the percentage of cells with severe DNA damage comprised 33% of the total compared with only 12.6% in the lactulose-fed rats ([248]).

Klinder *et al*. ([249]) also showed that the prebiotic effect of ITF and probiotic supplementation (8 months) caused a reduction in the genotoxicity of faecal and caecal samples obtained from azoxymethane-treated rats.

Rafter *et al* ([247]) investigated the influence of 12 weeks synbiotic supplementation (Lactobacillus rhamnosus GG (LGG) + *Bifidobacterium lactis* Bb12 + ITFmix) on selected cancer biomarkers in patients with resected colonic polyps or cancer. Synbiotic supplementation resulted in significant reductions in DNA damage in the colonic mucosa of polyp patients. The results provide evidence that both supplementation of LAB and prebiotic effects may be protective against the early stages of colon cancer.

Another important aspect to be considered in relation to the anti-toxic potential associated with a prebiotic effect is the formation of reducing equivalents, such as glutathione.. Food-borne carcinogens such as heterocyclic amines and polycyclic aromatic hydrocarbons are often conjugated with glutathione and thus inactivated. The enzyme involved, glutathione transferase (GSH) is found in the liver and in other tissues including the gut. Challa *et al* ([234]) showed in a study of the effect of a synbiotic (*B. longum* and lactulose) on azoxymethane (AOM)-induced aberrant crypt foci (ACF) in the rat colon that GSH in the colonic mucosa was inversely realted to the ACF numbers and higher with the synbiotic intervention Such an effect would be effective against a wide range of oxidative damage.

4.5.5.2 Effects on bacterial enzymes, metabolite production

As described in the section *Microbiota of the gastro-intestinal tract* of this paper, the increase in concentration of lactic acid bacteria (LAB) in the gut as a consequence of consumption of ingredients showing a prebiotic effect leads to decreases in certain bacterial enzymes purported to be involved in synthesis or activation of carcinogens, genotoxins and tumour promoters. This would appear to be due to the low specific activity of these enzymes in LAB ([218]). Such changes in enzyme activity or metabolite concentration have been suggested to be responsible for the decreased level of preneoplastic lesions or tumours seen in carcinogen-treated rats given pro and pre biotics ([219; 220]). Although a causal link has not been demonstrated, this remains a plausible hypothesis.

4.5.5.3 Production of anti cancer metabolites

Luminal SCFAs, in particular butyrate, are potential anti-carcinogenic agents within the gut. Butyrate is the preferred energy source of colonocytes and has been implicated in the control of the machinery regulating apoptosis and cellular differentiation. Perrin *et al*. ([250]) studied the effect of different forms of dietary fibre, a starch free wheat bran, a type 3 resistant starch and ITF on the prevention of ACF in rats. Their hypothesis was that, only fibres capable of releasing butyrate *in vitro* would be capable of preventing colon cancer. The resistant starch diet and the ITF diet both produced large quantities of butyrate and inhibited ACF formation, in contrast to the wheat bran diet that neither generated large amounts of butyrate nor protected against ACF formation.

4.5.5.4 Stimulation of protective enzymes

Many of the food-borne carcinogens such as heterocyclic amines and polycyclic aromatic hydrocarbons are known to be conjugated to glutathione, which appears to result in inactivation. The enzyme involved, glutathione transferase (GSH), is found in the liver and in other tissues including the gut. Challa *et al* ([234]) investigated the effect of Bifidobacterium longum and lactulose on AOM-induced ACF in the colon and showed that the activity of GSH in the colonic mucosa was inversely related to the ACF numbers. Such a mechanism of protection would be effective against a wide range of dietary carcinogens.

4.5.5.5 Apoptotic effects

The control of gene expression, cell growth, proliferation and cell death in multi-cellular organisms is dependent upon the complex array of signals received and transmitted by individual cells. Apoptosis or programmed cell death is one of the primary mechanisms by which multi-cellular organisms control normal development and prevent aberrant cell growth. Upregulation of apoptosis has received some attention recently as a potential mechanism of action of probiotics and ingredients showing a prebiotic effect.

Hughes & Rowland ([251]) fed 3 groups of rats one of three diets: basal, basal with oligofructose (5%w/w) or basal with long chain inulin (5%w/w), for three weeks. All animals were then dosed with 1,2-dimethylhydrazine and killed 24 h later. The mean number of apoptotic cells per crypt was significantly higher in the colon of rats fed oligofructose (P=0.049) and long chain inulin (P=0.017) as compared with those fed the basal di*et al*one. This suggests that such ingredients exert protective effects at an early stage in the onset of cancer, as the supplements were effective soon after the carcinogen insult. Comparison of the apoptotic indices between the two oligosaccharide diets showed no significant difference even though the mean apoptotic index was higher in animals fed long chain inulin.

4.5.5.6 Effects on tight junctions

Other studies have looked at cellular and physiological events associated with tumour promotion in the colon. For example, one feature of colonic tumour promotion is a decrease in epithelial barrier integrity.

Commane *et al* ([252]) showed using an *in vitro* model of tight junction integrity (transepithelial resitance) that metabolic products (probably SCFAs) derived from probiotics and ingredients showing a prebiotic effect fermentations were capable of improving tight junction integrity, suggesting that synbiotics may have anti tumour promoting activity.

4.6 Summary and conclusion

- Data from animal models as well as preliminary evidence in human study suggest reduction in the risk of colon cancer development associated with the prebiotic effects.

- Data from animal models, with endpoints such as DNA damage, aberrant crypt foci and tumours in the colon, suggest that reduction in the risk of colon cancer development is associated with prebiotic effects.
- Limited animal studies also indicate that combinations of pre- and probiotics may be more effective than either agent alone
- A pre+probiotics study in human subjects using putative biomarkers of cancer risk showed improvements in some, including a reduction in DNA damage and cell proliferation in colon biopsies. Further studies are needed
- A number of potential mechanisms for reduction in cancer risk by prebiotic effect, including changes in gut bacterial enzyme activities , upregulation of apoptosis and induction of protective enzymes have been explored in animal models, but currently evidence for such effects in humans is lacking

5 Prebiotic effects and mineral absorption[6]

Accumulating knowledge prompted the scientific community to consider compounds showing prebiotic effects as a source for putative innovative dietary health intervention for improvement of mineral retention. This particular effect of ingredients showing a prebiotic effect is indeed especially challenging because, among the bone builders, calcium is critical in achieving optimal peak bone mass and modulating the rate of bone loss associated with ageing, and is the most likely to be inadequate in terms of dietary intakes. Consequently, this specific property of prebiotics has been investigated extensively because if the mineral is inadequate during growth, the full genetic program for skeletal mass acquisition cannot be achieved. Then, if calcium intake is not enough to offset obligatory losses, acquired skeletal mass cannot be maintained, leading to osteoporosis, a major public health problem.

Moreover, biological properties of ingredients showing a prebiotic effect could extend far beyond, with potential improvement of other minerals bioavailability, including magnesium, iron or zinc.

5.1 Rationale behind the prebiotic effects on mineral absorption

Calcium

The most compelling data have demonstrated that ingredients showing a prebiotic effect lead to increased calcium absorption. As such ingredients are resistant to hydrolysis by small intestinal digestive enzymes, they reach the colon virtually intact, where they are selectively fermented by the microbiota ([253; 254]). This colonic fermentation produces SCFAs and other organic acids that contribute to lower luminal pH in the large intestine which, in turn, elicits a modification of calcium speciation and hence solubility in the luminal phase so that its passive diffusion is improved ([255-257]). SCFAs are also likely to contribute directly to the enhancement of calcium absorption via a cation exchange mechanism (increased exchange of cellular H+ for luminal Ca2+) ([258]).

Further, these ingredients may also modulate transcellular active calcium transport by increasing calbindin D9K expression in the cecum and colorectum (the intracellular carrier protein involved in the translocation of calcium to the basolateral membrane of mucosal epithelial cells) ([259; 260]).

[6] The main authors of this section are Dr. Coxam, Dr. Davicco, Dr. Léotoing and Dr. Wittrant

Another way to contribute to the enhanced mineral absorption is the trophic effect of prebiotics on the gut (cell growth and functional enhancement of the absorptive area; ([261]). It has been suggested that this is mediated by an increased production of butyrate and/or certain polyamines ([253]). Rémésy *et al*. ([255]) have shown that inulin is able to stimulate ornithine decarboxylase, the rate-limiting enzyme for polyamine synthesis. Nevertheless, Scholz-Ahrens & Schrezenmeier ([262]) failed to show that polyamines mediate this effect.

In summary, ingredients showing a prebiotic effect help to increase calcium bioavailability by extending the site of mineral absorption (through the tight junctions between mucosal cells in the small intestine) towards the large intestine.

Other minerals

With regard to the magnesium, most of the potential of ingredients showing a prebiotic effect on its absorption are similar to those described for calcium, but less clear. They include increased magnesium solubility and absorption due to reduced colonic pH ([263]). Nevertheless, significant effects on magnesium retention have been demonstrated in dogs, despite the lack of any change in fecal pH ([264]). It is also possible that SCFAs affect magnesium absorption ([265]), butyrate being more efficient than propionate or acetate ([266]), probably via a cation exchange mechanism. Indeed, butyric acid is able to enhance the intestinal uptake by activation of an apical Mg2+/2H+ antiport through the provision of protons within the epithelial cell.

Iron and zinc balance can be improved by consumption of these ingredients however, animal studies have failed to show any significant effect on copper bioavailability ([267]).

5.2 Summary of key studies (Table 12)

5.2.1 Animal study (Table 13 & 14)

Animal studies targeting the effect of prebiotics on calcium absorption are listed on the Tables 13 and 14. The points arising from these studies are the following:

- -Different types of molecules have been studied, including ITF-$_{Dpav\ 3\text{-}4,}$ ITF-$_{Dpav\ 12,}$ ITF-$_{Dpav\ 25}$, ITF-$_{MIX}$, GOS, lactulose or resistant starch.

- -Dietary supplementation with ITF enhances the uptake of calcium, improves bone mineral content (BMC) in growing rats and alleviates the reduction in BMC and bone mineral density (BMD) which follows ovariectomy or gastrectomy in rats.

5.2.2 Clinical trials (Table 15& 16)

In infants

The only available study targeting the prebiotic effect on mineral metabolism in infants was conducted in 6 to 12 months healthy formula-fed babies. Even though, ITF did not elicit any modulation of faecal SCFAs concentration, a beneficial effect on both iron and magnesium absorption and retention was reported. No significant difference was observed for calcium, copper or zinc ([268]).

In adolescents

As far as adolescents are concerned, in 1999, Van den Heuvel *et al*. ([269]) demonstrated that a daily consumption of 15g of ITF for 9 days stimulated fractional calcium absorption by 10% in young boys (14-16y). Later on, Griffin *et al*. ([270]) provided the evidence that modest intake of ITF_{mix}, corresponding to 8g per day, stimulated calcium absorption in 60 girls at or near menarche. The increase reached about 30% after 3 weeks of consumption, when compared with oligofructose only or placebo intakes. This effect was mostly observed in girls with lower calcium absorption status ([271]). Moreover, when given for 36 days to adolescent girls (12-14y), 10 g of $ITF\text{-}_{Dpav\ 3\text{-}4}$ were able to stimulate magnesium absorption (18%), without affecting calcium absorption, vitamin D or parathyroid (PTH) serum concentration or urine concentration which are used as markers of bone resorption ([272]).

The longest and most compelling study, is a 1 year intervention trial on pre-pubertal girls and boys (n= 100) that found significantly increased calcium absorption in the group receiving $ITF\text{-}_{MIX}$ (8g per day) after 8 weeks. The effect lasted throughout the intervention period resulting, after 1 year, in improved whole body BMC and significantly increased BMD, compared to the controls ([273]). This demonstrates a beneficial effect on long-term use of this particular mixture on calcium absorption and bone mineralization in young adolescents. ([274]). A further study by Abrams *et al.*showed that responders to the “treatment” had greater calcium absorption and increased accretion of calcium to

the skeleton, and thus concluded on the importance of such a strategy to enhance peak bone mass, as the extra absorbed calcium is deposited in bones ([275]).

In adults

It has been previously shown, using the metabolic balance methodology, that addition of up to 40g per day of ITF and sugar beet fibres, to a normal mixed diet for 28 days improved calcium balance, without adverse effects on the retention of other mineral ([276]). However, a study carried out by Van den Heuvel *et al*. ([277]) in healthy young adults, found no significant differences in mineral absorption, irrespective of the treatment (which consisted of a constant basal diet supplemented for 21 days with 15g/d ITF, or galacto-oligosaccharide, or not supplemented) followed by a 24 hour urine collection. It was hypothesised that a 24 h period of urine collection, used in the study, was too short to include the colonic component of calcium absorption and thus to make up a complete balance necessary to detect the effect of ITF. In a similar way, Teuri *et al*. ([278]), investigated a combination of 15g of ITF and 210mg of calcium added to 100g of cheese given at breakfast to 15 adult healthy women with an average age of 23 years old. The study failed to show any significant influence of the diet on blood ionized calcium or PTH concentration over the 8h assessment period. Nevertheless, measuring serum PTH and ionised calcium do not provide direct information about calcium absorption, as do isotope techniques, and it has been suggested that he length of the trial was probably too short. Moreover, the addition of 1.1 g ITF-$_{Dpav\ 3\text{-}4}$ or caseinophosphopeptides to calcium-enriched milks, a valuable source of well-absorbed calcium, did not significantly increase calcium absorption in adults (25-36y), independently of sex ([279]). Finally, Abrams *et al*. ([280]) gave to 13 young adults (average age of 23y) a supplementation containing 8g of ITF-$_{MIX}$ for 8 weeks. Eight of the 13 volunteers were classified as responders, based on their level of calcium absorption.

In postmenopausal women

Ducros *et al*. ([281]) carried out a clinical trial in postmenopausal women (age between 50-70 years with at least 2 years of menopause). The volunteers were provided with 10g/d ITF-$_{Dpav\ 3\text{-}4}$ or a placebo for 5 weeks using a cross-over design. They demonstrated that consumption of ingredients showing a prebiotic effect was associated with increased copper absorption, while no significant effect could be demonstrated on zinc or selenium bioavailability.

In a similarly designed double-blind randomised, crossover design, post-menopausal women without HRT (*please explain abbreviation)* were given 10g of ITF-$_{Dpav\ 3-4}$ daily for 5 weeks. Magnesium absorption and status was determined using mass spectrometer analysis in faeces, urine and blood. Results showed that the ITF-$_{Dpav\ 3-4}$ -enriched diet increased magnesium absorption by 12.3%, compared to the placebo sucrose control group ([282]). In the same experiment, Tahiri *et al*. ([283]) showed that over 5 weeks of a moderate daily dose (10 g) of ITF-$_{Dpav\ 3-4}$ failed to modify intestinal calcium absorption in the early postmenopausal phase, while, in the subgroup of late phase (women who had been going through menopause for more than 6 years), an increase in calcium absorption was observed.

Twelve older postmenopausal women (of at least 5 years past the onset of menopause) drank 100 ml of water containing 5 or 10 g of lactulose or a reference substance at breakfast for 9 days. True fractional calcium absorption was calculated using calcium isotope ratios and consumption of lactulose was found to increase calcium absorption in a dose-response way ([284]).

In a crossover trial, 12 postmenopausal women were given a 200 ml yogurt to drink twice a day (at breakfast and lunch) containing either GOS (20g) or sucrose for 9 days; a greater true calcium absorption (16%) was observed after consumption of a product rich in GOS. In addition, no increased urinary calcium excretion was observed, suggesting that GOS could also indirectly increase the uptake of calcium by bones and/or inhibit bone resorption ([285]).

Adolphi *et al*., ([286]) tested, the hypothesis that, in postmenopausal women (between 48 and 67 y and who had been postmenopausal for 10.5 ± 0.7 y), consumption of fermented milk (supplemented with calcium) at bedtime could prevent the nocturnal peak of bone resorption by decelerating its turnover, and that this effect could be improved by adding calcium absorption enhancers. Actually, they showed that indeed such a practice can reduce the nocturnal bone resorption and that supplementation with calcium had no additional effect unless absorption enhancers such as ITF and caseinphosphopeptides were added.

Kim *et al*. ([287]) who investigated the effects of ITF supplementation (8g/d for 3 months) in postmenopausal women (mean age: 60 y) showed that apparent calcium absorption was significantly increased by 42% in the ITF group, while a 29% decrease was robserved in the placebo group. This was associated with lower alkaline phosphate plasma levels (a parameter which is actually not specific of bone formation) and a trend toward a slight reduction in urinary deoxypyridinolin (a

biomarker for bone resorption). As expected, due to the very short length of exposure, BMD was not modified by the treatment.

Finally, 15 women (who were a minimum of 10 y past the onset of menopause and had taken no hormone replacement therapy for the past years) were treated with 10g/d of a specific mixture of ITF for 6 weeks, according to a double-blind placebo controlled crossover design. True fractional calcium absorption, measured by dual isotopes before and after treatment, was significantly increased (+7%) in women with lower initial BMD ([288]).

In institutionalized patients

Bone resorption, used as indicator of calcium retention, remained unchanged in institutionalized adults after 3 weeks of treatment with 13g per day of ITF-fortified beverages ([289]).

5.3 Outline of general rules

5.3.1 Involvement of the colon

The main points arising from the available studies are that the calcium sparing effect elicited by a prebiotic effect involves colonic absorption. Indeed, using *in vitro* Ussing chambers Raschka & Daniel ([261]) provided the evidence of the effect of ITF-$_{MIX}$ on transepithelial calcium fluxes in rat large intestine.

Levrat *et al*. ([290]) showed that dietary ITF given in the range of 0 to 20% in the diet stimulated intestinal calcium absorption in a dose dependent manner, coinciding with a progressive decrease in caecal or ileal pH, hypertrophy of caecal walls and a rise in caecal pool of SCFA.

Moreover, Ohta *et al*. ([256]) demonstrated that in rats fed a ITF-containing diet, but not in those given a control diet, the ratio of calcium or magnesium to chromium (chromium being used as an unabsorbable marker to calculate apparent absorption of calcium and magnesium) were correlated with the fractional length of transit along the colon and rectum, indicating linear disappearance of calcium and magnesium during the colorectal passage. Consequently, in cecectomized rats, ITF failed to increase calcium absorption ([291]).

Similarly, in patients with conventional ileostomy, data analysis of ITF effects on mineral absorption and excretion (Mg, Zn, Ca, Fe) showed no significant influence ([292]).

This offers an explanation as to why Van den Heuvel *et al*. ([277]) found no significant differences in mineral absorption in healthy young adults, irrespective of the treatment they received (consisting of a constant basal diet supplemented for 21 days with 15g/d ITF, or galacto-oligosaccharide, or not supplemented), as the 24 h period of urine collection used in this study was too short to include the colonic component of calcium absorption and thus to make up a complete balance necessary to detect the effect of fructans.

Indeed, Abrams *et al*. ([280]) gave young adults (average age of 23y) 8 g of ITF-$_{MIX}$ for 8 weeks, and confirmed that calcium absorption after treatment occurred principally in the colon (69.6 ± 18.6%).

Nevertheless, it is still unclear whether the calcium sparing effect results from induction of specific bacterial strains or from their "colonic food" activity ([293]).

5.3.2 Dose effect

Various doses of ITF have been investigated ranging from 1.1 g/d to 17 g/d (and even 40g/d in one case). A minimum level of 8 g/d seems to be required to elicit an improvement on both calcium absorption and bone mineralisation. Indeed, Lopez-Huertas *et al*. ([279]) explained the lack of effect of the addition of 1.1g ITF or caseinophosphopeptides to calcium-enriched milks in adults by the very low dose provided in the diet.

However, with regards to animal studies, ITF appears to exhibit a dose-dependent effect on calcium absorption, as well. Levrat *et al*. ([290]) showed that dietary ITF given in the range of 0 to 20% in the diet stimulated intestinal calcium absorption in a dose dependent manner. Similarly, in the study carried out by Brommage *et al*. ([294]), a near linear increase in calcium absorption was demonstrated in rats fed a 5 and 10% lactulose containing diet. Nevertheless, it appears that when a minimum is reached, calcium absorption enhancement occurs whatever the dose, as a diet supplemented with either 10% of ITF ([267]) or 5% of oligofructose or other non-digestible carbohydrates ([294]) leads to a similar increase (about 60-65%) of the apparent absorption of calcium, even though, raising the content of oligofructose in the diet from 2.5 to 10% in ovariectomized rats, a bone sparing effect has been shown, independent of the dose by Scholz-Ahrens *et al*. ([295]).

5.3.3 Test substances

Various substances such as the different types of ITF, GOS, soy-oligosaccharides, lactulose, or resistant starch have provided evidence of a positive effect on calcium absorption, at least in the rat. However, the biological effect is likely to be related to the rate of fermentation which is mainly dependent on the degree of polymerisation, as well as the solubility and the structural arrangement of the carbohydrates. In rats fed ITF with different degrees of polymerisation (ITF-$_{Dpav\ 3\text{-}4}$, ITF-$_{Dpav\ 25}$, ITF-$_{MIX}$), Kruger *et al*. ([296]) showed that the various ITF do not have the same effect on calcium retention, femoral bone density, bone calcium content and excretion of collagen degradation products in the urine.

From the available data, it can be concluded that the higher biological effects were elicited by a combination of ingredients showing a prebiotic effect with different chain length. Indeed, ITF-$_{MIX}$ outperformed the traditional molecules given alone with regard to calcium absorption. Indeed, in adolescent girls, such a combination increased the true calcium absorption by almost 20%, while oligofructose alone did not show any significant effect ([270]). This conceptual rule is even more apparent in animal experiments. Coudray *et al*. ([297]) compared different types of fructans which differed in both sugar chain length and chain branching, and found a synergistic effect of a combination of ITF with different chain lengths in adult male rats.

A potential mechanism for the improved efficiency of such a mixture could be the larger distribution of fermentation along the colon, depending on the chain length, which is critical to obtain maximum efficacy at low daily doses. Actually, the short chain components such as oligofructose are most active in the proximal part of the colon, while the long-chain molecules have their effect in the distal part. The combination of both molecules offers a synergistic effect on calcium absorption, the fermentation process taking place over the full length of the colon, thus maximising the mucosal surface through which the extra solubilised calcium can migrate ([298]).

5.3.4 Influence of physiological status

It appears that some subjects are more likely to benefit from consumption of inulin, according to their physiological status.

5.3.4.1 ***Initial status in calcium***.

First of all, Griffin *et al*. ([271]) demonstrated that the most consistent identifiable determinant of a beneficial effect on calcium absorption was the fractional calcium absorption at baseline with those individuals with lower absorption during placebo period showing the greatest benefit. This data was corroborated by data published by Holloway *et al*. ([288]) who showed that, in 15 postmenopausal women (who were a minimum of 10 y past the onset of menopause) treated with 10g/d of ITF-$_{MIX}$ for 6 weeks, true fractional calcium absorption, measured by dual isotopes before and after treatment, was significantly increased only in those with lower initial BMD.

5.3.4.2 Estrogen permeation.

From human data we can conclude that an improvement in calcium absorption is possible in adolescents or young adults. Similarly, a positive effect has been reported in older women. However;, ITF failed to modulate calcium absorption during the first 5 years after the onset of menopause, a period, actually, predominantly characterized by hormonal disturbances. In fact, menopausal status is the overriding factor in determining bone loss in women in their early fifties. Thus, given the tremendous impact of gonadal hormones on bone health, a high calcium intake will not offset osteopenia that occurs immediately following menopause.

However, ITF could still remain a source for putative innovative dietary health intervention to prevent post-menopausal osteoporosis by modulating phytoestrogens bioavalability. Setchell *et al*. ([299]) have found that intestinal metabolism of isoflavones (the major class of phytoestrogens) would be the more important clue to the clinical efficacy of soy foods in preventing osteopenia. Thus, because a greater efficacy of phytoestrogens can be expected if converted into equol by the intestinal microbiota, there is a good rationale for considering non-digestible carbohydrates with prebiotic effects, targeting an increase of isoflavones bioavailability. Nevertheless, available data are still conflicting. In animal studies, it has been shown that dietary oligofructose may increase β-glucosidase activity in the large intestine, leading to an enhancement of the large intestinal absorption of these compounds ([300]). Furthermore, in ovariectomized mice ([301]) or rats ([302]), two experimental models for postmenopausal osteoporosis, oligofructose consumption has been shown to augment the bone sparing effect of isoflavones by improving equol production. Again, Devareddy *et al*. ([303]) demonstrated that although the combination of ITF and soy had no additive effect on BMD, it had a greater effect in reversing the

loss of certain microarchitectural parameters such as tibial trabecular number, separation and thickness. By contrast, Zafar *et al*. ([304]) concluded from a rat experiment that isoflavones could enhance calcium absorption, without synergy from ITF, and that actually ITF decreased equol production.

In postmenopausal women, Piazza *et al*. ([305]) showed that the presence of ITF in the diet (3.6g twice a day) facilitated the absorption of isoflavones. As far as bone metabolism is concerned, Mathey *et al*. ([302]) demonstrated that ITF consumption was able to improve the protective effect of isoflavones on bone resorption.

5.4 From mineral absorption to health benefits

The key question of whether the extra absorption of minerals may exhibit substantial benefits needs to be addressed.

5.4.1 Minerals

Ohta *et al*. ([306]) showed that, in rats fed ITF-$_{Dpav\ 3\text{-}4}$ (1 or 5%in the diet), apparent magnesium absorption was increased, as compared to controls. The highest dose (and sufficient magnesium in the diet, i.e. 0.5 mg/g) resulted in a reduction of auricular and facial peripheral hyperemia and hemorrhage and improved inflammation in magnesium-deficient rats. Similarly, in iron-deficient animals, ITF-$_{Dpav\ 3\text{-}4}$ feeding not only increased iron, calcium and magnesium absorption but improved recovery from anemia, as well ([307]). Kobayashi also found that soy polysaccharides could enhance iron absorption and improve anemia ([308]).

Consequently, these studies provide the evidence that ITF are able to elicit health improvement by enhancing mineral and calcium absorption. Further studies are necessary to assess this possibility.

5.4.2 Calcium and bone health

The adequate consumption of calcium in conjunction with optimisation of its absorption is likely to optimise bone mass. It is thus necessary to prove that the benefits of ingredients showing a prebiotic effect on calcium absorption persist and can be translated into benefits to bone health, in other words whether the extra absorbed calcium is deposited in bones, as such a substantial bone benefit may have important implications for future preventative strategies for osteoporosis.

Even though animal data provide promising results on the role of ingredients showing a prebiotic effect on bone health, they need to be confirmed by human intervention trials. Most of the scientific evidence of the bone sparing is based on animal studies, in which they not only improve calcium absorption, but also prevent bone loss in conditions of estrogen deprivation. Actually, the major available data comes from the Abrams's team ([273]) and the study with ITF-$_{MIX}$ is the only published data dealing with long term effect. Thus, because when targeting bone mineralization process, calcium is the most likely to be inadequate in terms of dietary intake, the enhancement of calcium accretion in bones, and hence BMD, in adolescents given ITF-$_{MIX}$ for 1 year, is very interesting. Indeed, adequate calcium intake in childhood is critical for the formation and retention of a healthy skeleton. However, if those molecules may help to optimise peak bone mass, their effect in older people, when bone turnover is increased needs to be acertained.

Moreover, because bone strength is the ultimate hallmark of bone quality, the issue of persistence of the beneficial effect on the skeleton is antoher important to consider, in order to assess their potential in the prevention of the risk of fracture.

5.5 Key points

- Ingredients showing a prebiotic effect are able to improve mineral absorption (and especially calcium) in the animals.
- Most data are available for ITF, in particular ITF-$_{Dpav\ 3\text{-}4}$ as well asITF-$_{MIX}$.
- ITF have been found to increase magnesium absorption in humans, nevertheless available data are very limited.
- These ingredients are able to enhance calcium absorption in human, depending from their physiological status (no effect in early postmenopausal women).
- The benefits on calcium absorption can be translated into benefits to bone health in animals.
- More interestingly, ITF-$_{MIX}$ given for 1 year to adolescents was able to elicit not only an enhancement of calcium accretion in bones, but also BMD. In this light, such or similar may have important implications for future preventative startegies for osteoporosis.

- A combination of molecules with different degrees of polymerization appears to be more efficient as shown with the research on ITF-$_{MIX}$ in comparison with the small and high MW fractions given alone.

5.6 Recommendations (future targets for research)

- Further studies are required to investigate the underlying mechanisms of the prebiotic effects on absorption of minerals, with special attention to the role of the specific changes in gut microbiota. Indeed the question still remains open of wether theses effects are due to the changes in colonic microbiota composition (prebiotic effect) or any other mechanisms.In this regard, high throughput methodologies such as metabolomics, for example, are warranted.
- Results from ITF, in particular ITF-$_{MIX}$ need to be confirmed in other ingredients showing a prebiotic effect for a generalisation.
- Further long term well designed clinical trials need to be implemented to prove that the benefits of these ingredients persist in the longer term (because bone strength is the ultimate hallmark of bone quality, the issue of persistence of the effect of ITF-$_{DPav\ 3\text{-}4}$ on the skeleton is important to consider) to assess their potential in the prevention of the risk of fracture
- With regards to the bone target, it is interesting to focus on relevant populations, i.e. during childhood and during ageing
- It is still challenging to investigate the potential synergy between the prebiotic effect and other nutrients (such as phytoestrogens for example) endowed with bone sparing effect.

6 Prebiotic effects in weight management and obesity-related disorders[7]

Several reviews report the interest of non digestible carbohydrates – which are prone to be fermented by the gut microbiota in the control of obesity and related metabolic disorders. Carbohydrates showing a prebiotic effect have received special attention in this context, since they have been shown - mostly in experimental animal studies - to regulate food intake and weight gain, as well as metabolic disorders

[7] The main authors of this section are Prof. Delzenne, Dr. Cani and Dr. Neyrinck

associated with obesity, such as liver steatosis, dyslipidemia, diabetes, and/or even hypertension ([309]). Most of the data published to date have been obtained through the supplementation with ITF as prebiotics. The relevance of changes in gut microbiota in the modulation of obesity and related disorders is discussed, taking into account both animal and human studies published so far.

6.1 Description of the prebiotic effects on obesity and related metabolic disorders

6.1.1 Prebiotic effects and regulation of food intake, fat mass and body weight

6.1.1.1 Animal studies

Numerous data have described the effect of prebiotics (5-10% in feed) feeding on the evolution of body weight and fat mass in experimental animal models (Table 16). The observed decrease in fat mass had sometimes occurred without significant effect on body weight, and has been observed in all types of white adipose tissue (epididymal, visceral and or subcutaneous). In numerous studies of rodent models (lean, genetic or nutritional induced obese mice or rats) this decrease in fat mass following feeding with ingredients showing a prebiotic effect was associated with a reduction of food/energy intake. The decrease in food/energy intake is not observed when ITF prebiotics are substituted by non fermentable dietary fibre (microcrystalline cellulose), suggesting that at least the colonic fermentation plays a role in the modulation of food intake ([310; 311]).

6.1.1.2 Potential mechanism

The decrease in food intake associated with prebiotics feeding in animals might be linked to the modulation of GI peptides involved in the regulation of food intake. Endocrine cells present in the intestinal mucosa secrete peptides involved in the regulation of energy homeostasis. Among those

peptides, GLP-1, PYY, Ghrelin and oxyntomodulin have recently been proposed as important modulators of food intake and energy expenditure ([312-315]).

Several data obtained in rats and mice show that of ITF-$_{DPav\ 3-4}$ reduce food intake, body weight gain and fat mass development, these features being associated with a significant increase in the portal plasma levels of anorexigenic peptides GLP-1 and PYY; some data also report a decrease in the serum level of orexigenic ghrelin upon prebiotics feeding ([316-320]). Dietary intervention with ingredients showing a prebiotic effect in post-natal diets causes a rapid increase in GLP-1 in rats, and this influences fat mass and glycemia in adulthood ([321]).

Prebiotics feeding promotes GLP-1 synthesis (mRNA and peptide content) in the proximal colon namely by a mechanism linked to the differentiation of precursor cells into enteroendocrine cells ([322]). The overproduction of GLP-1 of mice supplemented with short chain ITF could constitute a key event explaining several systemic effects of prebiotics, since the decrease in food intake and in fat mass after fructans treatment is abolished in GLP-1 Receptor knock-k out mice or in mice treated chronically with a GLP-1 receptor antagonist - Exendin 9-39 ([323]).

6.1.1.3 Human Data

In healthy humans, feeding 16g/d of ITF-$_{\mathbf{DPav\ 3-4}}$ (short chain ITF) promotes satiety following breakfast and diner, and reduces hunger and prospective food consumption after the dinner. This is accompanied by a significant 10% lower total energy intake ([324]). Similarly, Archer *et al*. have demonstrated that the gut microbiota fermentation of ITF, added to food as fat-replacer, is able to lower energy intake during a test day ([325]). ITF feeding (20g/d) increased plasma GLP-1 in one interventional study performed in patients presenting gastric reflux. This study was not aimed at demonstrating an effect on food intake and/ or satiety ([326]). The authors suggested that the “kinetics” of fermentation – assessed by hydrogen breath test – is important to take into account when assessing the influence of fermented nutrients on circulating gut peptides. The increase in hydrogen expired (marker of fermentation), correlates with the modulation of plasma GLP-1 level, which could explain the link between intestinal fermentation and gut peptide secretion.

According to this observation, we have recently demonstrated that the prebiotics-induced gut microbiota fermentation was associated with increased postprandial GLP-1 and PYY and subsequent changes in appetite sensations ([327]).

A recent study demonstrated that supplementation with ITF-$_{MIX}$ not only benefited bone mineralization, but also had a significant benefit on the maintenance of an appropriate body mass index (BMI), and fat mass in primarily non obese young adolescents ([328]). Daily intake of yacon syrup, allowing to bring 0.14g FOS per kg per day, over 120 days, resulted in an increase in satiety sensation and a decrease in body weigth, waist circumference and BMI in obese pre-menopausal women ([329]). Interestingly, the relevance of gut hormone modulation in the management of obesity and metabolic syndrome in humans is supported by some data. A recent clinical trial supports the evidence that ITF-$_{DPav\ 3\text{-}4}$ (short chain ITF) decrease food intake, body weight gain and fat mass development in obese subjects. The authors found a higher plasma PYY levels as well as a drop in ghrelin following meal, however, they failed to observe an increase GLP-1 plasma concentrations over a 6-hour meal tolerance test ([330]). The effect of acute treatment with 8g ITF with or without 0.3g β-glucans over 2 days did not have any effect on appetite, satiety or food intake, suggesting that an adaptative process (linked to the modulation of gut microbiota?) may be necessary to observe the satietogenic effect of prebiotics ([331]).

6.1.2 Prebiotic effects and glucose homeostasis

6.1.2.1 Animals.

An improvement of glucose homeostasis by ingredients showing a prebiotic effect has been observed in rats or mice in several nutritional, genetic, or toxic conditions leading to glucose intolerance and/or diabetes : high-fructose ([332]) or high fat diet -fed animals ([333-336]), genetically obese or diabetic mice ([337]), streptozotocin-induced diabetic rats ([338]). The improvement of glycemic response can be explained on either increase insulin secretion or insulin sensitivity, depending on the model.

In streptozotocin treated-rats (STZ), characterized by a diabetes linked to the destruction of β-cells, prebiotics feeding improve glucose tolerance and increase plasma insulin. In this model, the treatment with ITF allows a partial restoration of pancreatic insulin and β–cells mass. Endogenous GLP-1

production is increased in diabetic rats received ITF as compared to other groups ([338]). This GLP-1 overproduction might be part of the protective effect of dietary ITF because:

1) it has been shown that in diabetes prone-BB rats that are characterized by a default of production of gut peptides, no effect of ITF was shown ([339]),

2) GLP-1 has been shown to increase β-cells differentiation and

3) That beneficial effect of ITF is not due to the satietogenic effect alone, since the improvement of glucose tolerance and pancreatic β–cell mass observed in STZ-ITF fed rats is not reproduced through the sole pair-feeding restriction.

It is likely that a more direct effect of GLP-1 could be due to its effect on pancreatic β–cells differentiation.

ITF improve hepatic insulin sensitivity and increases plasma insulin in diet induced diabetes and obesity (high fat fed mice) ([340]). As shown by an increase in food intake and body mass, genetic and pharmacological disruption of the GLP-1 receptor action abolished the beneficial effect of the treatment on both glucose tolerance and insulin sensitivity, suggesting a key role for this gut peptide ([341]). In diet-induced obese dogs, 1% short chain fructans given in the diet for 6 weeks resulted in a decrease in insulin resistance assessed by euglycemic/hyperinsulinemic clamp, and these effects occurred in parallel with changes in the expression of genes involved in glucose and lipid metabolism in the adipose tissue ([342]).

Altogether, these data support the relevance of the prebiotic modulation of gut microbiota by using dietary in the control of glucose homeostasis in different models of diabetes. The implication of gut peptides may be involved in this effect, however, other metabolic mechanisms, - such as a decrease in inflammatory tone - could also contribute to the improvement of glucose homeostasis upon treatment with ingredients showing a prebiotic effect (see below).

6.1.2.2 Human studies

Several papers have been published, which have focused on the influence of ingredients showing a prebiotic effect on glucose homeostasis in humans. Luo *et al.* ([343]) has shown that 20g short chain fructans given for 4 weeks to healthy subjects decreased basal hepatic glucose production, but had no detectable effect on on insulin-stimulated glucose metabolism. They tested the same approach in

type 2 diabetic patients but no significant modification of glucose homeostasis (plasma glucose level, hepatic glucose production) occurred in the prebiotics treated patients ([344]). In a similar study conducted in hypercholesterolemic patients, prebiotics (short chain fructans) treatment reduced the post-prandial insulin response, but the clinical relevance of this effect remains unclear ([345]). In a recent study, a 2-week supplementation with 16g/day ITF, compared with the same amount of maltodextrin used as placebo, increased GLP-1 production and lessen the post-prandial glucose response after a standardized breakfast ([327]).

6.1.3 Prebiotic effects and lipid homeostasis, including steatosis and hepatic alterations.

6.1.3.1 Animal Studies

Ingredients showing a prebiotic effect are able to modulate hepatic lipid metabolism in rats or hamsters, resulting in changes in either triglyceride accumulation in the liver (steatosis), and/or serum lipids ([346]). In non-obese rats and/or hamsters fed a high carbohydrate diet, a decrease in hepatic and serum triglycerides was observed, when ITF were added to the diet at concentrations ranging from 2.5 to 10% for several weeks (from 2 to 12 weeks) ([347]). In animals, reduced triglyceridaemia or steatosis is often linked to a decrease in de novo lipogenesis in the liver ([348]). In rats fed a lipid-rich diet containing fructans, a decrease in triglyceridaemia also occurs without any protective effect on hepatic triglyceride accumulation and lipogenesis, suggesting a possible peripheral mode of action ([333]). By contrast, in obese Zucker rats, dietary supplementation with ITF lessens hepatic steatosis, with no effect on post-prandial triglyceridaemia when added to the standard diet ([349]). This effect is likely to be mainly the of a lower availability of non-esterified fatty acids coming from adipose tissue, since fat mass and body weight are decreased by the treatment. In obese dogs, a 6 weeks treatment with short chain fructans was able to increase uncoupling protein 2 and carnitine palmitoyltransferase 1 expression in the adipose tissue, thereby suggesting a higher substrate oxidation in adipocyte, that occurred without any significant change in triglyceridemia ([342]).

The decrease in triglyceride synthesis and accumulation of dietary prebiotics compounds could be linked to several events. First, a decrease in glycemia could be part of the process, since glucose (together with insulin) is a driver of lipogenesis. Second, the SCFAs produced through the fermentation process, could play a role in the regulation of lipid metabolism. The high proportion of

propionate produced in the caecum, which reaches the liver through the portal vein, is, at least in animals, a key event in explaining a lower hepatic triglyceride synthesis ([350; 351]). Interestingly, acetate, when supplied in the diet of diabetic mice at a dose of 0.5% for 8 weeks, activates AMPkinase in the liver, a phenomenon that is related to the inhibition of de novo lipogenesis ([352]). The incubation of rat hepatocytes with acetate (0.2 mM) activates AMPkinase and decreases sterol response element binding protein (SREBP-1c) expression, two factors clearly implicated in the regulation of lipogenesis. Therefore, the classical deleterious role attributed to acetate as a precursor of lipogenesis might be modulated taking into account its regulatory effect on key molecular factors involved in fatty acid synthesis in the liver.

Several studies have also reported a decrease in total serum cholesterol after dietary supplementation with inulin (10%) in mice or rats ([353-357]). Experiments in apoE deficient mice support the fact that dietary inulin (mainly long chain inulin) significantly lowers total cholesterol levels by about one third. This is accompanied by a significant decrease in the hepatic cholesterol content. The authors suggest that the decrease in serum cholesterol could reflect a decrease in TAG-rich lipoproteins which are also rich in cholesterol in apo-E deficient animals ([356]).

With regard to the hypocholesterolemic effect of prebiotics, several mechanisms have been proposed. The modulation of the intestinal metabolism of bile acids, (e.g. steroid-binding properties) may be involved, which are independent of the fermentation of the ingredient showing a prebiotic effect in the lower intestinal tract ([358-360]). A recent study, performed in rats supplemented with GOS/FOS, did not support the involvement of changes in the bile salt pool size and kinetics in the modulation of lipid and energy metabolism ([361]).

6.1.3.2 Human data

Reported effects of prebiotics on circulating blood lipids in both normo- and moderately hyperlipidemic humans are variable ([362]). Both positive and negative outcomes have been obtained from a small number of well designed human studies, devoted to analyse the effect of dietary supplementation with fructans (doses ranging from 8 to 20g per day) exhibiting prebiotic properties. The effect of ITF supplementation on lipogenesis has been shown in human volunteers: the hepatic capacity of triglycerides synthesis is lowered by this ingredients showing a prebiotic effect as previously shown in

rats ([363]). In patients with non alcoholic steatohepatitis, short chain ITF supplementation lead to a decrease in serum activity of amino-transferases, suggesting an improvement of hepatic alterations in those patients ([364]), thereby suggesting that a prebiotic approach could be useful in the management of hepatic disease associated with obesity.

6.1.4 Prebiotic effects and obesity-associated inflammation.

Obesity and insulin resistance are associated with a low grade inflammation (for review, see ([309; 365]). The gut microbiota takes part of this component of the metabolic disorder associated with obesity. In fact, LPS has been considered to be the triggering factor for the early development of inflammation and metabolic diseases ([366]). The excessive intake in dietary fat facilitates the absorption of highly pro-inflammatory bacterial LPS from the gut, thereby increasing plasma LPS level leading to "metabolic endotoxemia" ([367]). Interestingly, several reports have shown that obesity induced following dietary manipulations (high-fat feeding) ([368-371]) or genetic deletion (leptin deficient models) ([372]) is characterized by changes in gut microbiota towards a decreased number of bifidobacteria. Importantly, this group of bacteria has been shown to reduce intestinal LPS levels in mice and to improve the mucosal barrier function ([373-376]). Feeding mice with ITF-$_{DPav\ 3\text{-}4}$ restores the number of intestinal bifidobacteria and reduces the impact of high-fat diet induced-metabolic endotoxaemia and inflammatory disorders ([377; 378]). With regard to the possible mechanism of action of these ingredients, data obtained in obese ob/ob mice showed that they increase the production of a gut peptide secreted by endocrine cells of the colon, namely glucagon-like peptide-2 (GLP-2), which plays a role on the intestinal tissue itself, by restoring tight junction protein expression and repartition, and thereby decreasing gut permeability, endotoxemia, and associated metabolic disorders ([379]) .

The relevance of endotoxemia on metabolic disorders due to fat excess, and diabetes in human is supported by several recent studies. However, the impact of the prebiotic approach on endotoxemia and inflammation in obese and diabetic patients has not yet been demonstrated. This area of research may be very interestingimportant, since inflammation is considered as an important event

that drives a lot series of metabolic alterations linked to obesity (cardiovascular diseases, NASH, insulin resistance…).

6.2 Relation between prebiotic effects and improvement of obesity and associated disorders

Relative specificity of prebiotics effects versus other "dietary fibres" on physiological targets regulating appetite and metabolic disorders

It has been proposed before that the secretion of gut peptides might be part of the effects of fermentable carbohydrates with prebiotics properties. Some of those effect can also been driven by dietary compounds for which a prebiotic effect has not yet been shown. Resistant starch has also been shown to increase GLP-1 and PYY in several rodent studies, with consequences on fat mass development ([380; 381]).

An increase in the post-prandial response of GLP-1 was observed after ingestion of β-glucan-rich rye bread by healthy subjects ([382]). The administration of guar gum (together with galactose) promoted the increase in GLP-1 in women, and this was related to a significant increase in satiety ([383]). An increase in the level of non-ndigestible carbohydrates (barley-kernel bread) in the evening meal resulted in an increase in satiety and in a decrease glucose response following breakfast, an event that can be linked to an increase in GLP-1, to the extent of fermentation (assessed through the hydrogen breath test) and which is related to a lower proinflammatory cytokine level (IL6) ([384]).

These data suggest that some effect described for "well established" prebiotics can also be the attribute of other non-digestible/fermentable carbohydrates. The relevance of the gut microbiota composition and activity in this process remains poorly explored. In that view, recent data suggest that butyrate is able to improve insulin sensitivity and energy expenditure in rodents ([385]) thereby supporting the hypothesis that besides the changes in the composition of the microbiota, the gut microbiota, the pattern of fermentation could also be important to take into account.

What is the contribution of changes in gut microbiota composition in the improvement of metabolic alterations by prebiotics?

A recent study has shown, for the first time in humans, that differences in specific "healthy" bacteria in gut microbiota may precede the development of becoming overweight ([386]). The authors found that *Bifidobacterium* spp. during the first year of life was higher in number in children who exhibited a normal weight at 7 years than in children becoming overweight. More importantly, and according to the results obtained in experimental models, they found that the faecal numbers of *S. aureus* were lower in children remaining normal weight than in children becoming overweight. These results unequivocally imply that the gut microbiota profile in favour of a higher number bifidobacteria and a lower number of *S. aureus* in infancy may provide protection against overweight and obesity development. The authors proposed that *S. aureus* may act as a trigger of low-grade inflammation ([387]), contributing to the development of obesity. Experimental data in mice suggest that the promotion of Bifidobacteria by the intake of ingredients showing a prebiotic effect - may be helpful *per se*. On one hand, intervention studies relating concomitantly the changes in gut microbiota composition (and activity), and, on the other hand, behavioural (appetite) or physiological changes are therefore necessary to proof the relevance of the gut microbial changes in the effects.

6.3 Methodological aspects

Key questions remain open concerning the adequacy of the experimental protocol to estimate the relevance of ingredients showing a prebiotic effect in the management of obesity and associated disorders. The choice of a placebo is rather difficult, and the type of placebo compounds is different when experiments are conducted in animals or in humans. There may also be differences when considering endpoints such as fat mass development or satiety, or glucose/lipid homeostasis.

In animal studies, the authors often add ingredients showing a prebiotic effect at a relatively high dose (1 to 10% wt/wt in the diet) and to compare the data obtained in animals receiving the basal diet alone. The interpretation of results would then require the difference in energy/nutrients intake and/or an experimental group with the same intake of energy upon the treatment (pair-fed animals) to be

taken into account. Other authors propose to replace the amount of ingredients showing a prebiotic effect by a non digestible-non fermentable carbohydrate such as microcrystalline cellulose as placebo. This allows a comparison based on differential fermentation properties.

For human studies, the dose of ingredients showing a prebiotic effect is much lower (from 1 to 30g per day). The organoleptic and physico-chemical properties of the placebo are very important to take into account. Several placebos are proposed in the literature. eg a digestible carbohydrate, such as maltodextrin - i.e. alone ([324; 327]), or in combination with aspartame ([345]) - or saccharose ([343; 344]). dietary fibres such as oat fibre ([331]).

The choice of the adequate placebo is really difficult and will depend on the end-point and duration of the treatment. When estimating the influence on glucose/lipid metabolism, one must consider a placebo that does not change post-prandial glucose level or has a minor impact as lipogenic substrate, for example.

For studies aiming at controlling appetite and energy, one has to choose an adequate placebo which does not exert an effect per se. When estimating a long term effect on body weight composition, the consequence of placebo treatment on global energy intake must be taken into account.

There are, therefore, several possibilities and the interpretation and discussion of the results might also take into account the differences that could be due to the placebo effect in a specific context.

6.4 Conclusions and future trends

Collectively, these studies provide support for the beneficial effect of prebiotics on energy homeostasis and body weight gain. Only a few human studies are available to date, but some of them support a role of gut peptide modulation by ingredients showing a prebiotic effect as a potential mechanism occurring in the gut, and appetite regulation. The question of the relevance of gut microbiota modulation in these effects remains unexplored in most of the studies performed in humans. In mice, an inverse relationship has been established between the level of faecal bifidobacteria and some features of the metabolic alterations linked to obesity (endotoxemia, fat mass, glucose intolerance). Some other non digestible carbohydrates or dietary fibres (i.e. resistant starch, insoluble fibre form barley) - for which prebiotic effect has not yet been established - would be able to modulate gut peptides production with consequences on appetite, inflammation, and other

components of the metabolic syndrome. The analysis of the gut microbiota changes will be crucial in further research and clinical approach, in order to clearly relate those changes with the improvement of metabolic alterations of the host. This will be the way to propose a “targeted approach in the modulation of gut microbiota by ingredients showing a prebiotic effect” as relevant in the context of obesity.

7 Conclusion and perspectives: Which data to support the hypothesis of a causal relationship between a prebiotic effect and health effects/benefits?[8]

A prebiotic effect exists and is now a well established scientific fact. A large number of human intervention studies have demonstrated that dietary consumption of food products/ingredients/supplements results in statistically significant changes in the composition of the faecal (and in some cases, the mucosal) gut microbiota. Most of the available data concern the selective stimulation of bifidobacteria (but also lactobacilli). Other purportedly beneficial genera such as *Roseburia, Eubacterium* may be more fully investigated in the future – although further evidence of their beneficial effects is required. Some, but not all, studies have reported a reduction in the concentration of pathogenic bacteria such as clostridia and salmonella. The more data are accumulating, the more it will be recognized that such changes in the composition of the fecal microbiota, especially increase in bifidobacteria can be regarded as a marker of intestinal health. This is already supported by scientific publications ([388-392]).

Research on the impact of the prebiotic effect on the activity (metabolic, regulatory, signaling) of the microbiota is ongoing and appropriate relevant methodologies are being developed, validated and applied.

1. Results from experimental models but also in a few human studies, food products/ingredients/supplements with a demonstrated prebiotic effect have been shown to modulate certain immunological biomarkers and affect activity(ies) of the immune system. Whether changes in immune function markers or immune-health benefits are related to a prebiotic induced change in the composition of the gut microbiota is an area for future investigation. While several studies report changes in the fecal microbial composition alongside changes in immune markers, only one study sofar has correlated these findings. Although these observations make the link between immuno-modulation and microbiota changes likely, convincing evidence needs to be established by further studies showing clear correlations between parameters of immune function and changes in the microbiota. Although a *causal* relationship is virtually impossible to

[8] The author of this section is Prof. Marcel B. Roberfroid.

establish in human subjects, current plausible hypotheses and future correlative findings will help to establish the correlation between prebiotic modulation of the intestinal microbiota and changes in immune function

2. The effect of breast feeding on infant gut microbiota composition is well established and mother's milk is known to contain a complex mixture oligosaccharides with prebiotic (especially bifidogenic) effects, therefore, infant formulae/foods have been supplemented with prebiotics. Confirming the studies in adults, it has been demonstrated that such supplementation increases the faecal concentration of bifidobacteria. This concomitantly, improves stool quality (soft and loose stools), reduces the risk of gastro-enteritis, improves general well-being, and reduces the frequency of atopic eczema. It is plausible that these effects were microbiota-induced changes.

3. Changes in the gut microbiota composition are classically considered as one of the many factors involved in the pathogenesis of either IBD or IBS. The use of particular food products/ingredients/ supplements with prebiotic effects has thus been tested in clinical trials with the objective to improve the well-being of patients with such disease states. Promising beneficial effects have been demonstrated in some but still preliminary studies with changes in gut microbiota composition (especially increase in bifidobacteria concentration) being associated. Again, it is feasible to conclude that the mechanism of these effects is linked to the prebiotic effect.

4. Colon cancer is another pathology for which a possible role of gut microbiota composition has been hypothesized. Numerous experimental studies in mice and rats have reported reduction in incidence of tumours and cancers after feeding specific food products / ingredients / supplements with prebiotic effects. Some of these studies (including one human trial) have also reported that, in such conditions, gut microbiota composition was modified (especially due to increased concentration of bifidobacteria), however, role of such changes in the eventual anti-cancer effect of these specific food products / ingredients / supplements remains to be definitively proven.

5. Dietary intake of particular food products/ingredients/supplements with a prebiotic effect has been shown, especially in adolescents, but also tentatively in postmenopausal women, to increase Ca absorption as well as bone calcium accretion and BMD. No correlation has been reported between such a beneficial effect and changes in gut microbiota composition - although this is plausible but not exclusive. However other food products/ingredients/supplements that do not show prebiotic effect (e.g. lactose, miscellaneous dietary fibres) have also been reported to exert similar effects. Moreover a study in adolescents revealed the existence of a genetic component in response (with 1/3rd of non responders) to increased calcium absorption. It is thus likely that improved calcium absorption is not uniquely caused by changes in gut microbiota composition and might be a consequence of a combination of different effects. Preliminary data have reported, mainly in experimental models, that specific food products/ingredients/supplements with prebiotic effects could also increase the absorption of other minerals (e.g. Mg, Fe). More research is needed to confirm these data and, eventually, to demonstrate if their mechanism involves changes in gut microbiota composition.

6. Recent data, both from experimental models and human studies, support the beneficial effects of particular food products / ingredients / supplements with prebiotic properties on energy homeostasis, satiety regulation and body weight gain. Together with data that correlate obesity with differences in gut microbiota composition, these studies have led to hypothesize that gut microbiota composition (especially the number of bifidobacteria) may contribute to modulate metabolic processes associated with syndrome X, especially obesity and diabetes type II. In a study on the mechanism of action of a prebiotic food ingredient in reducing obesity, an inverse correlation between bifidobacteria fecal concentration, and gut permeability and metabolic endotoxemia (plasmatic LPS), has been reported. However and since non-prebiotic dietary fibres have also shown some similar effects, the question of the specific benefits that can specifically be attributed to prebiotic effects remains open.

   By reference to the present kowledge (mostly based on the data obtained with the various ITFs and the GOS) on the prebiotic effect and its possible multiple physiological consequences it appears likely that different compounds (food ingredients or food

supplements) including chemically-identical compounds with eg different chain lengths (like in the ITF group) will have:

- different prebiotic effects will influence differently the composition of the microflora in the different segments of the intestine, especially in the large bowel
- different physiological effects and thus will not affect similarly the same functions (as this is clearly the case for Ca absorption, a function that is more influenced by ITF-$_{MIX}$ than by the different ITFs given separately.

Any effect of one particular compound with a prebiotic effect can never be generalized to another compound, unless this has been scientifically substantiated for each particular food ingredient/supplement. ([78])

The majority of successful human trials on the prebiotic effects show significantly increased intestinal levels of bifidobacteria. Often, these are associated with improvement in well characterised and accepted markers of health.as shown by the extensive and growing body of evidence, outlined in this report. This strongly associates prebiotic-induced increases in numbers of bifidobacteria in the gut with a range of GI and systemic health benefits. Although it could be argued that these studies alone do not necessarily indicate causality, when considered with the results of trials in human subjects and animals supplemented with live bifidobacteria they do indeed provide compelling evidence that the relationship between intestinal bifidobacteria and health might well be causal. ([388-392])

Even so, key questions still remain such as:

- Which effect(s) (see Table 2) is/are causally linked to selective change(s) in gut microbiota composition?
- Which of the physiological and/or pathophysiological well-being and health benefits are directly linked with a particular composition of the gut microbiota or (a) selective change(s) therein?
- Which, amongst the physiological and/or pathophysiological well-being and health benefits, is (are) not linked to a particular composition of the gut microbiota or (a)

selective change(s) therein but is (are) the consequence(s) of other mechanism(s) of the product claimed to have a prebiotic effect?

- Which protocol(s) is (are) now validated to demonstrate change(s) in microbiota composition

- Which protocol(s), methodology(ies) is (are) now available and validated to demonstrate links between a particular composition of the gut microbiota or a selective change therein and a particular physiological and/or pathophysiological well-being and health benefit?

Over the last 2 decades, data has and continues to accumulate improving our knowledge of the gut microbiota composition but also, through the metabonomic approaches, gut microbiota activities. It has convincingly demonstrated that particular food products/ingredients/supplements can, upon feeding, selectively modulate that composition and possibly these activities. Dietary consumption of some of these specific food products/ingredients/supplements has also been reported to exert a series of beneficial health effects that may justify improved function and/or reduction of disease risk claims ([21; 393]). A causal relationship between the induced change(s) in gut microbiota composition and/or activity(ies) and these health effects is more than plausible – given our knowledge that prebiotics are known to be specifically metabolized by the gut microbiota. The more we understand the complexity of the gut microbiota, its interactions with the gut epithelium, its roles in modulating epithelial cell differentiation and epithelial cell functions and, beyond, in the whole body, the more we will be in a position to recommend these food ingredients for their health promoting values. It is becoming more and more clear that gut microbiota plays key roles in modulating human/animal physiology even far beyond the GI tract. Specific food products/ingredients/supplements with prebiotic properties are unique tools to study such effects but also offer unique opportunity to develop new functional foods/food ingredients/food supplements to improve host health. One major contribution of this review article summarizing the state of the art in the research on the metabolic and health effects of these compounds is to recommend where research efforts should be concentrated to improve understanding of

the activities and the physiological roles of the gut microbiota and in particular the importance of its qualitative composition and the consequences of that modulation. Through this, it should be possible to better address the continuing burden of gastro intestinally mediated disorders. Importantly, tools exist to underpin this with mechanistic explanations of effect leading to effective hypothesis driven research.

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Table 1: Developing definitions of the prebiotic concept

"A non-digestible food ingredient that beneficially affects the host by selectively stimulating the growth and/or activity of one or a limited number of bacteria in the colon, and thus improves host health" *Gibson, G. R., Roberfroid, M. B. Dietary modulation of the human colonic microbiota: introducing the concept of prebiotics, J. Nutr. 125, 1401-1412, 1995*
'A selectively fermented ingredient that allows specific changes, both in the composition and/or activity in the gastrointestinal microflora that confers benefits upon host well being and health.' *Gibson G.R., Probert H.M., Van Loo J.A.E., Roberfroid M.B. Dietary Modulation of the Human Colonic Microbiota: Updating the Concept of Prebiotics, Nutr. Res. Rev. 17, 259-275, 2004*
'A dietary prebiotic is a selectively fermented ingredient that results in specific changes, in the composition and/or activity of the gastrointestinal microbiota, thus conferring benefit(s) upon host health.' *ISAPP (2008) 6th Meeting of the International Scientific Association of Probiotics and Prebiotics. London, Ontario.*

Table 2: Summary of the main physiological and patho-physiological targets for prebiotic effects i.e effects associated with a selective stimulation of growth and/or activity(ies) of one or a limited number of gut microorganisms.

Improvement and/or stabilization of gut microbiota composition
Improvement of intestinal functions (stool bulking, stool regularity, stool consistency)
Increase in mineral absorption & improvement of bone health (bone Ca content, bone mineral density)
Modulation of gastro-intestinal peptides production, energy metabolism & satiety
Initiation (after birth) and regulation/modulation of immune functions
Improvement of intestinal barrier functions, reduction of metabolic endotoxemia
Reduction of risk of intestinal infections
and tentatively
Reduction of risk of obesity, type II diabetes, metabolic syndrome…
Reduction of risk and/or improvement in the management of intestinal inflammation
Reduction of risk of colon cancer

Table 3: Description and usual nomenclature of the main products with established prebiotic effect.

Generic name and structural characteristics (Abbreviation used in text[9])	Usual names and average DP (DP_{av})
INULIN-TYPE FRUCTANS **ITF** **Linear β(2→1) fructosyl-fructose.** **$G_{py}F_n$ and/or $F_{py}F_n$**	**Fructo-oligosaccharides, FOS**
Oligomers (DP 2-8) **ITF-$_{DPav\ 3-4}$**	**Short-chain fructo-oligosaccharides, scFOS** (enzymatic synthesis from sucrose) **(DP_{av} 3.6)** **Oligofructose** (enzymatic partial hydrolysis of inulin) **(DP_{av} 4)**
Short and medium size polymers **(DP 2-60)** **ITF-$_{DPav\ 12}$**	**Inulin (especially chicory inulin)** **(DP_{av} 12)**
(DP 10-60) **ITF-$_{DPav\ 25}$**	**High molecular weight inulin** (physical purification) **(DP_{av} 25)**
Mixtures **(DP 2-8) + (DP 10-60)** **ITF-$_{MIX}$**	**Mixture of oligomers and medium size polymers**
GALACTANS **Mixture of** **β(1→6); β(1→3); β(1→4) galactosyl-galactose** **GOS**	**Galacto-oligosaccharides,** **Trans-galactooligosaccharides,** (enzymatic transgalactosylsation of lactose)
(DP 2-8)	(**DP_{av} 3**)
Mixture of galactans and inulin-type fructans **GOS-FOS**	**Galacto-oligosaccharides and high molecular weight inulin,** **Usually known as GOS-FOS or scGOS-lcFOS**

[9] The abbreviations mentioned in this table will be used throughout the documents to identify the different compounds used in the studies.

Table 4: Microbial diversity of the mucosa of the human small intestine as determined by 16S rRNA gene sequence analysis

Subject	Biopsy	No. of clones examined	No. of OTUs identified	Phylum: species identified*	Reference
35-year-old healthy female	Distal ileum	Unknown	Unknown	*Bacteroidetes*: *Bacteroides vulgatus*, uncultured *Bacteroides* sp. adhufec51 and *Parabacteroides* spp. *Firmicutes*: *Clostridium* cluster XIVa (uncultured bacteria mpn group 24 and 66.25) and *Streptococcus salivarius*	Wang *et al.*, 2003 ([12])
54-year-old healthy female	Jejunum	88	22	*Actinobacteria*: *Micrococcus mucilaginosus* (1 %) *Bacteroidetes*: *Prevotella* sp. oral clone and *P. melaninogenica* (3 %) *Firmicutes*: *Streptococcus mitis*, *S. salivarius*, *S. oralis*, *S. parasanguis* and *S. anginosus* (68 %); *Clostridium* clusters XI (*Mogibacterium neglectum* and *Peptostreptococcus anaerobius*) and IX (*Veillonella atypica* and *V. parvula*) (3 and 7 %, respectively) *Fusobacteria*: *Fusobacterium* sp. BS011 (3 %) *Proteobacteria*: *Haemophilus parainfluenzae*, *Pseudomonas putida*, *Acinetobacter johnsonii*, *A. lwoffii* and *A. haemolyticus* and *Neisseria subfla*va (13 %) Others (2 %)	Wang *et al.*, 2005([13])
	Distal ileum	85	33	*Bacteroidetes*: *Bacteroides vulgatus*, *Bacteroides* spp., *B. thetaiotaomicron*, *B. ovatus*, *B. uniformis* and *Alistipes putredinis* (49 %) *Firmicutes*: *Streptococcus mitis* and *S. oralis* (2 %); *Clostridium* clusters XIVb (*Clostridium lactatifermentans*), IX (*Dialister invisus*), IV (*Faecalibacterium prausnitzii*, *Oscillospira guilliermondii* and *Clostridium orbiscindens*) and XIVa (*Clostridium* spp., *Clostridium symbiosum*, *Coprococcus catus*, *Dorea formicigenerans*, *Ruminococcus gnavus*, *R. obeum*, *Ruminococcus* spp. and *Roseburia intestinalis*) (5, 5, 7 and 20 %, respectively) *Fusobacteria*: *Fusobacterium varium* (1 %) *Proteobacteria*: *Sutterella wadsworthensis* (1 %) *Verrucomicrobia*: *Verrucomicrobium* spp. (5 %) Others (5 %)	
74-year-old male at autopsy	Jejunum	92	9	*Firmicutes*: *Veillonella parvula* (4 %), *Lactobacillus reuteri* (1 %), *L. lactis* (11 %), *L. mali* (73 %), *Streptococcus salivarius* (4 %) and *S. pneumoniae* (1 %) *Proteobacteria*: *Actinobacillus actinomycetemcomitans* (5 %)	Hayashi *et al.*, 2005([15])
	Ileum	89	17	*Firmicutes*: *Veillonella parvula* (15 %), *Clostridium lituseburense* (1 %), *Abiotrophia* sp. (1 %), *Lactobacillus reuteri* (1 %), *L. mali* (20 %), *L. lactis* (14 %), *Streptococcus salivarius* (9 %), *S. constellatus* (1 %) and *S. pneumoniae* (9 %) *Fusobacteria*: *Leptotrichia buccalis* (1 %) and *Fusobacteria* spp. (1 %) *Proteobacteria*: *Neisseria gonorrhoeae* (1 %) and *Actinobacillus actinomycetemcomitans* (22 %) Others (1 %)	

Subject	Biopsy	No. of clones examined	No. of OTUs identified	Phylum: species identified*	Reference
85-year-old female at autopsy	Jejunum	90	13	*Bacteroidetes*: *Bacteroides fragilis* (1 %) *Fusobacteria*: *Phascolarctobacterium faecium* (1 %), *Eubacterium ventriosum* (1 %), *E. cylindroides* (1 %), *Clostridium purinolyticum* (3 %), *C. leptum* (1 %) and *Enterococcus* group (5 %) *Proteobacteria*: *Escherichia coli* (4 %) and *Klebsiella* subgroup (67 %) Others (2 %)	Hayashi *et al.*, 2005([15])
	Ileum	94	4	*Firmicutes*: *Enterococcus* group (13 %) *Proteobacteria*: *Klebsiella* subgroup (85 %)	
87-year-old female at autopsy	Jejunum	91	3	*Firmicutes*: *Enterococcus* group (7 %) *Proteobacteria*: *Actinobacillus actinomycetemcomitans* (1 %) and *Klebsiella planticola* (92 %)	Hayashi *et al.*, 2005([15])
	Ileum	89	15	*Firmicutes*: *Rumincococcus gnavus* (2 %), *Peptostreptococcus anaerobius* (6 %), *P. micros* (2 %), *Enterococcus* group (33 %), *Streptococcus salivarius* (8 %) and *Clostridium leptum* (3 %) *Proteobacteria*: *Actinobacillus actinomycetemcomitans* (1 %), *Escherichia* subgroup (16 %), *Klebsiella* subgroup (2 %), *Klebsiella planticola* (21 %) and *Xenorhabdus* subgroup (5 %)	

*Numbers in parentheses represent proportion of clones ascribed to a particular phylum/genus/cluster where known. Names of nearest phylogenetic relatives are given.

Table 5: Bacteria, their substrates and products in the human large intestine
Taken from Salminen *et al.* (1998).([389])

Bacteria	Gram reaction	Mean concn [$\log_{10}$ (g dry weight faeces)$^{-1}$]	Mode of action on substrate(s)	Fermentation product(s)
Bacteroides	–	11.3	Saccharolytic	Ac, Pr, Su
Eubacteria	+	10.7	Saccharolytic, some aa-fermenting species	Ac, Bu, La
Bifidobacteria	+	10.2	Saccharolytic	Ac, La, f, e
Clostridia	+	9.8	Saccharolytic, some aa-fermenting species	Ac, Pr, Bu, La, e
Lactobacilli	+	9.6	Saccharolytic	La
Ruminococci	+	10.2	Saccharolytic	Ac
Peptostreptococci	+	10.1	Saccharolytic, some aa-fermenting species	Ac, La
Peptococci	+	10.0	aa fermentation	Ac, Bu, La
Methanobrevibacter	+	8.8	Chemolithotrpohic	CH_4
Desulfovibrio	–	8.4	Various	Ac
Propionibacteria	+	9.4	Saccaharolytic, lactate fermentation	Ac, Pr
Actinomyces	+	9.2	Saccharolytic	Ac, Pr
Streptococci	+	8.9	Carbohydrate and aa fermentation	La, Ac
Fusobacteria	–	8.4	aa fermentation, assimilation of carbohydrates	Bu, Ac, La
Escherichia	–	8.6	Carbohydrate and aa fermentation	Mixed acids

aa, amino acid; Ac, acetate; Pr, propionate; Su, succinate; Bu, butyrate; La, lactate; f, formate; e, ethanol.

Table 6: Microbial diversity of the mucosa of the human large intestine as determined by 16S rRNA gene sequence analysis

Subject	Biopsy	No. of clones examined	No. of OTUs identified	Phylum: species identified*	Reference
35-year-old healthy female	Ascending colon	27		*Bacteroidetes*: *Bacteroides vulgatus*, *Bacteroides* spp. *Firmicutes*: *Clostridium* cluster XIVa (uncultured bacteria mpn group 24 and 66.25, *Ruminococcus gnavus*)	Wang *et al.*, 2003([12])
	Descending colon	27		*Bacteroidetes*: *Bacteroides vulgatus*, uncultured *Bacteroides* sp. adhufec51 and *Parabacteroides* spp. *Firmicutes*: *Clostridium* cluster XIVa (uncultured bacteria mpn group 24 and 66.25)	
68-year-old female with mild sigmoid diverticulosis	Descending colon	190		*Bacteroidetes* (17.3 %): *Bacteroides vulgatus*, uncultured *Bacteroides* sp. HUCC30 and *Parabacteroides* spp. *Firmicutes* (1 %): *Streptococcus pneumoniae* *Proteobacteria* (39.6 %): *Shigella flexneri*, *S. sonnei*, *Stenotrophomonas maltophila*, *Leptothrix cholodnii*, *Herbaspirillum lemoignei*, *Methylobacterium* sp., *Sphingomonas* sp. and *Haemophilus influenzae* *Firmicutes*: *Bacillus–Lactobacillus–Streptococcus* (1.3 %); *Clostridium* cluster I (*Clostridium perfringens*), IV (*Faecalibacterium prausnitzii*, *Ruminococcus* spp., *Anaerofilum* spp. and uncultured bacterium CB25), IX (*Veillonella atypica*) and XIVa (uncultured bacteria mpn group 24 and AF54, *Lachnospira pectinoschiza* and *Clostridium xylanolyticum*) (1.3, 17.9, 1.8, and 15.3 %, respectively)	Wang *et al.*, 2003‡([12])
54-year-old, healthy female	Ascending colon	86	37	*Bacteroidetes*: *Bacteroides vulgatus*, *Bacteroides* spp., *B. thetaiotaomicron*, *B. ovatus*, *B. uniformis* and *Alistipes putredinis* (27 %) *Firmicutes*: *Clostridium* clusters XIVb (*Clostridium lactatifermentans*), IX (*Dialister invisus* and *Propionispira arboris*), IV (*Faecalibacterium prausnitzii*, *Clostridium sporosphaeroides*, *C. orbiscindens* and *Oscillospira guilliermondii*) and XIVa (*Eubacterium halii*, *E. elegans*, *E. ramulus*, *Dorea formicigenerans*, *Ruminococcus lactaris*, *R. gnavus*, *Ruminococcus* sp., *Clostridium symbiosum*, *Clostridium* spp., *C. xylanolyticum* and *Roseburia intestinalis*) (6, 9, 13 and 33 %, respectively) *Fusobacteria*: *Fusobacterium varium* (1 %) *Proteobacteria*: *Escherichia coli*, *Acinetobacter johnsonii* and *Sutterella wadsworthensis*) (4 %) *Verrucomicrobia*: *Verrucomicrobium* spp. (5 %) Others (1 %)	Wang *et al.*, 2005†([13])
	Rectum	88	32	*Bacteroidetes*: *Bacteroides vulgatus*, *Bacteroides* spp., *B. thetaiotaomicron*, *B. uniformis* and *Alistipes putredinis* (44 %) *Firmicutes*: *Clostridium* clusters XI, XIVb, IX, IV and XIVa (*Clostridium* spp., *Eubacterium halii*, *Dorea formicigenerans*, *Ruminococcus lactaris*, *R. torques*, *Ruminococcus* spp. and *Roseburia intestinalis*) (1, 1, 5, 8 and 29 %, respectively) *Fusobacteria*: *Fusobacterium varium* (1 %) *Proteobacteria*: *Escherichia coli* (2 %) *Verrucomicrobia*: *Verrucomicrobium* spp. (9 %)	

Subject	Biopsy	No. of clones examined	No. of OTUs identified	Phylum: species identified*	Reference
74-year-old male at autopsy	Caecum	90	41	*Bacteroidetes*: *Bacteroides fragilis* (3 %) and *Prevotella nigrescens* (1 %) *Firmicutes*: *Veillonella parvula* (2 %), *Clostridium xylanolyticum* (2 %), *C. polysaccharolyticum* (2 %), *C. leptum* (23 %), *C. lituseburense* (1 %), *C. glycolicum* (1 %), *Ruminococcus hansenii* (8 %), *R. gnavus* (4 %), *Butyrivibrio fibrisolvens* (22 %), *Eubacterium ventriosum* (1 %), *Lachnospira multipara* (4 %), *Lactobacillus reuteri* (1 %), *Streptococcus salivarius* (1 %), *S. pneumoniae* (3 %) and unclassified (14 %) *Proteobacteria*: *Actinobacillus actinomycetemcomitans* (3 %)	Hayashi *et al.*, 2005([15])
	Recto-sigmoid colon	90	38	*Bacteroidetes*: *Bacteroides fragilis* (4 %) and unclassified (1 %) *Firmicutes*: *Veillonella parvula* (1 %), *Phascolarctobacterium faecium* (3 %), *Ruminococcus hansenii* (9 %), *R. gnavus* (6 %), *Butyrivibrio fibrisolvens* (4 %), *Eubacterium ventriosum* (4 %), *Clostridium polysaccharolyticum* (2 %), *C. leptum* (30 %), unclassified (6 %) *Proteobacteria*: *Desulfovibrio desulfuricans* (2 %) and *Escherichia* subgroup (13 %) Other (2 %)	
85-year-old female at autopsy	Caecum	91	11	*Bacteroidetes*: *Bacteroides fragilis* (3 %) *Firmicutes*: *Ruminococcus gnavus* (2 %), *Clostridium lituseburense* (2 %), *Enterococcus* group (35 %) *Proteobacteria*: *Klebsiella* subgroup (36 %) *Actinobacteria*: *Bifidobacterium infantis* (2 %)	Hayashi *et al.*, 2005([15])
	Recto-sigmoid colon	90	27	*Firmicutes*: *Clostridium xylanolyticum* (1 %), *C. purinolyticum* (1 %), *C. ramosum* (1 %), *C. leptum* (11 %), *Eubacterium cylindroides* (1 %), *Ruminococcus hansenii* (2 %), *R. gnavus* (1 %), *Lactobacillus reuteri* (1 %), *Enterococcus* group (19 %), unclassified (7 %) *Proteobacteria Desulfovibrio desulfuricans* (1 %), *Escherichia* subgroup (7 %), *Klebsiella* subgroup (22 %) *Actinobacteria*: *Bifidobacterium infantis* (2 %) Others (19 %)	
87-year-old female at autopsy	Caecum	92	22	*Bacteroidetes*: *Bacteroides fragilis* (2 %) *Firmicutes*: *Veillonella parvula* (1 %), *Clostridium leptum* (4 %), *Ruminococcus hansenii* (1 %), *R. gnavus* (3 %), unclassified (12 %), *Lactobacillus delbrueckii* (1 %), *L. mali* (8 %), *Enterococcus* group (1 %), *Streptococcus salivarius* (41 %), *S. pneumoniae* (16 %) *Proteobacteria*: *Escherichia* subgroup (7 %), *Klebsiella planticola* (1 %)	Hayashi *et al.*, 2005([15])
	Recto-sigmoid colon	92	26	*Bacteroidetes*: *Bacteroides fragilis* (2 %) *Firmicutes*: *Clostridium xylanolyticum* (2 %), *C. leptum* (1 %), *Ruminococcus hansenii* (2 %), *R. gnavus* (5 %), *Lactobacillus delbrueckii* (7 %), *L. reuteri* (27 %), *L. mali* (14 %), *Streptococcus salivarius* (11 %), *S. pneumoniae* (1 %) and unclassified (11 %) *Proteobacteria*: *Escherichia* subgroup (1 %) *Actinobacteria*: *Actinomyces–Bifidobacterium catenulatum* subgroup (9 %), *B. bifidum* (3 %), *B. infantis* (2 %)	

*Numbers in parentheses represent proportion of clones ascribed to a particular phylum/genus/cluster where known. Names of nearest phylogenetic relatives are given.

Table 7: Details of some TGGE and DGGE studies of the faecal microbiota

Target population	Subject	Investigation	Overall results	Reference
All bacteria	7 males, 9 females	Interindividual variation; stability over 6 months monitored for two subjects	Differences in fingerprints among individuals demonstrated that each individual harboured a unique microbiota (interindividual variation); TGGE profiles were highly consistent over time for individuals, demonstrating intraindividual stability	Zoetendal *et al.* (1998)([9])
Lactic acid bacteria	2 males, 2 females	Development and validation of group-specific primers for human studies	Detection of *Lactobacillus* at $>1\times10^5$ cfu (g wet weight faeces)$^{-1}$; interindividual variation; intraindividual variation over 6 months	Walter *et al.* (2000)([394])
	2 adults on probiotic trial	Monitor changes in LAB population during *Lactobacillus* feeding	Amplicon for the probiotic strain only seen during feeding period; one donor had stable fingerprint over time, while the other showed variation	
Bifidobacteria	3 males, 3 females	Stability of bifidobacterial population over 4 weeks	Multiple bifidobacterial biotypes seen in 5 of 6 subjects; no amplicon could be generated for one of the subjects	Satokari *et al.* (2001)([395])
Lactobacilli, leuconostocs and pediococci	12 adults 1 baby boy	*Lactobacillus* population stability over time (0, 6 and 20 months for adults; 0–5 months for baby boy)	Interindividual variation and variable intraindividual stability in adults (stable in some individuals, but more dynamic in others); no amplicons prior to day 55 for baby, indicating that *Lactobacillus* were below the detection limit, but complexity of fingerprint increased after introduction of solid foods to the diet	Heilig *et al.* (2002)([396])
All bacteria	50 adults of varying relatedness plus four different primates	Impact of genetic relatedness on composition of the faecal microbiota	Positive linear relationship between host genetic relatedness and similarity of fingerprints; significantly higher similarity between unrelated humans when compared with other primates	Zoetendal *et al.* (2002)([11])
All bacteria	13 pairs of identical twins, 7 pairs of fraternal twins and 12 unrelated control pairs (4 months–10 years of age)	Examine faecal samples from related and unrelated children	Profiles for the unrelated group had the lowest similarity; highest levels of similarity seen between profiles from genetically identical twins; significant differences between profiles from fraternal and paternal twins, strongly suggesting a genetic influence over the composition of the faecal	Stewart *et al.* (2005)([34])

Target population	Subject	Investigation	Overall results	Reference
			microbiota	
Clostridium leptum group (cluster IV)	6 adults (23–43 years of age) and 5 children (5.5–10 years of age) 7 faecal samples from a 10-year-old child over 3 years	Investigate the diversity of the *Clostridium leptum* subgroup in human faeces	Showed host-specific profiles for the adults, but at least four bands were seen in 8/11 subjects Demonstrated structural succession of the over the first 2 years, with stabilization in the third year	Shen *et al.* (2006)([397])
All bacteria *Bacteroides fragilis* subgroup *Clostridium coccoides*/ *Eubacterium rectale* group (cluster XIVa) *Clostridium lituseburense* group (cluster XI)	3 groups of 10 healthy humans	Effect of a prebiotic substrate and a probiotic organism and their synbiotic combination on the faecal microbiota over 120 days	All populations examined remained fairly stable over the course of the study, with interindividual variation observed; intraindividual stability, with minor changes attributed to diet; one band appeared or intensified in the universal profiles after ingestion of lactulose (attributed to *Bifidobacterium adolescentis*)	Vanhoutte *et al.* (2006)([398])

Table 8: Human studies (healthy persons) designed to determine the prebiotic effect of short-chain fructooligosaccharides (scFOS), fructooligosaccharides (FOS), galactooligosaccharides (GOS) and inulin.

Prebiotic	Subject	Dose	Duration	Effect	References
Inulin	8 healthy humans, placebo controlled	34 g/d	64 days	Significant increase in bifidobacteria established by FISH	Kruse et al., 1999([399])
scFOS	40 healthy humans	2.5 to 20 g/d	14 days	Significant increase in bifidobacteria levels without excessive gas production	Bouhnik et al., 1999([400])
Inulin and FOS	4 or 8 healthy humans	15 g/d	45 days	Bifidobacteria becoming predominant in faeces with both inulin and oligofructose	Gibson et al., 1995([401])
Inulin	35 elderly constipated humans	20 g/d and 40 g/d	19 days	Significant increase in bifidobacteria, decreases in enterococci and fusobacteria	Kleessen et al., 1997([402])
FOS in biscuits	31 healthy humans, double blind placebo controlled	7 g/d	42 days	Significant increase in bifidobacteria established via FISH. No change in total bacterial levels	Tuohy et al., 2001a([403])
FOS	12 healthy adult humans	4 g/d	42 days	Significant increase in bifidobacteria, no change in total bacteria levels	Buddington et al., 1996([404])
FOS	8 healthy humans, placebo controlled	8 g/d	5 weeks	Significant increase in faecal bifidobacteria and decrease in fecal pH	Menne et al., 2000([405])
GOS	12 healthy humans	15 g/d		Significant increase in faecal lactic acid bacteria	Teuri et al., 1998([406])
GOS plus FOS	90 term infants, placebo controlled	0.4 g/d and 0.8 g/d	28 days	Dose-dependent stimulating effect on the growth of bifidobacteria and lactobacilli and softer stool with increasing dosage of supplementation	Moro et al., 2002([407])
scFOS or GOS	40 healthy adults, controlled, double blind, parallel group	10 g/d	6 weeks	Significant increase in faecal bifidobacteria	Bouhnik et al., 2004([408])
scFOS	12 healthy persons, +65y	8g/d	4 weeks	Well tolerated and lead to a significant increase in faecal bifidobacteria in healthy elderly subjects	Bouhnik et al., 2007([409])
Inulin	14 healthy adults	9g/d	2 weeks	FISH probes show increased bifidobacteria	Harmsen et al., 2002([8])
Inulin	45 healthy adults	7.7g then 15.4g/d	3 weeks	Increased bifidobacteria and decreased bacteroides	Kleesen et al., 2007([410])
Inulin	40 adults	8g/d	2 weeks	FISH showed an increase in bifidobacteria	Tuohy et al., 2001b([411])
Inulin/FOS	19 adults	10g/d	4 weeks	Bifidobacteria increased	De Preter et al. 2008([412])
scFOS	19 elderly persons	8g/d	3weeks	Increased bifidobacteria	Guigoz et al., 2002([108])
scFOS	10 healthy adults	4g/d	2 weeks	Increased bifidobacteria and lactobacilli	Williams et al., 1994([413])
Inulin	30 healthy volunteers	5 or 8g/d	2 weeks	Both doses increased bifidobacteria, a higher percent of volunteers responded to 8g/d	Kolida et al., 2007([199])
GOS	30 healthy adults	3.6 or 7g/d	7 days	Selective bifidogenic effect	Depeint et al., 2008([414])

Table 9: The prebiotic effect on immune markers

Subject	Trial design	Groups	N	Duration	Key findings of the prebiotic intervention on immune parameters and effect on microbiota	Reference
Healthy elderly (> 70y)	RPC parellel	(a) daily vitamin & protein supplement with 6g oligofructose/inulin (b) daily vitamin & protein supplement	(a) 23 (b) 20	28 weeks	- no effect on secretory IgA, - no effect on serum titers after vaccination (influenza A and B and pneumococcus) - no effect on secretion of IL-4, IFNg, and lymphocyte proliferation in cultured PBMC stimulated with phytohemagglutinin and influenza antigen	([102])
Newborn non-breastfed infants	RDBPC parallel	(a) standard infant formula (b) prebiotic formula containing mixture of 0.6 g GOS/FOS)/100 ml formula (c) probiotic formula containing 6.0x109 cfu B. animalis/100 ml formula	(a) 19 (b) 19 (c) 19	32 weeks	- trend towards higher fecal sIgA (significant at week 16) - *trend towards higher percentage of fecal Bifidobacteria* - *significantly lower fecal pH* ([415])	([106])
Peruvian breast-fed infants 6-12 mo	1) RDBPC parallel 2) idem	(a) cereal supplemented with oligofructose with of average 0.67g OF/day (b) control cereal (a) cereal supplemented 1 mg zinc/d and with oligofructose (average 0.67g OF/day) (b) cereal supplemented 1 mg zinc/d	(a) 141 (b) 141 (a) 174 (b) 175	6 months 6 months	- no effect on antibody titers after H.influenza B vaccination - no effect on antibody titers after H.influenza B vaccination - *effect on microbiota not adressed*	([104])
Nursing home elderly (77-97 yr)	uncontrolled	8g oligofructose /day	19	3 weeks	Compared to baseline: - increase in % CD4 and CD8 lymphocytes - decrease in phagocytic activity (mean fluorescence) in granulocytes and monocytes - reduced IL-6 mRNA expression in PBMC - *increase in fecal Bifidobacteria and Bacteroides* - *no effect on fecal Enterobacteriae, Enterococci and Lactobacilli*	([108])
Newborn healthy infants	RDBPC parallel	(a) infant milk formula with 6 g/L short-chain GOS and long-chain FOS ratio 9:1 (b) infant formula without prebiotics	(a) 21 (b) 25	26 weeks	- increase in fecal sIgA in those exclusively formula fed -*increase in % of fecal bifidobacteria and decrease in % of fecal Clostridia*	([107])
Adult males	RDBPC semi CO	(a) bread (placebo) (b) bread supplemented with inulin, linseed and soya fibre	(b) 19 (c) 19	5 weeks	- increase of % lymphocyte expressing surface markers CD19 and CD3+HLA-DR+ - decrease of % lymphocyte expressing ICAM-1 - decrease of % CD3+ NK+ cells	([416])

		(c) idem with antioxidants			- no change in phagocytosis and oxidative burst - *effect on fecal microbiota not assessed*	
Elderly (64-79 yr)	DPRPC, CO	(a) galacto-oligosaccharide 5.5g/day (b) maltodextrin	44	10 wks with 4 wks washout	- increase in ex-vivo NK cell activity - increase in ex-vivo phagocytosis - increase in ex vivo IL-10 production by PBMC - decrease in ex-vivo IL-6, TNFa and IL-1 b production by PBMC - *positive correlation between numbers of Bifidobacterium spp., Lactobacillus-Enterococcus spp., and the C. coccoides–E. rectale group with % and total number of phagocyting cells.* - *negative correlation between numbers of* *Bacteroides spp. and E. coli d with % and total number of phagocyting cells.*	([110])
Pregnant women	RDBPC	(a) 9 g/d GOS/lcFOS (b) maltodextrin	48	From week 25 of gestation until delivery	- no change of fetal (cord-blood) immune parameters (lymphocyte subsets, cytokine secretion) - *increased proportions of bifidobacteria in maternal fecal samples* - *no change in the proportion of lactobacilli* - *no change in bifidobacteria and lactobacilli percentages in infants*	([109])
Newborn infants at risk for allergy	RDCPC	(a) hypoallergenic whey formula with 8 g/l GOS/FOS in a 9 : 1 ratio (b) hypoallergenic whey formula with 8 g/l maltodextrine (placebo)	(a) 41 (b) 43	6 months	- significant reduction in plasma levesl of total IgE, IgG1, IgG2 and IgG3 - no effect on IgG4 - Cows milk protein-specific IgG1 was significantly decreased. - no effect on response to DTP vaccine - *significant increase in the number of fecal bifidobacteria* - *no effect on fecal lactobacilli counts ([113])*	([105])

Table 10: Comparison of faecal microbiota between IBS and healthy control subjects

Subject (n)	Results of IBS versus control subjects	Reference
IBS subjects (20) Control subjects (20)	Lower number of coliforms, lactobacilli and bifidobacteria	(5)
IBS subjects (Rome II criteria) (25) Control subjects (25)	Lower number of Bifidobacteria Higher number of *Clostridium perfringens* Higher number of Enterobacteriaceae Lower Bifidobacteria/ Enterobacteriaceae ratio	(6)
IBS subjects (Rome II criteria) (26) Control subjects (25)	Higher number of coliforms Higher proportion of aerobic bacteria	(27)
IBS subjects (Rome II criteria) (27) Control subjects (22)	Lower number of *Lactobacillus spp* in diarrhoea predominant IBS Higher number of *Veillonella spp* in constipation predominant IBS Lower number of *Bifidobacterium catenulatum* and *Clostridium coccoides*	(7)
IBS subjects (Rome II criteria) Control subjects	Lower number of *Lactobacillus spp*, Bifidobacteria and lactate-utilizing bacteria Higher number of Sulphate-reducing bacteria	(8)
IBS subjects (Rome II criteria) (16) Control subjects (16)	Lower proportion of *Clostridium coccoides* and *Eubacterium rectale* in constipation predominant IBS	(11)
IBS subjects (Rome II criteria) (24) Control subjects (23)	Lower number of *Collinsella;* Lower prevalence of *Collinsella aerofaciens*; Lower number of *Coprococcus eutactus* Lower number of *Bifidobacterium catenulatum*	(9)
IBS subjects (Rome II criteria) (41) Control subjects (26)	Lower number of Bifidobacteria Lower number of *Bifidobacterium catenulatum*	(10)

Table 11: Clinical trials on the prebiotic effect in inflammatory bowel disease

Subjecfs	Trial design[1]	Groups	N[2]	Duration	Key findings	Reference
Pouchitis (active)	Open label	(a) FOS (1 tablet/d) *L. rhamnosus GG* (1 tablet/d)	(a) 10	-	'Clinical and endoscopic remission'	Friedman et al (2000)([204])
Pouchitis (remission)	DB-RCT, CO	(a) Inulin (24 g/d) contained in drink (b) Placebo drink	(a/b) 20	3 weeks	Compared with baseline, the prebiotic: Reduced pouchitis activity Reduced *B. fragilis* Had no effect on bifidobacteria Increased faecal butyrate	Welters et al (2002)([205])
UC (active)	DB-RCT	(a) Oligofructose / inulin (12 g/d) *B. longum* ($4x10^{11}$ cells/d) (b) Maltodextrose placebo (12 g/d)	(a) 9 (b) 9	1 month	Compared with placebo, the synbiotic: Reduced sigmoidoscopy score Compared to baseline, the synbiotic: Increased mucosal bifidobacteria Reduced human beta defensin mRNA Reduced TNF α, IL-1α Reduced mucosal inflammation	Furrie et al (2005) ([159])
UC (active)	DB-RCT	(a) Oligofructose / inulin (12 g/d) (b) Maltodextrose placebo (12 g/d) Both groups started Mesalazine 3 g/d	(a) 10 (b) 9	2 weeks	Compared with placebo, the prebiotic: Did not result in greater reduction in disease activity Reduced faecal calprotectin Compared to baseline, the prebiotic: Reduced disease activity Reduced dyspepsia	Casellas et al (2007)([160])
CD, paediatric (active)	Open label	(a) Oligofructose / inulin (mean 8.4 g/d) (semi-elemental) Enteral nutrition	(a) 10	6 weeks	Compared with baseline, the prebiotic enteral formula: Reduced disease activity Reduced inflammation (ESR, WBC scan) Increased quality of life	Hussey et al (2003)([209])
CD (active)	Open label	(a) Oligofructose / inulin (15 g/d)	(a) 10	3 weeks	Compared with baseline, the prebiotic: Reduced disease activity Increased faecal bifidobacteria Did not affect mucosal bifidobacteria Increased dendritic cell IL-10 Increased dendritic cell TLR-2 and TLR-4 expression	Lindsay et al (2006) ([111])

CD (remission)	DB-RCT	(a) Synbiotic 2000 (inulin, resistant starch, pectin, β-glucans, 2.5g each, *P. pentoseceus, L. raffinolactis, L. paracasei, L. plantarum*) (b) Placebo	(a) 20 (b) 10	24 months	Compared with placebo, the synbiotic: Did not influence relapse rates	Chermesh et al (2007) ([211])
CD (active)	DB-RCT	(a) Oligofructose / inulin (15 g/d) (b) Maltodextrose placebo (15 g/d)	(a) 54 (b) 49	4 weeks	Compared with placebo, the prebiotic: Did not lower disease activity Did not result in greater reduction in disease activity Did not result in greater numbers in remission	Benjamin et al (2009)([210])

[1] DB-RCT, double-blind randomised controlled trial
[2] Numbers recruited to each group

Table 12: Published reviews on the prebiotic effect on mineral metabolism

Model	Dietary fibres	Mineral	Results	References
- Human	Fibres	Ca, Mg, Fe, Zn	Mineral metabolism	([417])
- Rat	Phytic acid			
- Rat	Prebiotics (FOS)	Ca	Bioavailability	([418])
- Human	Oligosaccharides	Ca, Mg, Fe, Zn	Ca absorption	([419])
- Rat			Ca absorption Methodology concerns	
- Human	Oligosaccharides	Ca	Bioavailability	([277])
- Human	Prebiotics	Ca, Mg, P, Fe, Zn	Mineral metabolism	(Schaafsma *et al.*, 1998)([420])
- Rat				
- Human	Prebiotics	Ca, Mg, Fe, Zn	Bioavailability	([254])
- Rat	Synbiotics		Functional foods	
- Human	Prebiotics	Ca, Mg, Fe, Zn	Bioavailability	([421])
- Rat	Probiotics			
- Human	Prebiotics	Ca, Mg, Fe, Zn	Mineral absorption	([422])
	(oligofructose, inulin)			
- Human	Prebiotics	Ca	Ca absorption	([423])
- Rat				
- Human	Prebiotics	Ca, Mg, Fe, Zn	Mineral absorption	([424])
- Rat	(FOS, GOS)			
- Human	Prebiotics	Ca, Mg, Fe, Zn	Mineral metabolism	([293])
- Rat	(oligofructose, oligosaccharides)		Ca metabolism	
			Bone structure	
			Mechanisms of action	
- Human	Prebiotics	Ca	Ca absorption	(Roberfroid, 2002)([425])
	(oligofructose, inulin)			
- Human	Prebiotics	Ca	Ca absorption	(Cashman, 2002)([426])
- Rat	(oligofructose, inulin)		Functional foods	
- Human	Prebiotics	Ca, Mg, P	Ca bioavailability	(Kaur & Gupta, 2002)([427])
- Rat	(oligofructose, inulin)			
- Rat	Prebiotics	Ca, Mg	Mineral metabolism	(Scholz-Ahrens & Schrezenmeir, 2002)([295])
	(oligofructose, inulin , TOS)		Bone structure	
			Mechanisms of action	
- Rat	Prebiotics	Ca	Ca bioavailability	(Cashman, 2002)([428])
- Human	(oligofructose, inulin, GOS)		Bone structure	
			Mechanisms of action	
- Human	Prebiotics	Ca	Ca bioavailability	(Cashman, 2002)([426])

- Human - Rat	Prebiotics	Mineral and trace elements	Mineral absorption, mechanisms of action	A. Bongers & E.G.H.M.van den Heuvel (2003)([429])
- Human - Rat	Prebiotics	Ca	Ca absorption, Bone health, Mechanisms of action, Osteoporosis	(Cashman, 2003)([430])
- Human - Rat	Prebiotics	Ca	Ca absorption	(Caers, 2003)([431])
- Human - Rat	Prebiotics (FOS, GOS, oligofructose, inulin)	Mg	Mg absorption	(Coudray *et al.*, 2003)([432])
- Human	Prebiotics	Mg	Mg absorption	(Coudray, 2004)([433])
- Human - Rat	Prebiotics (oligofructose, IF + oligofructose)	Ca	Ca balance, Bone health, Osteoporosis	(Coxam, 2005)([298])
- Rat	Prebiotics (oligofructose, inulin)	Ca, Mg	Ca absorption, Mg retention, Bone health	(Weaver, 2005)([434])
- Human - Rat	Prebiotics (oligofructose, inulin)	Ca	Ca absorption, Bone health, Osteoporosis	(Abrams, 2005)([273])
- Human - Rat	Prebiotics (oligofructose, inulin)	Ca	Ca absorption, Bone health	(Franck, 2006)([435])
- Human - Rat	Prebiotics (oligofructose, inulin)	Ca	Ca absorption, Bone health, Osteoporosis	(Bosscher, Van Loo & Franck, 2006)([436])
- Human	Prebiotics	Ca	Ca absorption, Bone mineralization, Mechanisms of action	(Cashman, 2006)([274])
- Human	Prebiotics (oligofructose, inulin) Phytoestrogens	Ca	Ca Bioavailability, Bone health, Phytoestrogens bioavailability	(Coxam, 2007)([437])
- Rat	Prebiotics (oligofructose, inulin) (impact of polymerization degree of prebiotics)	Ca, Mg P, Fe, Zn	Mineral metabolism, Ca metabolism, Bone health, Mechanisms of action	(Scholz-Ahrens & Schrezenmeir, 2007)([438])
- Human - Rat	Prebiotics Probiotics Synbiotics	Ca	Ca absorption, Bone health, Mechanisms of action	(Scholz-Ahrens *et al.*, 2007)([439])
- Human - Rat	Prebiotics (oligofructose, inulin)	Ca, Mg	Ca absorption, Bone health	(Alexiou & Franck, 2008)([440])
- Human - Rat	Prebiotics (oligofructose, inulin)	Ca	Ca absorption, Bone health, Osteoporosis	(Gibson & Delzenne, 2008)([441])

- Human	Prebiotics	Ca	Ca absorption	(De Vresse & Schrezenmeir, 2008)([158])
-Rat -Dog	Prebiotics	Ca	Ca absorption	(Griffin & Abrams, 2008)([442])
- Human - Rat	Prebiotics	Ca	Ca absorption, Bone mineralization	(Hawthorne & Abrams, 2008)([443])
- Human	Prebiotics (oligofructose, inulin)	Ca, Mg, Fe, Zn	Mineral metabolism, Bone remodelling, Mechanisms of action	(Kelly, 2009)([444])
- Human	Prebiotics Probiotics	Ca	Ca absorption, Osteoporosis	(De Vrese, 2009)([445])

FOS: Fructo- oligosaccharides

GOS: Galacto- oligosaccharides
TOS: Transgalacto- oligosaccharides

Table 13: The prebiotic effects on bone metabolism in the rat

Substance	Amount g/100g diet length of treatment	Bone Effect	Study design Animals (n) Method analysis	Reference
GOS	20 d	↑ tibia Ca content	OVX Wistar rats AAS	(Chonan *et al*., 1995)([446])
FOS (Meioligo-P, Japan)	5 60 d	↑ femoral Ca content ↑ bone volume	Growing Wistar rats (16 males) AAS Histomorphometric method	(Takahara *et al*., 2000)([447])
Oligofructose (Orafti) or Inulin (Orafti)	10 13 weeks	Both ↑ femoral Ca content	Growing Fisher rats (30 males, 4 week-old) ICPMS	(Richardson *et al*., 2002)([448])
Ca + Inulin (Raftiline HP, Orfati)	0.2 + 5 or 0.2 + 10 or 0.5 + 5 or 0.5 + 10 or 1 + 5 or 1 + 10 or From 4 to 22 weeks	↑ Whole body BMC ↑ Whole body BMD Ns Whole body bone area In each case (whatever Ca concentration and at all stage)	Growing Wistar rats (36 males, 4 week-old) DEXA	(Roberfroid *et al*., 2002)([425])
Ca + FOS (Raftilose P95, Orfati)	0.5 + 2.5 or	Ns L1-L4 Ca content ↑ trabecular tibial thickness	OVX Fisher 344 rats (96 females, 6 week-old) AAS Histomorphometric method	(Scholz-Ahrens *et al*., 2002)([295])
	0.5 + 5.0 or	Ns L1-L4 Ca content ↑ trabecular tibial perimeter		
	0.5 + 10 or	↑ L1-L4 Ca content ↑ trabecular tibial perimeter		
	1.0 + 50 or 16 weeks	↑ L1-L4 Ca content ↑ trabecular number		
-Oligofructose FOS (DP2-8, Orafti) or Inulin (Orafti) + FOS (DP2-8, Orafti)	5	Ns femoral BMC Ns femoral BMD ↑ spine BMC	Growing Sprague-Dawley rats (40 males, 7 week-old) DEXA ELISA	(Kruger *et al*., 2003)([296])
-Inulin (DP>23)	5 4 weeks	↑ femoral BMD ↑ spine BMC ↓ bone resorption		
-HP Inulin (DP 10-65) + ITF_{-MIX} (OF)	5+5	Ns tibial Ca content	Growing Wistar rats (10 males, 6 week-old) AAS	(Coudray *et al*., 2003)([297])
-HP Inulin (DP 10-65) + Oligofructose	5+5	Ns tibial Ca content		
- HP Inulin (DP 10-65)	10	Ns tibial Ca content		
- ITF_{-MIX}	10	Ns tibial Ca content		
- BC (branched –chain) inulin	10 28 d	Ns tibial Ca content		

ITF-$_{MIX}$	5.5 21 d	↑ femoral BMC ↑distal femur BMD	OVX Sprague-Dawley rat (26 females, 6 month-old) Ca^{45} kinetics method AAS	(Zafar *et al*., 2004a)([449])
-Inulin	5	Ns femoral Ca content ↑ femoral bone Ca content *vs* inulin	Growing Sprague-Dawley rats (48 males, 6 week-old) Ca^{45} kinetics method AAS	(Zafar *et al*., 2004b)([304])
- Inulin + IF	5 + 0.8 21 d			
IF (Prevastein, Eridania Beghin Say)+FOS (Actilight, Beghin Meiji)	10(μg/gwt/d) + 7.5	↑ Femoral BMD *vs* IF	Intact or OVX Wistar rat (88 females, 3 month-old) DEXA 3-point bending test RIA	(Mathey *et al*., 2004)([302])
	20 + 7.5	↑ Femoral BMD *vs* IF ↑ Femoral failure load ↓ urinary DPD		
	40 + 7.5	↑ Femoral BMD *vs* IF ↑ Femoral failure load ↓ urinary DPD		
	80 + 7.5	↑↑ Femoral BMD *vs* IF *vs* (IF10 + FOS) ↑ Femoral failure load ↓ urinary DPD		
Difructose anhydride III (DFAIII) (Nippon Beet sugar Mfg)	3 months 1.5 or 3 8 weeks	In intact rats Ns Maximum breaking force Ns distal femoral BMD In OVX rats ↑femoral Ca content ↑distal femoral BMD with 3% DFAIII ↑Maximum breaking force ↓ urinaryDPD in DFAIII groups (trend)	Intact or OVX Sprague-Dawley rats (50 females, 6 week-old) DEXA, 3-point bending test ELISA	(Mitamura & Hara, 2005)([450])
-Difructose anhydride III (DFAIII) (Nippon Beet sugar Mfg) - DFAIII + vitamin D-deficient	1.5 8 weeks	In intact rats Ns femoral Ca content In OVX rats ↑ femoral Ca content	Intact or OVX Sprague-Dawley rats (64 females, 6 week-old, vitamin D deficient or not) AAS	(Mitamura & Hara, 2006)([451])
-Oligofructose (chicory roots, Cosucra)	5	↑ Femur BMD ↑cancellous tibia area	Growing Wistar rats (38 males, 6 week-old) DEXA (pQCT) ELISA	(Nzeusseu *et al*., 2006)([452])
-Inulin (chicory roots, Cosucra)	5	↑ Femur BMD ↑ femoral BMC ↑ cancellous L3 area ↓ CTX1		
FOS (Raftilose P95, Orfati)	3 months 5 23 d	Ns Femur BMD ↑ Femur biomechanical properties	Growing Wistar rats (16 males, 4 week-old) DEXA 3-point bending test	(Lobo *et al*., 2006)([453])

FOS or IF+FOS	4 months	↑Whole body BMD vs control OVX ↑tibial BMC *vs* control OVX ↑lumbar BMD and BMC *vs* control OVX (no additive effects with IF+FOS) ↑tibial microarchitectural properties in IF+FOS (↑trabecular number *vs* OVX control)	OVX Sprague-Dawley rat (69 females, 9 month -old) DEXA Tomography	(Devareddy *et al*., 2006)([303])
Lc Inulin (Beneo HP, Orafti)	5 8 weeks	Ns BMD ↑ femoral BMC Ns bone markers (OC , CTX1)	Growing Sprague-Dawley rats (48 females, 3 week-old) DEXA ELISA	(Jamieson *et al*., 2008)([454])
-Inulin long – chain (Cosucra) or Inulin short – chain (Cosucra)	7.5	Trend to ↑ diaphysal femoral BMD and BMC Ns bone markers (OC ,DPD)	Growing Wistar rats (40 males, 3 month-old) DEXA 3-point bending test RIA	(Demigne *et al*., 2008)([455])
-Chicory (Cosucra)	7.5	↑diaphysal femoral BMD and BMC ↑ Femoral failure load Ns bone markers (OC , DPD)		
	3 months			
-SO (soybean oil) + ITF-$_{MIX}$	15 + 10.87	Ns femoral Ca content	Growing Wistar rats (24 males rats, 6 week-old) AAS 3-point bending test	(Lobo *et al*., 2009)([456])
- SO + Fish oil + ITF-$_{MIX}$	15 +11.5 + 10.87	↑ femoral Ca content ↑ tibial Ca content ↑ tibial bone strength		
	15 d			
IF or FOS or IF + FOS (Meioligo-P, Meiji)	0.2 5 0.2 + 5	↑distal femoral BMD and trabecular femur *vs* control OVX (additive effects with IF+FOS)	OVX mice (64 females ddY strain, 6week -old) Tomography	(Ohta *et al*., 2002)([301])
	6 weeks			
Inulin (Orafti)	10 2 weeks	↑ Mg bone content	C57B16J mice (24 males, 4 month-old) AAS	(Rondon *et al*., 2008)([457])

AAS: Atomic absorption spectrophotometry
DEXA: Dual- energy X ray absorptiometry
Femoral mechanical testing (3- point bending test)
FOS: Fructo-oligosaccharides
Galacto-oligosaccharides (GOS)
IF: Isoflavones

Table 14: The prebiotic effects on mineral absorption in the rat

Substance	Amount g/100g diet length of treatment (n)	Mineral absorption	Study design Animals (n) Method analysis	References
Raftilose P95 (Orafti)	5 3 d	↑ fractional Ca^{47} absorption	Fisher 344 (40 males, 38 week-old) Ca^{47} method Sc^{47} method Gamma counter	(Brommage *et al.*, 1993)([294])
FOS (Meioligo-P, Meiji)	5 28d	↑ apparent Ca and Mg absorption in intact rats ↑ apparent Mg absorption in cececomized rats	Intact or cececomized rats AAS	(Ohta *et al.*, 1994a)([291])
FOS (Meioligo-P, Meiji) (low Mg, High Ca and High P)	1 5	↑ apparent Mg absorption	Mg- deficient rats AAS	(Ohta *et al.*, 1994b)([306])
FOS (Meioligo-P, Meiji)	5 2 weeks	↑ apparent Ca, Mg and Fe absorption Improve recovery from anemia	Fe - deficient rats for 3 weeks (anemic rats) AAS	(Ohta *et al.*, 1995a)([307])
FOS (Meioligo-P, Meiji) (chromium-mordanted cellulose as an unabsorbable marker)	5 1d	↑ apparent Ca and Mg absorption And Colorectal absorption of Ca and Mg	Growing Sprague-Dawley rats (28 males, 6 week-old) (colon and rectum) AAS	(Ohta *et al.*, 1995b)([256])
GOS	20 d	↑ apparent Ca absorption	OVX wistar rats AAS	(Chonan *et al.*, 1995)([446])
TOS (Meioligo-P, Meiji)	5 10 10d	↑ apparent Ca absorption	Growing Wistar rats (males) AAS	(Chonan & Watanuki, 1995)([458])
Raftilose P95 (Orafti) or Raftiline ST (Orafti)	10 24d	Both ↑ apparent Ca, Mg and Zn retention Ns on Cu absorption Raftilose ↑ apparent Fe	Wistar rats (30 males, 100g) ICPMS	(Delzenne *et al.*, 1995)([267])
-Lactilol-oligosaccharide (LO) -Galactooligosaccharides (GL)	5 2 weeks	↑ apparent Ca absorption in LO ↑ apparent Mg absorption in LO and GL	Growing Sprague-Dawley rats (males, 8 week-old) AAS	(Yanahira *et al.*, 1997)([459])y
FOS (Meioligo-P, Meiji)	10 10d	↑ apparent Ca absorption	Growing gastrectomized Sprague-dawley rats (17 males, 4 week-old) AAS	(Ohta *et al.*, 1998)([259])
FOS (Meioligo-P, Meiji)	5 3 d	↑ true and apparent Ca absorption ↑ Ca balance	Growing Wistar rats (16males, 6 week-old) Ca^{45} kinetics study AAS	(Morohaschi *et al.*, 1998)([460])
-FOS short – chain (Meioligo-P, Meiji) (normal and Ca deficient diet)	10 10d	↑ CaBP levels Independent of 1,25(Oh)2D3 action	Rats (intestinal CaBP levels) AAS	(Takasaki *et al.*, 2000)([260])

FOS (DP 3-50) (Cosucra)	10	↑ apparent Ca, Mg, Fe, Cu absorption ↑ cecal Ca, Mg Ns Ca status	Growing Wistar rat (32 males, 6 week-old) AAS	(Lopez *et al*., 2000)([257])
FOS + PA (phytic acid)	10+7 21 d	↑ cecal Ca Ns cecal Ca vs PA		
FOS (Meioligo-P, Meiji)	5 60d	↑ apparent Ca absorption ↑ fractional Ca absorption	Growing Wistar rats (16 males, 6 week-old) AAS	(Takahara *et al*., 2000)([447])
-Inulin (Orafti)	10	↑ apparent Ca absorption ↑Ca retention	Adult Wistar rats (32 males, 8 week-old) AAS	(Younes *et al*., 2001)([461])
-Inulin + resistant starch	5 21d	(higher effect with inulin+resistant starch)		
-Difructose anhydride III (DFAIII) (Nippon Beet sugar Mfg)	3 4 weeks	↑ apparent Ca absorption	-Intact or OVX growing Sprague-Dawley rats (20 females, 6 week-old) - OVX or OVX cecocolonectomy growing Sprague-Dawley rats (20 females, 6 week-old) AAS	(Mitamura *et al*., 2002)([462])
- Difructose anhydride III (DFAIII) (Nippon Beet sugar Mfg)	1.5 3 4 weeks	-↑ Ca absorption rate was higher in cecolonectomized rats		
Ca + Oligofructose	0.5 + 2.5	↓ apparent Ca absorption (after 4 wk)	OVX Fisher 344 rats (96 females, 6 week-old) AAS	(Scholz-Ahrens *et al*., 2002)([295])
	0.5 + 5.0	Ns apparent Ca absorption ↑ apparent Ca absorption Vs OVX (wk 8)		
	0.5 + 10	↑ apparent Ca absorption Vs OVX (wk 4)		
	1.0 + 50 (16 weeks)	Vs OVX (wk 8) Vs OVX (wk 16)		
-HP Inulin (DP 10-65) + ITF-$_{MIX}$ (OF)	5+5	↑ apparent Ca and Mg absorption ↑Ca and Mg balance	Growing Wistar rats (10 males, 6 week-old) AAS	(Coudray *et al*., 2003)([297])
-HP Inulin (DP 10-65) + Oligofructose	5+5	OF+HP : additive effect		
- HP Inulin (DP 10-65)	10			
- ITF-$_{MIX}$	10			
- BC (branched –chain) inulin	10			
-Oligofructose FOS (DP2-8, Orafti) or	28 d 5	Ns urinary Ca excretion	Growing Sprague-Dawley rats (40 males, 7 week-old) ICPOES (vista model inductively coupled plasma optical emission spectroscopy)	(Kruger *et al*., 2003)([296])
-Inulin (DP>23)	5	Ns urinary Ca excretion ↑Ca bioavailability		
-Inulin (Orafti) + FOS (DP2-8, Orafti)	5 4 weeks	↑ urinary Ca excretion		

ITF-$_{MIX}$	5.5 21 d	↑ true Ca absorption ↑ Ca balance	OVX Sprague-Dawley rat (26 females, 6 month-old) Ca^{45} kinetics method AAS	(Zafar *et al*., 2004a)([449])
-Inulin	5	Ns true Ca absorption *vs* IF	Growing Sprague-Dawley rats (48 males, 6 week-old) AAS, Ca^{45} kinetics method	(Zafar *et al*., 2004b)([304])
- Inulin + IF	5 + 0.8 21d			
-FOS short – chain (Meioligo-P, Meiji) -Four non digestible saccharides (DFAIII, Nippon Beet Sugar MFG)	3 4 weeks Measurement after 10-14 days	↑ apparent Ca, Mg, Fe absorption	Growing Sprague-Dawley rats (48 males) AAS	(Asvarujanon, 2005)([463])
-FOS short – chain (Meioligo-P, Meiji) -Four non digestible saccharides (DFAIII, Nippon Beet Sugar MFG)	3 4 weeks Measurement after 24-28 days	↑ apparent Ca, Mg absorption Higher effect with DFAIII DFAIII ↑ Fe absorption		
-FOS short – chain (Meioligo-P, Meiji) -Four non digestible saccharides (DFAIII, Nippon Beet Sugar MFG)	3 5 weeks	-Ns apparent Ca absorption in OVX rats -↑ apparent Ca absorption vs FOS in OVX rats	Growing OVX Sprague-Dawley (68 females, 6 week-old) AAS	
Difructose anhydride III (DFAIII) (Nippon Beet sugar Mfg)	1.5 or 3 8 weeks	Both doses restore the reduced Ca absorption in OVX rats and Mg absorption in both OVX and SH rats	Intact or OVX Sprague-Dawley rats (50 females, 6 week-old) AAS	(Mitamura & Hara, 2005)([450])
ITF-$_{MIX}$	10 21 d	↑ Net transepithelial Ca transport (large intestin) ↑ Ca absorption rate (caecum)	Growing Sprague-Dawley rats (48 males) (transepithelial Ca in vitro) AAS	(Raschka, 2005)([261])
Ca + inulin (Raftiline, Orafti)	0.25 + 10 0.50 + 10 0.75 + 10 40 d	After 13 d ↑ apparent Ca absorption higher effect when Ca is low (0.25) or high (0.75) After 40 d ↑ apparent Ca absorption higher effect when Ca is low (0.25)	Growing rats, 10 weeks (10 males wistar) AAS	(Coudray et al., 2005a)([464])
Inulin (Raftaline, Orafti)	7.5 3 weeks	-↑ true Ca absorption Higher effect in 10 and 20 month-old animals vs those aged 2 and 5 month-old	Wistar rats (18 males -2 month-old -5 month-old -10 month-old -20 month-old Ca44 method, AAS ICPMS	(Coudray et al., 2005b)([465])
-Difructose anhydride III (DFAIII) (Nippon Beet Sugar MFG)	3	↑ Fe absorption	Growing Sprague-Dawley rats (18 males, 4 week-old)	(Shiga et al., 2006)([466])
-FOS (Meioligo-P, Meiji)	3 4 weeks	DFAIII restores gastrectomy-induced Fe malabsorption	Growing gastrectomized Sprague-Dawley rats (32 males, 4 week-old) AAS	

Shoyu polysaccharides (SPS)		↑ iron absorption in organs	Anemics rats (*in vivo*, *in vitro*)	(Kobayashi *et al*., 2006)([308])
FOS (Raftilose P95, Orfati)	5 23 d	↑ apparent Ca absorption ↑ apparent Mg absorption	Growing Wistar rats (16 males, 4 week-old) AAS	(Lobo *et al*., 2006)([453])
-Oligofructose (chicory roots, Cosucra)	5	↑ apparent Ca absorption (Higher effect with inulin which could be related to an ↑ calbindin-9K)	Growing Wistar rats (38 males, 6 week-old) AAS	(Nzeusseu *et al*., 2006)([452])
-Inulin (chicory roots, Cosucra)	5 3 months			
-Difructose anhydride III (DFAIII) (Nippon Beet sugar Mfg) - DFAIII + vitamin D-deficient	1.5 8 weeks	In intact rats Ns apparent Ca absorption ↑ apparent Ca absorption in vitamin D-deficient rats In OVX rats ↑ apparent Ca absorption (higher effect in vitamin D-deficient rats)	Intact or OVX Sprague-Dawley rats (64 females, 6 week-old, vitamin D deficient or not) AAS	(Mitamura & Hara, 2006)([451])
Inulin (Raftaline, Orafti)	7.5 3 weeks	-↑ true Cu and Zn absorption lower effect in 10 and 20 month-old animals *vs* those aged 2 and 5 month-old	Wistar rats (18 males - 2 month-old -5 month-old -10 month-old -20 month-old Cu^{65} Zn^{67} method, AAS ICPMS	(Coudray *et al*., 2006)([467])
-Inulin long – chain (Cosucra) or -Inulin short – chain (Cosucra) - Chicory (Cosucra)	7.5 3 months	↑ apparent Ca absorption (1 month) Ns 3 month	Growing Wistar rats (40 males, 3 month-old) AAS	(Demigne *et al*., 2008)([455])
Inulin (Orafti)	10 2 weeks	↑ Mg absorption	C57B16J mice (24 males, 4 month-old) AAS	(Rondon *et al*., 2008)([457])
-GR inulin (Orafti) -Artichoke inulin - ITF-$_{MIX}$ -Artichoke + P95 oligofructose	0.1 (0.82g/d human equivalent dose) 75 d	-Ns on calcemia level -↑ calcemia -Ns on calcemia level -Ns on calcemia level	Growing Sprague-Dawley rats (36 females, 6 week-old) Colorimetric assay	(Azorin-Ortuno, 2009)([468])
-SO (soybean oil) + ITF-$_{MIX}$ - SO + Fish oil + ITF-$_{MIX}$	15 + 10.87 15 +11.5 + 10.87 15 d	↑ apparent Ca absorption ↑ apparent Ca absorption (higher effect)	Growing Wistar rats (24 males rats, 6 week-old) AAS	(Lobo *et al*., 2009)([456])
Inulin HPX (Orafti)	2.5 5 d	Ns apparent Ca absorption	Wistar rats (24 males, 6 week-old) AAS	(Klobukowski *et al*., 2009)([469])
FOS FOS + PA (phytic acid) (Shandong Zibo Jiyun Biotechnology)	0.08 or 0.25 0.08 + 1 or 0.25 + 1 4 weeks	FOS↑ apparent Ca, Mg and Fe absorption and counteract the deleterious effects of PA	Kung-Ming mice (60 males, 4 week-old) AAS	(Wang *et al*., 2009) (with mice)([470])

Apparent absorption: Ca intake (I) –Ca fecal excretion (F)

AAS: Atomic absorption spectrometry
Ca balance: 4-7 days balance period (I, F, U using metabolic cages) % Ca^{45} absorption: % Ca^{45} oral dose / % Ca^{45} IP dose x 100

Fractional Ca absorption: Ca^{47}, Sc^{49} ratio (I – F)
GOS: Galactooligosaccharides
ICPMS: Inductively coupled plasma mass spectrometry
Net retention: Ca intake (I) – [Ca fecal excretion (F) + Ca urinary excretion (U)]
TOS: Transgalactosylated oligosaccharides
True intestinal Ca absorption: (Ca^{45} Ca^{44}) = (I – F)+ f (endogenous net Ca excretion)

Table 15: The prebiotic effects on mineral absorption in the human

Substance	Amount (g/d) length of treatment (n)	Mineral absorption	Study design Subjects (n)	Reference
Sc Inulin (infant formula)	0.75, 1 or 1.25	Ns apparent Ca absorption (↑ apparent and net iron retention with 1g/d) (↑ apparent and net Mg retention with O.75 & 1. 25g/d)	R study Formula-fed Infants (6-12 month-old) (36) AAS	(Yap *et al*., 2005)([268])
Oligofructose (Raftilose P95, Orafti)	15 9 days	↑ true fractional Ca absorption	R, DB, CO study Male adolescents (24) Kinetic technique (Ca^{44}, Ca^{48}) ICPMS	(Van den Heuvel *et al*., 1999a)([269])
Oligofructose (Raftilose P95, Orafti) or Sc-FOS + ITF_{MIX}	8 3 weeks	Ns with oligofructose ↑ true Ca absorption with Synergy 1	DB, CO study Young Girls (29) Kinetic technique (Ca^{46}, Ca^{42}) TIMMS	(Griffin *et al*., 2002)([270])
Sc-FOS + ITF_{MIX}	8 3 weeks	↑ true Ca absorption	R, CO study Young girls (54) Kinetic technique (Ca^{46}, Ca^{42}) TIMMS	(Griffin *et al*., 2003)([271])
Sc-FOS + ITF_{MIX}	8 1 year	↑ fractional Ca absorption	DB study Male & female adolescents (48) Kinetic technique (Ca^{46}, Ca^{42}) TIMMS	(Abrams *et al*., 2005b)([273])
Sc-FOS + ITF_{MIX}	8 1 year	↑ true fractional Ca absorption (32 responders & 16 non-responders)	DB, PC, Sex stratification study Male and female adolescents (48) Kinetic technique (Ca^{46}, Ca^{42}) TIMMS	(Abrams *et al*., 2007b)([275])
Sc-FOS (Actilight, Beghin Meiji)	10 37 days	Ns true fractional Ca absorption (↑ true Mg absorption)	R, DB, CO study Adolescent girls (14) Low Ca intake (Ca^{44}, Ca^{48}) ICPMS	(Van den Heuvel *et al*., 2009)([272])
Inulin (Chicory roots)	40 28 days	↑ apparent Ca absorption	3x3 Latin square Young men (9) AAS	(Coudray *et al*., 1997)([276])
Inulin (Raftiline ST, Orafti) OF (Raftilose P95, Orafti)	17 3 days	Ns mineral (Ca, Mg, Zn, Fe) excretion because of ileostomy	DB, CO study ileostomised patients (5 men and 5 women) AAS	(Ellegard *et al*., 1997)([292])
Inulin, FOS, or GOS (Orafti)	15 21 days	Ns true fractional Ca or iron absorption *(Methodologic concern : analysis after 24h urines)*	DB, CO study Young men (12) Kinetic technique (Ca^{44}, Ca^{48}) ICPMS	(Van den Heuvel *et al*., 1998)([277])
Inulin (Raftiline, Orafti) + Ca (210 mg/d)	15 5 days	Ns urinary Ca excretion (lower iPTH lower →later increase in Ca absorption)	R, DB, CO study Young woman (50) AAS IRMA	(Teuri *et al*., 1999)([278])

Shoyu polysaccharides (SPS)	0.6 4 weeks	↑ in plasma iron in the SPS group	R, DB, PC parallel study Young woman (45) AAS	(Kobayashi *et al*., 2006)([308])
FOS (Ebro-Puleva) in milk	0.75g/100ml 1d	Ns true fractional Ca absorption	R, DB, CO study Young men (8) and women (7) Kinetic technique (Ca^{44}, Ca^{42}) ICPMS	(Lopez-Huertas *et al*., 2006)([279])
Sc-FOS + ITF_{MIX}	8 8 weeks	↑ true fractional Ca absorption (responders /non responders) Colonic absorption	Young adults (13) Kinetic technique (Ca^{42}, Ca^{46}) TIMMS	(Abrams *et al*., 2007a)([280])
Lactulose	5 or 10 9 days	Ns true fractional Ca absorption with 5g/d ↑ true Ca absorption with 10g/d	R, DB, CO study POM (12) Kinetic technique (Ca^{44}, Ca^{48}) ICPMS	(Van den Heuvel *et al*., 1999b)([284])
Transgalactooligosaccharide TOS (Elix'or)	20 9 days	↑ true Ca absorption	R, DB, CO study POM (12) Kinetic technique (Ca^{44}, Ca^{48}) ICPMS	(Van den Heuvel *et al*., 2000)([285])
Sc FOS (Beghin-Say)	10 35 days	↑ Mg absorption, accompanied by an ↑ in plasma Mg^{25} and higher Mg excretion	R, DB, CO study POM (12) Kinetic technique (Mg^{25}) ICPMS	(Tahiri *et al*., 2001)([282])
Sc FOS (Beghin-Say)	10 35 days	-Ns true Ca absorption -Trend for ↑ in women > 6 yr POM subgroup	R, DB, CO study POM (12) Kinetic technique (Ca^{44}) ICPMS	(Tahiri *et al*., 2003)([283])
Chicory fructan fiber (Cosucra)	8 3 months	↑ apparent Ca absorption ↑ apparent iron absorption	DB parallel design POM (13) AAS	(Kim *et al.*, 2004)([287])
Sc FOS (Actilight, Beghin-Say)	10 35 days	↑ Cu absorption No effect on ZN and Se	R, DB, CO study POM (12) Kinetic technique (Cu^{65} Zn^{67} Se^{74}) ICPMS	(Ducros *et al*., 2005)([281])
Sc-FOS + ITF_{MIX}	10 6 weeks	↑ fractional Ca absorption	R, DB, PC, CO study POM (50) Kinetic technique (Ca^{46}, Ca^{42}) ICPMS	(Holloway *et al*., 2007)([288])
Sc-FOS + ITF_{MIX} + Ca + CPP + fermented milk	1.75g/cup 14 days	-↑ intestinal Ca absorption with Synergy 1 + Ca + CPP	Parallel DB, PC study POM (85) HPLC Colorimetric assay (Kone)	(Adolphi *et al*., 2009)([286])

AAS: Atomic Absorption Spectrometry
Fractional Ca: (Ca^{44}, Ca^{43}) ratio ; (Ca^{46}, Ca^{42}) ratio
ICPMS: Inductively Coupled Plasma Mass Spectrometry
R, randomized; DB, double-blind, PC, Placebo Control; CO, crossover
TIMMS: Thermal Ionisation Magnetic sector Mass Spectrometry

Table 16: The prebiotic effects on human bone health

Substance	Amount g/d length of treatment (n)	Bone Effect	Study design Subjects (n) Method analysis	References
Sc-FOS + ITF-$_{MIX}$	8 1 year	↑ BMC ↑ BMD	DB, PC, Sex stratification study Male and female adolescents (48) DEXA	(Abrams *et al*., 2005b)([273])
Sc-FOS + ITF-$_{MIX}$	8 1 year	Higher Ca accretion in responders (Ca absorption ↑ by at least 3%)	DB, PC, Sex stratification study Adolescents (48) 32 responders & 16 non-responders DEXA	(Abrams *et al*., 2007b)([275])
Sc-FOS (Actilight, Beghin Meiji)	10 37 days	Ns bone resorption (DPD) Ns PTH Ns Vitamin D	R, DB, CO study Adolescent (40) HPLC	(Van den Heuvel *et al*., 2009)([272])
Inulin (Raftiline, Orafti) + Ca (210 mg/d)	15 5 days	Ns PTH	R, DB, CO study Young woman (50) IRMA	(Teuri *et al*., 1999)([278])
Sc FOS (Beghin-Say)	10 35 days	Ns bone turnover (OC-DPD) ↘1,25(OH)2D in early POM subgroup	R, DB, CO study POM (12) Kinetic technique (Ca^{44}) ICPMS, RIA	(Tahiri *et al*., 2003)([283])
Chicory fructan fiber (Cosucra)	8 3 months	Ns lumbar spine or femoral neck BMD (short term study) Ns bone turnover markers Trend to ↘ DPD	DB parallel study POM (13) DEXA, IRMA, ELISA	(Kim *et al*., 2004)([287])
Sc-FOS + ITF-$_{MIX}$	10 6 weeks	↑ Bone turnover (OC-DPD)	R, DB, PC, CO design POM (50) IRMA –ELISA	(Holloway *et al.*, 2007)([288])
Isoflavones + prebiotics or Isoflavones +sc FOS (Actilight, Beghin-Meiji)	7 30 days	Ns bone formation (b-ALP) ↘bone resorption (DPD) compared to when isoflavones are given alone Higher effects in early POM *vs* late POM	Parallel DB, PC study POM (39) IRMA-RIA	(Mathey *et al.*, 2008)([471])
Sc-FOS + ITF-$_{MIX}$ ITF-$_{MIX}$ + Ca + CPP + fermented milk	1.75g/cup 14 days	Fermented milk ↘nocturnal bone turnover (↘DPD) Additional effect of Synergy 1 + Ca + CPP	Parallel DB, PC study POM (85) HPLC	(Adolphi *et al.*, 2009)([286])
Inulin (Fruitifit Sensus Inc)	15 3 weeks	Ns bone resorption (urinary NTx)	DB, CO study Institutionalized adults (less than 60 year-old) (15) ELISA	(Dahl *et al*., 2005)([289])

MD: Bone Mineral Density, BMC: Bone Mineral Content, PP: Caseinophosphopetide, DPD: Deoxypyridinoline, ELISA: Enzyme-Linked Immunosorbent Assay, IRMA: Immunoradiometric assay
OC: Osteocalcin, POM: Postmenopausal women, PTH: Parathormone, RIA: Radioimmunoassay

Table 17. Experimental data supporting the prebiotic effects on body weight and fat mass development

Animal model	Study design	Results	Reference
Male Wistar rats	10% FOS or GOS – 50d	↓BW gain (NS)	[472]
Male obese Zucker rats	10% FOS – 7 wk	↓BW gain	[473]
Male Wistar rats	10% FOS – 3 wk	daily BW gain =	[474]
Male obese Zucker rats	10% fructan (ITF_{-MIX}) – 8 wk	↓BW gain	[475]
Male Wistar-Han rats fed either high fructose diet or starch-based diet	10% FOS – 4 wk	↓BW gain (NS)	[476]
Male Wistar rats	10% FOS or FOS+inulin or inulin alone – 3 wk	↓BW gain (NS) ↓EAT for FOS and inulin	[477]
Male Wistar rats fed a HF–HC diet	pretreatment with standard diet or FOS-enriched (10%) standard diet for 35 d followed by 15 d of HF-HC diet with or without FOS (10%)	↓BW gain ↓EAT	[478]
Male Wistar rats	5% high and low-molecular inulin versus 5% cellulose– 4 wk	BW gain =	[479]
Male C57Bl/6J mice fed a HF– carbohydrate free diet	10% FOS – 4 wk	↓BW gain ↓EAT	[480]
Male Wistar rats	5% or 10% inulin – 4wk	↓final BW (NS)	[481]
Male C57Bl/6J mice fed a HF–carbohydrate free diet	10% FOS – 4 wk	↓BW gain ↓EAT	[482]
Male C57Bl/6J mice fed a HF–HC diet	10% FOS – 4 wk	↓BW gain (NS) EAT =	[483]
Male Wistar rats fed a HF and HC diet	5 % inulin – 8 wk	↓final BW	[484]
Male Wistar rats	10% FOS – 4 wk	↓BW gain ↓EAT, IAT, VAT	[485]
Male C57Bl/6J mice fed a HF–carbohydrate free diet	10% FOS – 14 wk	↓BW gain ↓EAT, VAT, SAT	[486]
Male obese (cp/cp) James C Russell corpulent rats	9 % inulin – 3 wk	↓final BW	[487]
Male C57Bl/6J mice	10% FOS or inulin-type fructans from Agavae - 5 wk	↓BW gain ↓EAT for fructans from Agave tequilana Gto	[488]
Female Sprague–Dawley rats	5% inulin + 5% cellulose versus 10% cellulose – 4 and 8 wk	↓BW gain (NS) ↓whole body fat mass	[489]
Male obese ob/ob mice	10% FOS – 5 wk	↓EAT, VAT, SAT	[490]

BW, body weight; d, days; EAT, epididymal adipose tissue; FOS, fructo-oligosaccharides; GOS, galacto-oligosaccharides; HC, high carbohydrate; HF, high fat; IAT, inguinal adipose tissue; NS, not significant; SAT, subcutaneous adipose tissue; VAT, visceral adipose tissue; wk, weeks.

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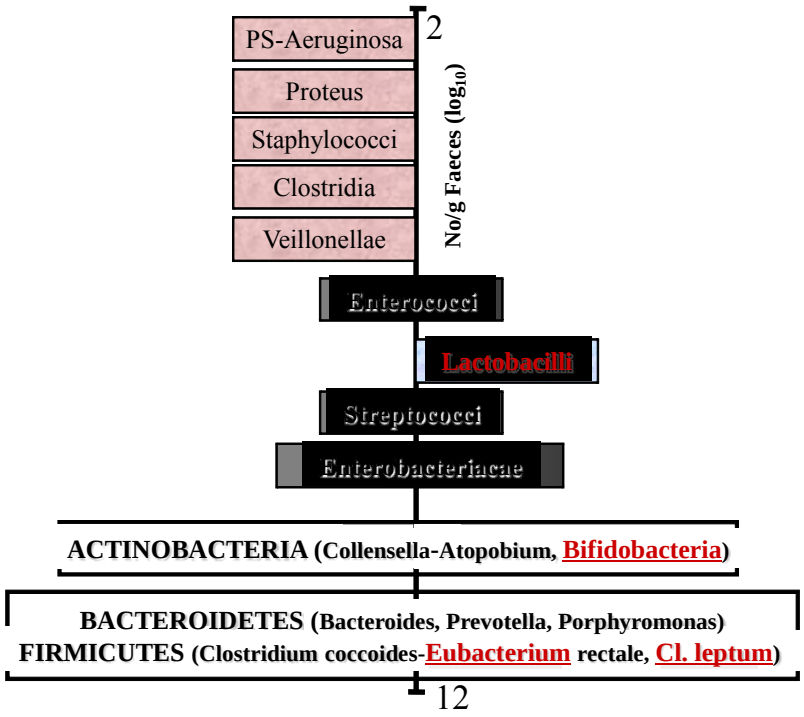

Figure 1: Schematic representation of gut microbiota

Major phylla and genera are located on a logarithmic scale as N° of CFU/g of faeces. Genera on the left site are likely to be potentially harmful whereas those on the right site are potentially beneficial to health. Those that sit both on the left site and the right site either contain species that are potentially harmful and species that are potentially beneficial to health or contain genera/species that still need to be classified. Indeed many of these have only recently been identified in the gut microbiota and their activity(ies) is/are still largely unknown.