

Prebiotic effects: metabolic and health benefits

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Prebiotic Effects: Metabolic and Health Benefits

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1	Table of contents	
2	1	Prebiotic effects in the gut 8
3	2	Prebiotic effects and immune system.....21
4	3	Prebiotic effects in paediatrics28
5	4	Prebiotic effects and Gastro-intestinal disorders.....35
6	5	Prebiotic effects and mineral absorption.....58
7	6	Prebiotic effects in weight management and obesity-related disorders69
8	7	Conclusion and perspectives: Which data to support the hypothesis of a causal relationship
9		between a prebiotic effect and health effects/benefits?..... 81
10	8	Acknowledgement86

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14

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

26

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28

1 **Running Title:** Prebiotic concept and health

2

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

6

7 **Abstract:**

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

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

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

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

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

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

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

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

20

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

26

1 Introduction¹

2

3 In the 1980s, Japanese researchers (^{1; 2}) had already demonstrated that specific non-digestible
4 oligosaccharides (especially fructo-oligosaccharides) were selectively fermented by bifidobacteria and
5 had the capacity, upon feeding, stimulating their growth in human faeces. These observations were
6 confirmed and further expanded by Gibson & Roberfroid who introduced the concept of prebiotics in
7 1995 (³) and have recently published a review of the research which includes the most recent
8 development (⁴) (Table 1). During the last fifteen years, this concept has attracted the interest of many
9 academic as well as industrial scientists and it has become a popular research topic in nutrition and,
10 more recently, in the biomedical fields.

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

23 A large part of the research activity has concentrated, and still does focus on the *in vitro* and *in vivo*
24 ability of selective modification in the composition of the complex gut microbiota, in particular research
25 has focused on the selective stimulation of growth of mainly bifidobacteria, but also lactobacilli. In the
26 future, it is likely this may be expanded towards other genera eg *Eubacterium*, *Faecalibacterium* and
27 *Roseburia*. It has become clear that products, causing such a selective modification in gut
28 microbiota's composition and/or activity(ies), could, in addition, either induce beneficial physiological
29 effects not only in the colon but also within the whole body and/or contribute towards reducing the risk
30 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.

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

3

4 The intensiver research of the past 15 years has contributed towards an improved understanding of
5 the complexity of the gut microbiota. This includes the discovery of new phyla/genera, their relative
6 concentration in the gut microbiota, the key role of diet in modulating its composition, the changes
7 associated with ageing or chronic diseases and the individual character of gut microbiota composition.
8 In addition, past research has given us insights into its roles in human physiology and miscellaneous
9 pathophysiological conditions. The gut microbiota is thus now perceived as a key player in health and
10 well-being with, as a principal condition, a composition in which potentially health promoting dominant
11 microorganisms (especially the saccharolytic genera/species e.g. bifidobacteria) are elevated and/or
12 more active than the potentially harmful ones (especially the proteolytic/putrefactive genera/species)
13 (^{3; 21}) a situation known as 'normobiotic' or 'eubiotic'. It is now well recognized that, within such a
14 potentially health beneficial dominant microbiota, the genus *Bifidobacterium*, plays an important role
15 although future research may show different genera/species to also be important. Indeed, it has been
16 hypothesized that increasing bifidobacteria in gut microbiota, might improve health status and reduce
17 disease risk.

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

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

24 with

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

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

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

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

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

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

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

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

11

12 **1 Prebiotic effects in the gut²**

13 **1.1 Microbiota of the gastro-intestinal tract**

14

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

26

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 1.1.1 The stomach

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

12 1.1.2 The small intestine (duodenum, jejunum and ileum)

13

14 The environment of the duodenum is acidic (pH 4–5) with lactobacilli and streptococci predominating,
15 and numbers of bacteria are higher than those found in the stomach [10^2 – 10^4 CFU (ml contents) $^{-1}$;
16 (²⁷)].

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

26 The microbiota changes markedly from the duodenum to the ileum, as the velocity of the intraluminal
27 content decreases, pH increases and oxidation–reduction potentials lower, with bacterial loads
28 increasing to 10^6 – 10^8 CFU (ml contents) $^{-1}$ (²³). As transit time in the small intestine is rather rapid (2–
29 4h) and the bacterial density relatively low, its impact in terms of overall fermentation is low

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

4

5 1.1.3 The large intestine

6

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

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

1 Early cloning studies examined relatively small numbers of clones to generate a phylogenetic
2 inventory of the faecal microbiota of healthy adults. Wilson & Blitchington ⁽²²⁾ generated two clone
3 libraries [one from a 9-cycle polymerase chain reaction (PCR) (50 clones, 27 operational taxonomic
4 units (OTUs)), the other from a 35-cycle PCR (39 clones, 13 OTUs)] from a faecal sample from a
5 healthy 40-year-old male. Of the clones they analysed, 35 % were related to the *Bacteroides* group,
6 10 % to the *Clostridium coccooides* group (*Clostridium* cluster XIVa) and 50 % to the *Clostridium*
7 *leptum* group (*Clostridium* cluster IV). Less than a quarter of the sequences analysed were derived
8 from known bacteria. Suau *et al.* ⁽⁵⁾ found that, of the 284 clones they generated from a faecal sample
9 from a 40-year-old male, the majority of the sequences fell into three phylogenetic groups:
10 *Bacteroides* (31 %), *Clostridium coccooides* (44 %) and *Clostridium leptum* (20 %). The remaining
11 clones were derived from *Streptococcus salivarius* and *Streptococcus parasanguinis* and bacteria
12 related to *Mycoplasma* spp., clostridia, the *Atopobium* group, *Verrucomicrobium spinosum* and the
13 *Phascolarctobacterium faecium* subgroup. Seventy-six per cent of the clones analysed were derived
14 from previously unknown bacteria. Blaut *et al.* ⁽³²⁾ used a cloning approach to demonstrate that
15 microbial diversity in faeces increases with age ⁽³²⁾. It was found that the number of OTUs
16 corresponding to known molecular species was highest in infants and lowest in the elderly, with 92 %
17 of sequences from the elderly subjects corresponding to previously unknown bacteria.

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

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

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

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

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

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

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

23

24 **1.2 Prebiotic effects and fermentation and physiology**

25 **1.2.1 Bacterial fermentation in the large gut**

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

1 to degrade available substrates (^{35; 36}). These may be derived from either the diet or by endogenous
2 secretions (³⁷).

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

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

14

15 **1.2.2 Substrate utilisation in the colon**

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

28

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

1 and resistant dextrins, non-starch polysaccharides (e.g. pectins, arabinogalactans, gum Arabic, guar
2 gum and hemicellulose), non-digestible oligosaccharides (e.g. raffinose, stachyose, ITF, galactans
3 and mannans) as well as undigested portions of disaccharides (e.g. lactose) and sugar alcohols (e.g.
4 lactitol and isomalt) (^{37; 43; 44}). Resistant starch, non starch polysaccharides, most dietary fibres but
5 also some non-digestible oligosaccharides (e.g. lactose) are fermented by a wide range of the colonic
6 bacterial although the degree of their breaking down might vary (⁴⁵). However, some non-digestible
7 oligosaccharides entering the colon are rapidly and quantitatively but selectively fermented (e.g.
8 raffinose, ITF and galactans) by a small number of bacteria (e.g. bifidobacteria and lactobacilli) (⁴⁶).
9 The overall intake of non-digestible carbohydrate in a Western diet is estimated between 20-30 g/day
10 (⁴⁷). Endogenous carbohydrates, chiefly from mucins and chondroitin sulphate, contribute about 2-3
11 g/day of fermentable substrate (⁴⁸). The main saccharolytic species in the colonic microflora belong to
12 the genera *Bacteroides*, *Bifidobacterium*, *Ruminococcus*, *Eubacterium*, *Lactobacillus* and *Clostridium*.

13

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

19

20 1.2.3 Products of microbial fermentation in the colon and their effects on the host

21 Carbohydrates in the colon are fermented to SCFAs, mainly, acetate, propionate and butyrate (⁵⁰⁻⁵²)
22 and a number of other metabolites such as the electron sink products lactate, pyruvate, ethanol,
23 succinate as well as the gases H₂, CO₂, CH₄ and H₂S (⁵³). As a whole, SCFAs acidify the luminal pH
24 which suppresses the growth of pathogens (⁵⁴). They are rapidly absorbed by the colonic mucosa and
25 contribute towards energy requirements of the host (^{50; 55; 56}). Acetate is mainly metabolised in human
26 muscle, kidney, heart and brain Propionate, that is cleared up by the liver, is a possible gluconeogenic
27 substrate and it might contribute to inhibition of cholesterol synthesis. It might also play a role in the
28 regulation of adipose tissue deposition (^{57; 58}).

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

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

5
6 Proteins reaching and/or produced in the colon are fermented to branched chain fatty acids such as
7 isobutyrate, isovalerate and a range of nitrogenous and sulphur-containing compounds. Unlike
8 carbohydrate fermentation products which are recognized as beneficial to health, some of the end
9 products of amino acids metabolism may be toxic to the host e.g. ammonia, amines and phenolic
10 compounds (⁴⁹). Consequently, excessive fermentation of proteins, especially in the distal colon, has
11 been linked with disease states such as colon cancer and inflammatory bowel diseases, which
12 generally start in this region of the large intestine before affecting more proximal areas. Thus, it is
13 favourable to shift the gut fermentation towards saccharolytic fermentation over a prolonged period of
14 time into the distal parts.

15

16 **Conclusions**

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

28

29 **1.3 In vitro tests for prebiotic effect**

30

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

11

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

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

22 • to show changes in faecal microbiota (e.g. increase in bifidobacteria) but also to compare the
23 efficacy of different substrates (e.g. ITF, starch, polydextrose, fructose and pectin, galactans,
24 xylo-oligosaccharides, soybean oligosaccharides (⁶³⁻⁶⁵))

25 • to measure and to compare the evolution of gas and SCFAs production as a result of the
26 fermentation of different substrates (⁶⁴).

27

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

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

6

7 Continuous culture systems inoculated with faecal slurries can be used to investigate fermentation
8 profiles showing for example that, in accordance with earlier studies, bifidobacteria, and to a lesser
9 extent lactobacilli preferred ITF to glucose, whereas bacteroides could not grow on these substrates
10 (^{67; 68}). By varying parameters in the chemostat, the conditions for growth of bifidobacteria and
11 inhibition of bacteroides, clostridia and coliforms can be further analyzed showing that low pH (pH
12 5.5), high culture dilution rate (0.3h⁻¹) and 1% (w/v) concentration of carbohydrate, (i.e. similar to the
13 physicochemical environment of the proximal colon) are optimum.

14

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

28

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

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

14

15 A more sophisticated *in vitro* model of fermentation in the proximal large intestine is the TIM-2 model
16 (^{76; 77}). This consists of a series of linked glass vessels containing flexible walls. This arrangement
17 allows simulation of peristalsis together with temperature regulation by means of pumping water
18 through the space between the glass and flexible walls. The flow is controlled by computer to more
19 accurately simulate peristaltic mixing. The vessels are further equipped with a hollow fibre membrane
20 in the lumen to simulate absorption of water and short chain fatty acids. TIM-2 has been used to
21 investigate the population changes on the fermentation of lactulose using culture-based methods
22 coupled with DGGE (⁷⁷). Increases in lactobacilli and enterococci were seen.

23

24 Conclusions

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

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

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

7

8 **1.4 Human studies showing prebiotics effect in healthy persons**

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

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

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

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

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

17

18 **1.5 Conclusion**

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

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

22

23 **2 Prebiotic effects and immune system³**

24 **2.1 Outline of benefit area**

25 To provide optimal resistance against a large variety of pathogenic encounters, the immune system
26 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.

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

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

28

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

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

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

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

22
23 Secondly, microbial products such as SCFAs may interact with immune cells and enterocytes and
24 modify their activity. G-protein coupled receptors (GPR) 41 and GPR 43 are identified as receptors for
25 SCFA and are expressed on leukocytes, especially polymorphonuclear cells, (^{91; 92}) as well as on
26 enterocytes and enteroendocrine cells in the human colon (^{93; 94}). SCFAs modulate chemokine
27 expression in intestinal epithelial cells (⁸⁶), differentially affect pro-inflammatory IL-2 and IFN γ and
28 immuno-regulatory IL-10 production by rat lymphocytes *in vitro* (⁹⁵) and a recent publication shows
29 the importance of ligation to GPR43 in mice to maintain intestinal homeostasis (⁹⁶).

30

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

4
5 In summary, there are plausible mechanisms by which prebiotic effects can modulate immune
6 function parameters. The inaccessibility of the human GI immune system complicates the
7 investigation in this area and most human studies rely on the measurement of *ex vivo* systemic
8 immune markers, of which the predictive value for overall resistance to infections or outcome of
9 immune-related disorders is limited.

10

11 2.2 Summary of key studies

12

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

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

27

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

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

11

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

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

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

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

7

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

17 In infants at high risk of allergies, a mixture of GOS/FOS supplemented for 6 months reduced plasma
18 level of total IgE, IgG1, IgG2 and IgG3, whereas no effect on IgG4 was observed. In addition, cow's
19 milk protein-specific IgG1 was significantly decreased (¹⁰⁵). This may be beneficial change in infants
20 at risk of allergies, and although no direct correlations were reported, the same study found a
21 significant reduction in the incidence of atopic dermatitis in a subpopulation of the GOS/FOS group
22 (¹¹³).

23

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

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

7 **2.3 Key points**

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

29

1 **2.4 Recommendations**

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

13

14 **3 Prebiotic effects in paediatrics** ⁴

15

16 **3.1 Oligosaccharides and prebiotic effects in infant formulae**

17

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

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

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

6
7 A number of studies have been published thereafter on the addition of ingredients showing a prebiotic
8 effect to dietetic products for infants and recently reviewed (^{119-121; 121}). These ingredients have been
9 used either as one compound or as a mixture of different neutral and acidic oligosaccharides (¹²²⁻¹²⁴).
10 Collectively, these studies confirm that the administration of oligosaccharides with prebiotic effects in
11 dietetic products have the potential to increase dose-dependently the total number of bifidobacteria in
12 feces, although at present, it is not possible to define the number of bifidobacteria that would
13 constitute normal/optimal microbiota, and to soften stools. Furthermore, prebiotic effects modulate
14 stool pH, SCFAs pattern similar to those of breast fed infants. Whether any of these effects per se is
15 of benefit is currently not well established. Clinical outcomes related to the use of dietetic products for
16 infants supplemented with prebiotic effects are discussed in the sections below (e.g. effect on allergic
17 diseases, infections).

18
19 Currently, the Directive 2006/141/EC on infant formulae and follow-on formulae specifically allows the
20 addition of GOS-FOS in a ratio of 9/1 and in a quantity of 0.8g/ 100 ml prepared product (¹²⁵). This
21 Directive also states that other combinations and maximum levels of FOS and GOS may be used if
22 they satisfy the nutritional requirements of infants in good health as established by generally accepted
23 scientific data.

24

25 **3.2 Use of prebiotic effects in complementary foods for children**

26

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

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

5
6 Another double blind RCT (¹²⁷) involving 35 infants aged 4 to 6 months studied the effect of adding
7 GOS/FOS to solid foods results in an increase in the fecal proportion of bifidobacteria in the intestinal
8 microbiota. Intention-to-treat analysis revealed no significant difference between the 2 study groups.
9 Only per-protocol analysis involving 20 children who complied with the protocol showed that the fecal
10 percentage of bifidobacteria increased from 43% to 57% (p=0.03) from week 0 to week 6 but did not
11 significantly change in the control group (36% and 32%, respectively, p=0.4). There were no
12 statistically significant differences in stool frequency and consistency.

13
14 More recently the prebiotic effect of IFT in children aged 7-8 years has also been reported (¹²⁸).

15 16 **3.3 Use of prebiotic effects for pediatric disorders**

17 **3.3.1 Diarrheal diseases**

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

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

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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,

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

3

4 **3.3.3 Antibiotic-associated diarrhea**

5 The rationale for the use of ingredients showing a prebiotic effect for the prevention of antibiotic-
6 associated diarrhea (AAD) is based on the assumption that the use of antibiotics leads to intestinal
7 dysbiosis and that this is a key factor in the pathogenesis of AAD (¹³²). In contrast to probiotics, (<sup>133-
8 137</sup>) there is a paucity of data on the prebiotic effects in preventing AAD. One pediatric double-blind
9 RCT (¹³⁸) involved 140 children (1 to 2 years of age) who were treated with amoxicillin for acute
10 bronchitis. This study revealed no significant difference in the incidence of diarrhea in children
11 receiving ITF administered in a milk formula (4.5g/L) for 21 days after completion of antibiotic
12 treatment compared with placebo (10% vs. 6%, RR 0.6, 95% CI 0.2-1.8). However, ingredients
13 showing a prebiotic effect in a milk formula increased fecal bifidobacteria early after amoxicillin
14 treatment.

15

16 **3.3.4 Respiratory tract infections**

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

23

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

28

1 3.4 Prebiotic effects and atopy

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

7

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

15

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

22

23 One of the included RCT (¹⁴⁰) investigated the effect of the prebiotic mixture (GOS/FOS; dosage:
24 0.8g/dl) on the intestinal flora and the cumulative incidence of atopic dermatitis during the first 6
25 months of life in infants at risk for allergy (with at least one parent with documented allergic disease
26 confirmed by physician). Two hundred six of 259 (79.5%) infants who were randomly assigned to
27 receive extensively hydrolyzed whey formula supplemented either with 0.8g GOS/FOS (experimental
28 group, n=102) or maltodextrin as placebo (control group, n=104) were included in the per-protocol
29 analysis. The frequency of atopic eczema in the experimental group was significantly reduced
30 compared with the placebo group (9.8% vs. 23.1%, RR 0.42 (95% CI 0.2-0.8)), number needed to

1 treat (NNT) 8 (95% CI 5-31). In a subgroup of 98 infants, the parents provided fresh stool samples for
2 microbiological analysis using plating techniques; the fecal counts of bifidobacteria were significantly
3 higher in the group fed the GOS/FOS formula compared to the placebo group. No significant
4 difference was found for the lactobacilli count between groups. Follow-up of this study, showed that
5 at 2 years the cumulative incidences of atopic dermatitis, recurrent wheezing, and allergic urticaria
6 were higher in the placebo group (27.9, 20.6, and 10.3%, respectively) than in the intervention group
7 (13.6, 7.6, and 1.5%) ($P<0.05$). This is the first observation that prebiotic effects are able to reduce
8 the incidence of atopic diseases, and that this effect persists beyond the intervention period. This
9 assessment is based on a Per Protocol (PP) evaluation which aims at testing efficacy; due to the high
10 drop-out rate (20% at 6 months and 48% at 2 years of age) and lacking ITT analysis, effectiveness for
11 field practice needs to be confirmed (¹⁴¹). (See section *Prebiotic effects and mineral absorption* of this
12 paper)

13

14 4.5 Conclusions

15

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

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

2 4 Prebiotic effects and Gastro-intestinal disorders⁵

3

4 4.1 Prebiotic effects and Gastro-intestinal infections

5

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

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

17

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

24

25 4.2 Prebiotic effects and IBS

26

27 The Irritable Bowel Syndrome (IBS) is a functional bowel disorder manifested by chronic, recurring
28 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).

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

8
9 A precise aetiology for IBS is not recognized. However, epidemiological studies have identified a
10 series of pathogenetic factors, including genetic and early environmental conditioning, cognitive
11 /emotional adaptation, altered response to stress and inflammatory post-infectious processes of the
12 gut mucosa, etc. (¹⁴⁵). It has been shown that IBS patients have abnormal reflexes and perception in
13 response to gut stimuli (¹⁴⁷). In subsets of patients the underlying defects appear to be altered GI
14 motility, visceral hypersensitivity, small bowel bacterial overgrowth, excess gas production,
15 abnormalities in the composition of the gut microbiota (Table 10) or combinations of them (¹⁴⁸).

16
17 Among the modifications of the gut microbiota, a decrease of Bifidobacteria and more specifically
18 *Bifidobacterium catenulatum*, has been observed in IBS patients in comparison to healthy subjects
19 (^{149-151; 151; 152; 153; 154; 155}).

20
21 Hypothetically, some of these disturbances may be corrected or counteracted by prebiotic effects.
22 Indeed compounds showing such effects are known to modulate the digestive microbiota and
23 particularly to stimulate the growth of Bifidobacteria especially when the initial level is low (¹⁵⁶).
24 Furthermore human studies with ITF or lactulose have shown that such prebiotics modulate gut transit
25 (^{148; 157}), decrease putrefactive activity within the gut lumen (¹⁵⁸), prevent GI infections (^{142; 144}), and
26 mitigate inflammatory responses (^{111; 159; 160}).

27
28 Indirect evidence for beneficial effects of ingredients showing a prebiotic effect on abdominal well-
29 being was initially obtained in human trials addressing other primary endpoints. For instance,
30 Cummings *et al* (¹⁴²) tested the effectiveness of ITF in preventing diarrhoea in 244 healthy subjects,

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

9
10 Few studies have investigated the effect of ingredients showing a prebiotic effect in patients with IBS.
11 The study by Olesen *et al* (¹⁶²) tested a large dose of finally 20g ITF during 12 weeks. The authors
12 hypothesized that IBS symptoms may be provoked by large quantities of fermentable carbohydrates
13 in the colon. After 4-6 weeks on treatment, IBS symptoms worsened, as expected, in patients on 20g
14 ITF per day and improved in patients on placebo. However, continuous treatment for 12 weeks
15 resulted in adaptation and there were no differences between groups: symptoms improved in 58% of
16 the ITF group and in 65% of the placebo group, and symptoms worsened in 8% of the ITF group and
17 in 13% of the placebo group. Large doses of any fermentable carbohydrates should not be
18 recommended to IBS patients.

19
20 Hunter and co-workers (¹⁶³) found no effect of 2g ITF (three times daily) against placebo in a reduced
21 group of IBS patients studied in a double blind crossover trial. The Rome team of experts on
22 functional bowel disorders do not recommend the use of a crossover design for IBS treatment trials as
23 they have the potential disadvantages of carryover effects and unmasking the study product by
24 differences in taste and palatability (¹⁶⁴). Dughera *et al* (¹⁶⁵) reported a positive effect of a synbiotic
25 (including short chain ITF at 2.5g per day) on clinical manifestations and intestinal function in patients
26 with IBS. However, this was an open-label and uncontrolled study and IBS studies with subjective
27 outcomes are prone to study bias (¹⁴⁸).

28
29 To date, there are two published studies of adequate study design reporting the effects of an
30 ingredient showing a prebiotic effect in IBS. The first study screened 2235 subjects and recruited and

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

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

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

13 **4.2.1 Recommendations:**

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

17

18 **4.2.2 Key Points:**

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

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

6

7 4.3 Prebiotic effects and IBD

8 4.3.1 Introduction

9 Inflammatory bowel disease (IBD) is a chronic relapsing and remitting disorder characterised by
10 inflammation, ulceration and stricturing of the GI tract. Ulcerative colitis (UC) and Crohn's disease
11 (CD) are the two main types of IBD. In Europe, the incidence ranges from 1.5 to 20.3 cases per
12 100,000 person-years for UC and from 0.7 to 9.8 cases per 100,000 person-years for CD, meaning
13 that up to 2.2 million people in Europe currently live with IBD ⁽¹⁶⁸⁾.

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

20 The primary treatment approach in IBD is usually drug therapy. Patients can be treated with a variety
21 of drugs, including 5-ASAs (e.g. mesalazine), steroids (e.g. prednisolone) and immunosuppressants
22 (e.g. azathioprine). In addition, patients with CD may also receive new biological drugs such as
23 monoclonal antibodies (e.g. the anti-TNF- α antibody infliximab) when standard drug treatment fails
24 ⁽¹⁷²⁾. Despite their general efficacy, such drugs can carry a significant burden. They are not only
25 expensive, but side effects are common, with an incidence of 28% for immunosuppressants, rising to
26 50% for steroids ⁽¹⁷³⁾. In addition, approximately 30% of patients with UC and 50% of patients with CD

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

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

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

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

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

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

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

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

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

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

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

17

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

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

6 **4.3.2 Prebiotic effects in pouchitis**

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

20 **4.3.3 Prebiotic effects in ulcerative colitis**

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

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

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

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

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

22 **4.3.4 Prebiotic effects in Crohn's disease**

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

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

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

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

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

21 **4.3.5 Limitations of existing studies on prebiotic effects in IBD**

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

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

9

10 **4.3.6 Key points**

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

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

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

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

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

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

3

4 **4.3.7 Recommendations**

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

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

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

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

15 **4.4 Prebiotic effects and colon cancer**

16 **4.4.1 Colon carcinogenesis- the role of diet and gut microbiota**

17

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

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

5

6 **4.4.2 Prebiotic effects and CCR (colorectal cancer)**

7

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

- 13 1- Effects on bacterial enzyme activities.
- 14 2- Antigenotoxic effects in vivo.
- 15 3- Effects on pre-cancerous lesions in laboratory animals.
- 16 4- Effects on tumour incidence in laboratory animals
- 17 5- Epidemiological and experimental studies in humans

18

19 **4.5 Prebiotic protective effects and bacterial activities**

20 **4.5.1 Prebiotic effects and secondary bacterial enzyme activities.**

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

26 Ingredients showing a prebiotic effect should not stimulate bacteria capable for such metabolism.

27 During *in vivo* experiments this should result in an overall decrease in toxic substances.

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

9 Studies in laboratory animals have in general shown that ITF and galactans decrease caecal enzyme
10 activities (^{221; 221; 222}). However, human studies have yielded inconsistent or negative results on such
11 enzyme activities or on production of toxic bacterial metabolites such as ammonia and phenols (^{65; 223;}
12 ²²⁴).

13

14 **4.5.2 Prebiotic and synbiotic effects on pre-cancerous lesions in laboratory animals**

15

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

20 Ingredients showing a prebiotic effect alone appear to give inconsistent results on carcinogen induced

21 ACFs which may be partly a consequence of differences in carcinogen and treatment regimes used.

22 For example Rao *et al* (²²⁶) reported that ITF (10% in diet) had no significant effect on total ACF in

23 colon, or their multiplicity, in F344 rats, although curiously a significant decrease in ACF/cm² of colon

24 was reported. A study by Gallaher *et al* (²²⁷) on Bifidobacterium spp and FOS (2% in diet) gave

25 inconsistent results with only 1 out of 3 experiments showing a decrease in DMH-induced ACF. In

26 contrast Verghese *et al* (²²⁸), reported a dose-dependent decrease the incidence of ACF and total

27 crypts (P<0.01) after ITF supplementation (0, 2.5, 5 and 10 g /100 g diets) in AOM challenged rats.

28 The effects of prebiotics on ACF may be dependent on the chain length of the ITF, since a number of

29 studies report more potent inhibition by longer than by shorter chains (²²⁹⁻²³¹). For example,

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

3

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

11

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

22

23 Both Challa *et al* (²³⁴) and Rowland *et al* (²²⁰) studied the effect of combined treatment of probiotic and
24 prebiotic on ACF numbers. The combination of *Bifidobacterium longum* and lactulose resulted in a
25 48% inhibition of colonic ACF, which was significantly greater than that achieved by either
26 *Bifidobacterium longum* or lactulose alone (²³⁴). Similarly Rowland *et al* reported a decrease in total
27 ACF of 74% in rats given *Bifidobacterium longum* + ITF (by comparison to 29% and 21% reduction
28 achieved by *Bifidobacterium longum* or ITF alone). Importantly, the combined administration of
29 probiotic and prebiotic reduced large ACF by 59% whereas the individual treatments had no effect
30 (²²⁰). Nakanishi *et al* (²³⁷) showed that supplementation with *Clostridium butyricum* (CB) in AOM

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

4

5 **4.5.3 Prebiotic effects and colon tumour incidence in laboratory animals**

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

22

23 Femia *et al* (²³⁹) investigated the protective effects of prebiotic (ITF), probiotic (*Bifidobacterium lactis*
24 Bb12 and *Lactobacillus rhamnosus* GG, (5×10^8 CFU/g diet) or synbiotic combination of the two,
25 against AOM-induced colon tumours in rats. Prebiotic fed groups (prebiotic and synbiotic groups)
26 resulted in lower adenoma ($P < 0.001$) and adenocarcinoma ($P < 0.05$) incidence than in the rats not
27 given prebiotic (probiotic & control). Interestingly, in the groups treated with probiotics (probiotic and
28 synbiotic groups) the proportion of cancers relative to the total number of tumours was significantly
29 lower ($P = 0.04$) (9 cancers out of 84 tumours [11%]) than in the control and prebiotic groups (19

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

3

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

22

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

30

1 4.5.4 Prebiotic effects in human intervention studies

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

6

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

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

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

28

1 4.5.5 Mechanisms of anticarcinogenicity and antigenotoxicity

2 4.5.5.1 Prebiotic effects and in vivo prevention of genotoxicity

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

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

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

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

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

29

1 **4.5.5.2 Effects on bacterial enzymes, metabolite production**

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

10

11 **4.5.5.3 Production of anti cancer metabolites**

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

20

21 **4.5.5.4 Stimulation of protective enzymes**

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

29

1 4.5.5.5 Apoptotic effects

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

8 Hughes & Rowland ⁽²⁵¹⁾ fed 3 groups of rats one of three diets: basal, basal with oligofructose
9 (5%w/w) or basal with long chain inulin (5%w/w), for three weeks. All animals were then dosed with
10 1,2-dimethylhydrazine and killed 24 h later. The mean number of apoptotic cells per crypt was
11 significantly higher in the colon of rats fed oligofructose (P=0.049) and long chain inulin (P=0.017) as
12 compared with those fed the basal diet *alone*. This suggests that such ingredients exert protective
13 effects at an early stage in the onset of cancer, as the supplements were effective soon after the
14 carcinogen insult. Comparison of the apoptotic indices between the two oligosaccharide diets showed
15 no significant difference even though the mean apoptotic index was higher in animals fed long chain
16 inulin.

17

18 4.5.5.6 Effects on tight junctions

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

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

26

27 4.6 Summary and conclusion

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

- 1 • Data from animal models, with endpoints such as DNA damage, aberrant crypt foci and
2 tumours in the colon, suggest that reduction in the risk of colon cancer development is
3 associated with prebiotic effects.
- 4 • Limited animal studies also indicate that combinations of pre- and probiotics may be more
5 effective than either agent alone
- 6 • A pre+probiotics study in human subjects using putative biomarkers of cancer risk showed
7 improvements in some, including a reduction in DNA damage and cell proliferation in colon
8 biopsies. Further studies are needed
- 9 • A number of potential mechanisms for reduction in cancer risk by prebiotic effect, including
10 changes in gut bacterial enzyme activities , upregulation of apoptosis and induction of
11 protective enzymes have been explored in animal models, but currently evidence for such
12 effects in humans is lacking
- 13

1 **5 Prebiotic effects and mineral absorption**⁶

2

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

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

15

16 **5.1 Rationale behind the prebiotic effects on mineral absorption**

17 Calcium

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

26 Further, these ingredients may also modulate transcellular active calcium transport by increasing
27 calbindin D9K expression in the cecum and colorectum (the intracellular carrier protein involved in the
28 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

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

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

10

11 Other minerals

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

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

22

23 5.2 Summary of key studies (Table 12)

24 5.2.1 Animal study (Table 13 & 14)

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

- 27 • -Different types of molecules have been studied, including ITF_{-Dpav 3-4}, ITF_{-Dpav 12}, ITF_{-Dpav 25},
28 ITF_{-MIX}, GOS, lactulose or resistant starch.

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

4

5 **5.2.2 Clinical trials (Table 15& 16)**

6 In infants

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

11

12 In adolescents

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

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

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

3

4 In adults

5 It has been previously shown, using the metabolic balance methodology, that addition of up to 40g
6 per day of ITF and sugar beet fibres, to a normal mixed diet for 28 days improved calcium balance,
7 without adverse effects on the retention of other mineral (²⁷⁶). However, a study carried out by Van
8 den Heuvel *et al.* (²⁷⁷) in healthy young adults, found no significant differences in mineral absorption,
9 irrespective of the treatment (which consisted of a constant basal diet supplemented for 21 days with
10 15g/d ITF, or galacto-oligosaccharide, or not supplemented) followed by a 24 hour urine collection. It
11 was hypothesised that a 24 h period of urine collection, used in the study, was too short to include the
12 colonic component of calcium absorption and thus to make up a complete balance necessary to
13 detect the effect of ITF. In a similar way, Teuri *et al.* (²⁷⁸), investigated a combination of 15g of ITF and
14 210mg of calcium added to 100g of cheese given at breakfast to 15 adult healthy women with an
15 average age of 23 years old. The study failed to show any significant influence of the diet on blood
16 ionized calcium or PTH concentration over the 8h assessment period. Nevertheless, measuring
17 serum PTH and ionised calcium do not provide direct information about calcium absorption, as do
18 isotope techniques, and it has been suggested that the length of the trial was probably too short.
19 Moreover, the addition of 1.1 g ITF_{Dpav 3-4} or caseinophosphopeptides to calcium-enriched milks, a
20 valuable source of well-absorbed calcium, did not significantly increase calcium absorption in adults
21 (25-36y), independently of sex (²⁷⁹). Finally, Abrams *et al.* (²⁸⁰) gave to 13 young adults (average age
22 of 23y) a supplementation containing 8g of ITF_{MIX} for 8 weeks. Eight of the 13 volunteers were
23 classified as responders, based on their level of calcium absorption.

24

25 In postmenopausal women

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

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

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

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

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

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

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

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

8

9 In institutionalized patients

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

12

13 5.3 Outline of general rules

14

15 5.3.1 Involvement of the colon

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

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

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

1 Similarly, in patients with conventional ileostomy, data analysis of ITF effects on mineral absorption
2 and excretion (Mg, Zn, Ca, Fe) showed no significant influence ⁽²⁹²⁾.
3 This offers an explanation as to why Van den Heuvel *et al.* ⁽²⁷⁷⁾ found no significant differences in
4 mineral absorption in healthy young adults, irrespective of the treatment they received (consisting of a
5 constant basal diet supplemented for 21 days with 15g/d ITF, or galacto-oligosaccharide, or not
6 supplemented), as the 24 h period of urine collection used in this study was too short to include the
7 colonic component of calcium absorption and thus to make up a complete balance necessary to
8 detect the effect of fructans.
9 Indeed, Abrams *et al.* ⁽²⁸⁰⁾ gave young adults (average age of 23y) 8 g of ITF_{MIX} for 8 weeks, and
10 confirmed that calcium absorption after treatment occurred principally in the colon (69.6 ± 18.6%).
11
12 Nevertheless, it is still unclear whether the calcium sparing effect results from induction of specific
13 bacterial strains or from their “colonic food” activity ⁽²⁹³⁾.
14

15 5.3.2 Dose effect

16 Various doses of ITF have been investigated ranging from 1.1 g/d to 17 g/d (and even 40g/d in one
17 case). A minimum level of 8 g/d seems to be required to elicit an improvement on both calcium
18 absorption and bone mineralisation. Indeed, Lopez-Huertas *et al.* ⁽²⁷⁹⁾ explained the lack of effect of
19 the addition of 1.1g ITF or caseinophosphopeptides to calcium-enriched milks in adults by the very
20 low dose provided in the diet.
21 However, with regards to animal studies, ITF appears to exhibit a dose-dependent effect on calcium
22 absorption, as well. Levrat *et al.* ⁽²⁹⁰⁾ showed that dietary ITF given in the range of 0 to 20% in the diet
23 stimulated intestinal calcium absorption in a dose dependent manner. Similarly, in the study carried
24 out by Brommage *et al.* ⁽²⁹⁴⁾, a near linear increase in calcium absorption was demonstrated in rats
25 fed a 5 and 10% lactulose containing diet. Nevertheless, it appears that when a minimum is reached,
26 calcium absorption enhancement occurs whatever the dose, as a diet supplemented with either 10%
27 of ITF ⁽²⁶⁷⁾ or 5% of oligofructose or other non-digestible carbohydrates ⁽²⁹⁴⁾ leads to a similar
28 increase (about 60-65%) of the apparent absorption of calcium, even though, raising the content of
29 oligofructose in the diet from 2.5 to 10% in ovariectomized rats, a bone sparing effect has been
30 shown, independent of the dose by Scholz-Ahrens *et al.* ⁽²⁹⁵⁾.

1

2 5.3.3 Test substances

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

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

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

26 5.3.4 Influence of physiological status

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

29

1 5.3.4.1 **Initial status in calcium.**

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

9

10 5.3.4.2 **Estrogen permeation.**

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

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

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

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

9

10 **5.4 From mineral absorption to health benefits**

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

13 **5.4.1 Minerals**

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

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

23

24 **5.4.2 Calcium and bone health**

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

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

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

15

16 5.5 Key points

17

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

- 1 • A combination of molecules with different degrees of polymerization appears to be more
2 efficient as shown with the research on ITF_{-MIX} in comparison with the small and high MW
3 fractions given alone.
4

5 **5.6 Recommendations (future targets for research)**

6

- 7 • Further studies are required to investigate the underlying mechanisms of the prebiotic effects
8 on absorption of minerals, with special attention to the role of the specific changes in gut
9 microbiota. Indeed the question still remains open of whether these effects are due to the
10 changes in colonic microbiota composition (prebiotic effect) or any other mechanisms. In this
11 regard, high throughput methodologies such as metabolomics, for example, are warranted.
- 12 • Results from ITF, in particular ITF_{-MIX} need to be confirmed in other ingredients showing a
13 prebiotic effect for a generalisation.
- 14 • Further long term well designed clinical trials need to be implemented to prove that the benefits
15 of these ingredients persist in the longer term (because bone strength is the ultimate hallmark of
16 bone quality, the issue of persistence of the effect of ITF_{-DPav 3-4} on the skeleton is important to
17 consider) to assess their potential in the prevention of the risk of fracture
- 18 • With regards to the bone target, it is interesting to focus on relevant populations, i.e. during
19 childhood and during ageing
- 20 • It is still challenging to investigate the potential synergy between the prebiotic effect and other
21 nutrients (such as phytoestrogens for example) endowed with bone sparing effect.
22

23 **6 Prebiotic effects in weight management and obesity-related disorders⁷**

24

25 Several reviews report the interest of non digestible carbohydrates – which are prone to be fermented
26 by the gut microbiota in the control of obesity and related metabolic disorders. Carbohydrates showing
27 a prebiotic effect have received special attention in this context, since they have been shown - mostly in
28 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

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

5

6 **6.1 Description of the prebiotic effects on obesity and related metabolic disorders**

7

8 **6.1.1 Prebiotic effects and regulation of food intake, fat mass and body weight**

9 **6.1.1.1 Animal studies**

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

19 **6.1.1.2 Potential mechanism**

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

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

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

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

15 6.1.1.3 Human Data

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

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

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

19

20 **6.1.2 Prebiotic effects and glucose homeostasis**

21 6.1.2.1 Animals.

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

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

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

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

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

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

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

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

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

24

25 6.1.2.2 Human studies

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

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

8

9 **6.1.3 Prebiotic effects and lipid homeostasis, including steatosis and hepatic alterations.**

10 6.1.3.1 Animal Studies

11 Ingredients showing a prebiotic effect are able to modulate hepatic lipid metabolism in rats or
12 hamsters, resulting in changes in either triglyceride accumulation in the liver (steatosis), and/or serum
13 lipids (³⁴⁶). In non-obese rats and/or hamsters fed a high carbohydrate diet, a decrease in hepatic and
14 serum triglycerides was observed, when ITF were added to the diet at concentrations ranging from
15 2.5 to 10% for several weeks (from 2 to 12 weeks) (³⁴⁷). In animals, reduced triglyceridaemia or
16 steatosis is often linked to a decrease in de novo lipogenesis in the liver (³⁴⁸). In rats fed a lipid-rich
17 diet containing fructans, a decrease in triglyceridaemia also occurs without any protective effect on
18 hepatic triglyceride accumulation and lipogenesis, suggesting a possible peripheral mode of action
19 (³³³). By contrast, in obese Zucker rats, dietary supplementation with ITF lessens hepatic steatosis,
20 with no effect on post-prandial triglyceridaemia when added to the standard diet (³⁴⁹). This effect is
21 likely to be mainly the of a lower availability of non-esterified fatty acids coming from adipose tissue,
22 since fat mass and body weight are decreased by the treatment. In obese dogs, a 6 weeks treatment
23 with short chain fructans was able to increase uncoupling protein 2 and carnitine palmitoyltransferase
24 1 expression in the adipose tissue, thereby suggesting a higher substrate oxidation in adipocyte, that
25 occurred without any significant change in triglyceridemia (³⁴²).

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

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

10

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

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

23

24 6.1.3.2 Human data

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

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

5

6 **6.1.4 Prebiotic effects and obesity-associated inflammation.**

7

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

24

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

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

3

4 **6.2 Relation between prebiotic effects and improvement of obesity and associated disorders**

5

6 *Relative specificity of prebiotics effects versus other “dietary fibres” on physiological targets regulating*
7 *appetite and metabolic disorders*

8

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

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

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

27

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

30

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

15

16 **6.3 Methodological aspects**

17

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

23 In animal studies, the authors often add ingredients showing a prebiotic effect at a relatively high dose
24 (1 to 10% wt/wt in the diet) and to compare the data obtained in animals receiving the basal diet
25 alone. The interpretation of results would then require the difference in energy/nutrients intake and/or
26 an experimental group with the same intake of energy upon the treatment (pair-fed animals) to be

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

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

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

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

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

18

19 **6.4 Conclusions and future trends**

20

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

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

<p><i>“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”</i></p> <p><i>Gibson, G. R., Roberfroid, M. B. Dietary modulation of the human colonic microbiota: introducing the concept of prebiotics, J. Nutr. 125, 1401-1412, 1995</i></p>
<p><i>‘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.’</i></p> <p><i>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</i></p>
<p><i>‘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.’</i></p> <p><i>ISAPP (2008) 6th Meeting of the International Scientific Association of Probiotics and Prebiotics. London, Ontario.</i></p>

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.

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

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})
<p style="text-align: center;"><u>INULIN-TYPE FRUCTANS</u> ITF Linear $\beta(2\rightarrow1)$ fructosyl-fructose. $G_{py}F_n$ and/or $F_{py}F_n$</p> <p style="text-align: center;"><u>Oligomers (DP 2-8)</u> ITF-_{DPav 3-4}</p> <p style="text-align: center;"><u>Short and medium size polymers</u></p> <p style="text-align: center;">(DP 2-60) ITF-_{DPav 12}</p> <p style="text-align: center;">(DP 10-60) ITF-_{DPav 25}</p> <p style="text-align: center;"><u>Mixtures</u></p> <p style="text-align: center;">(DP 2-8) + (DP 10-60) ITF-_{MIX}</p>	<p>Fructo-oligosaccharides, FOS</p> <p>Short-chain fructo-oligosaccharides, scFOS (enzymatic synthesis from sucrose) (DP_{av} 3.6)</p> <p>Oligofructose (enzymatic partial hydrolysis of inulin) (DP_{av} 4)</p> <p>Inulin (especially chicory inulin) (DP_{av} 12)</p> <p>High molecular weight inulin (physical purification) (DP_{av} 25)</p> <p>Mixture of oligomers and medium size polymers</p>
<p style="text-align: center;"><u>GALACTANS</u></p> <p style="text-align: center;">Mixture of $\beta(1\rightarrow6)$; $\beta(1\rightarrow3)$; $\beta(1\rightarrow4)$ galactosyl-galactose GOS</p> <p style="text-align: center;">(DP 2-8)</p>	<p>Galacto-oligosaccharides, Trans-galactooligosaccharides, (enzymatic transgalactosylation of lactose)</p> <p>(DP_{av} 3)</p>
<p style="text-align: center;"><u>Mixture of galactans and inulin-type fructans</u></p> <p style="text-align: center;">GOS-FOS</p>	<p>Galacto-oligosaccharides and high molecular weight inulin, Usually known as GOS-FOS or scGOS-lcFOS</p>

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	<i>Bacteroidetes: Bacteroides vulgatus</i> , uncultured <i>Bacteroides</i> sp. adhufec51 and <i>Parabacteroides</i> spp. <i>Firmicutes: Clostridium</i> cluster XIVa (uncultured bacteria mpn group 24 and 66.25) and <i>Streptococcus salivarius</i>	Wang <i>et al.</i> , 2003 ⁽¹²⁾
54-year-old healthy female	Jejunum	88	22	<i>Actinobacteria: Micrococcus mucilaginosus</i> (1 %) <i>Bacteroidetes: Prevotella</i> sp. oral clone and <i>P. melaninogenica</i> (3 %) <i>Firmicutes: Streptococcus mitis</i> , <i>S. salivarius</i> , <i>S. oralis</i> , <i>S. parasanguis</i> and <i>S. anginosus</i> (68 %); <i>Clostridium</i> clusters XI (<i>Mogibacterium neglectum</i> and <i>Peptostreptococcus anaerobius</i>) and IX (<i>Veillonella atypica</i> and <i>V. parvula</i>) (3 and 7 %, respectively) <i>Fusobacteria: Fusobacterium</i> sp. BS011 (3 %) <i>Proteobacteria: Haemophilus parainfluenzae</i> , <i>Pseudomonas putida</i> , <i>Acinetobacter johnsonii</i> , <i>A. lwoffii</i> and <i>A. haemolyticus</i> and <i>Neisseria subflava</i> (13 %) Others (2 %)	Wang <i>et al.</i> , 2005 ⁽¹³⁾
	Distal ileum	85	33	<i>Bacteroidetes: Bacteroides vulgatus</i> , <i>Bacteroides</i> spp., <i>B. thetaiotaomicron</i> , <i>B. ovatus</i> , <i>B. uniformis</i> and <i>Alistipes putredinis</i> (49 %) <i>Firmicutes: Streptococcus mitis</i> and <i>S. oralis</i> (2 %); <i>Clostridium</i> clusters XIVb (<i>Clostridium lactatifermentans</i>), IX (<i>Dialister invisus</i>), IV (<i>Faecalibacterium prausnitzii</i> , <i>Oscillospira guilliermondii</i> and <i>Clostridium orbiscindens</i>) and XIVa (<i>Clostridium</i> spp., <i>Clostridium symbiosum</i> , <i>Coprococcus catus</i> , <i>Dorea formicigenerans</i> , <i>Ruminococcus gnavus</i> , <i>R. obeum</i> , <i>Ruminococcus</i> spp. and <i>Roseburia intestinalis</i>) (5, 5, 7 and 20 %, respectively) <i>Fusobacteria: Fusobacterium varium</i> (1 %) <i>Proteobacteria: Sutterella wadsworthensis</i> (1 %) <i>Verrucomicrobia: Verrucomicrobium</i> spp. (5 %) Others (5 %)	
74-year-old male at autopsy	Jejunum	92	9	<i>Firmicutes: Veillonella parvula</i> (4 %), <i>Lactobacillus reuteri</i> (1 %), <i>L. lactis</i> (11 %), <i>L. mali</i> (73 %), <i>Streptococcus salivarius</i> (4 %) and <i>S. pneumoniae</i> (1 %) <i>Proteobacteria: Actinobacillus actinomycetemcomitans</i> (5 %)	Hayashi <i>et al.</i> , 2005 ⁽¹⁵⁾
	Ileum	89	17	<i>Firmicutes: Veillonella parvula</i> (15 %), <i>Clostridium lituseburense</i> (1 %), <i>Abiotrophia</i> sp. (1 %), <i>Lactobacillus reuteri</i> (1 %), <i>L. mali</i> (20 %), <i>L. lactis</i> (14 %), <i>Streptococcus salivarius</i> (9 %), <i>S. constellatus</i> (1 %) and <i>S. pneumoniae</i> (9 %) <i>Fusobacteria: Leptotrichia buccalis</i> (1 %) and <i>Fusobacteria</i> spp. (1 %) <i>Proteobacteria: Neisseria gonorrhoeae</i> (1 %) and <i>Actinobacillus actinomycetemcomitans</i> (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	<i>Bacteroidetes: Bacteroides fragilis</i> (1 %) <i>Fusobacteria: Phascolarctobacterium faecium</i> (1 %), <i>Eubacterium ventriosum</i> (1 %), <i>E. cylindroides</i> (1 %), <i>Clostridium purinolyticum</i> (3 %), <i>C. leptum</i> (1 %) and <i>Enterococcus</i> group (5 %) <i>Proteobacteria: Escherichia coli</i> (4 %) and <i>Klebsiella</i> subgroup (67 %) Others (2 %)	Hayashi <i>et al.</i> , 2005 ⁽¹⁵⁾
	Ileum	94	4	<i>Firmicutes: Enterococcus</i> group (13 %) <i>Proteobacteria: Klebsiella</i> subgroup (85 %)	
87-year-old female at autopsy	Jejunum	91	3	<i>Firmicutes: Enterococcus</i> group (7 %) <i>Proteobacteria: Actinobacillus actinomycetemcomitans</i> (1 %) and <i>Klebsiella planticola</i> (92 %)	Hayashi <i>et al.</i> , 2005 ⁽¹⁵⁾
	Ileum	89	15	<i>Firmicutes: Rumincococcus gnavus</i> (2 %), <i>Peptostreptococcus anaerobius</i> (6 %), <i>P. micros</i> (2 %), <i>Enterococcus</i> group (33 %), <i>Streptococcus salivarius</i> (8 %) and <i>Clostridium leptum</i> (3 %) <i>Proteobacteria: Actinobacillus actinomycetemcomitans</i> (1 %), <i>Escherichia</i> subgroup (16 %), <i>Klebsiella</i> subgroup (2 %), <i>Klebsiella planticola</i> (21 %) and <i>Xenorhabdus</i> 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 intestineTaken 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	Chemolithotrophic	CH ₄
Desulfovibrio	–	8.4	Various	Ac
Propionibacteria	+	9.4	Saccharolytic, 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		<i>Bacteroidetes: Bacteroides vulgatus, Bacteroides spp.</i> <i>Firmicutes: Clostridium</i> cluster XIVa (uncultured bacteria mpn group 24 and 66.25, <i>Ruminococcus gnavus</i>)	Wang <i>et al.</i> , 2003 ⁽¹²⁾
	Descending colon	27		<i>Bacteroidetes: Bacteroides vulgatus</i> , uncultured <i>Bacteroides</i> sp. adhufec51 and <i>Parabacteroides</i> spp. <i>Firmicutes: Clostridium</i> cluster XIVa (uncultured bacteria mpn group 24 and 66.25)	
68-year-old female with mild sigmoid diverticulosis	Descending colon	190		<i>Bacteroidetes</i> (17.3 %): <i>Bacteroides vulgatus</i> , uncultured <i>Bacteroides</i> sp. HUCC30 and <i>Parabacteroides</i> spp. <i>Firmicutes</i> (1 %): <i>Streptococcus pneumoniae</i> <i>Proteobacteria</i> (39.6 %): <i>Shigella flexneri</i> , <i>S. sonnei</i> , <i>Stenotrophomonas maltophilia</i> , <i>Leptothrix cholodnii</i> , <i>Herbaspirillum lemoignei</i> , <i>Methylobacterium</i> sp., <i>Sphingomonas</i> sp. and <i>Haemophilus influenzae</i> <i>Firmicutes: Bacillus–Lactobacillus–Streptococcus</i> (1.3 %); <i>Clostridium</i> cluster I (<i>Clostridium perfringens</i>), IV (<i>Faecalibacterium prausnitzii</i> , <i>Ruminococcus</i> spp., <i>Anaerofilum</i> spp. and uncultured bacterium CB25), IX (<i>Veillonella atypica</i>) and XIVa (uncultured bacteria mpn group 24 and AF54, <i>Lachnospira pectinoschiza</i> and <i>Clostridium xylanolyticum</i>) (1.3, 17.9, 1.8, and 15.3 %, respectively)	Wang <i>et al.</i> , 2003‡ ⁽¹²⁾
	Ascending colon	86	37	<i>Bacteroidetes: Bacteroides vulgatus, Bacteroides spp., B. thetaiotaomicron, B. ovatus, B. uniformis</i> and <i>Alistipes putredinis</i> (27 %) <i>Firmicutes: Clostridium</i> clusters XIVb (<i>Clostridium lactatifermentans</i>), IX (<i>Dialister invisus</i> and <i>Propionispira arboris</i>), IV (<i>Faecalibacterium prausnitzii</i> , <i>Clostridium sporosphaeroides</i> , <i>C. orbiscindens</i> and <i>Oscillospira guilliermondii</i>) and XIVa (<i>Eubacterium halii</i> , <i>E. elegans</i> , <i>E. ramulus</i> , <i>Dorea formicigenerans</i> , <i>Ruminococcus lactaris</i> , <i>R. gnavus</i> , <i>Ruminococcus</i> sp., <i>Clostridium symbiosum</i> , <i>Clostridium</i> spp., <i>C. xylanolyticum</i> and <i>Roseburia intestinalis</i>) (6, 9, 13 and 33 %, respectively) <i>Fusobacteria: Fusobacterium varium</i> (1 %) <i>Proteobacteria: Escherichia coli, Acinetobacter johnsonii</i> and <i>Sutterella wadsworthensis</i> (4 %) <i>Verrucomicrobia: Verrucomicrobium</i> spp. (5 %) Others (1 %)	
54-year-old, healthy female	Rectum	88	32	<i>Bacteroidetes: Bacteroides vulgatus, Bacteroides spp., B. thetaiotaomicron, B. uniformis</i> and <i>Alistipes putredinis</i> (44 %) <i>Firmicutes: Clostridium</i> clusters XI, XIVb, IX, IV and XIVa (<i>Clostridium</i> spp., <i>Eubacterium halii</i> , <i>Dorea formicigenerans</i> , <i>Ruminococcus lactaris</i> , <i>R. torques</i> , <i>Ruminococcus</i> spp. and <i>Roseburia intestinalis</i>) (1, 1, 5, 8 and 29 %, respectively) <i>Fusobacteria: Fusobacterium varium</i> (1 %) <i>Proteobacteria: Escherichia coli</i> (2 %) <i>Verrucomicrobia: Verrucomicrobium</i> spp. (9 %)	Wang <i>et al.</i> , 2005† ⁽¹³⁾

Subject	Biopsy	No. of clones examined	No. of OTUs identified	Phylum: species identified*	Reference
74-year-old male at autopsy	Caecum	90	41	<i>Bacteroidetes</i> : <i>Bacteroides fragilis</i> (3 %) and <i>Prevotella nigrescens</i> (1 %) <i>Firmicutes</i> : <i>Veillonella parvula</i> (2 %), <i>Clostridium xylanolyticum</i> (2 %), <i>C. polysaccharolyticum</i> (2 %), <i>C. leptum</i> (23 %), <i>C. lituseburense</i> (1 %), <i>C. glycolicum</i> (1 %), <i>Ruminococcus hansenii</i> (8 %), <i>R. gnavus</i> (4 %), <i>Butyrivibrio fibrisolvens</i> (22 %), <i>Eubacterium ventriosum</i> (1 %), <i>Lachnospira multipara</i> (4 %), <i>Lactobacillus reuteri</i> (1 %), <i>Streptococcus salivarius</i> (1 %), <i>S. pneumoniae</i> (3 %) and unclassified (14 %) <i>Proteobacteria</i> : <i>Actinobacillus actinomycetemcomitans</i> (3 %)	Hayashi <i>et al.</i> , 2005 ⁽¹⁵⁾
	Recto-sigmoid colon	90	38	<i>Bacteroidetes</i> : <i>Bacteroides fragilis</i> (4 %) and unclassified (1 %) <i>Firmicutes</i> : <i>Veillonella parvula</i> (1 %), <i>Phascolarctobacterium faecium</i> (3 %), <i>Ruminococcus hansenii</i> (9 %), <i>R. gnavus</i> (6 %), <i>Butyrivibrio fibrisolvens</i> (4 %), <i>Eubacterium ventriosum</i> (4 %), <i>Clostridium polysaccharolyticum</i> (2 %), <i>C. leptum</i> (30 %), unclassified (6 %) <i>Proteobacteria</i> : <i>Desulfovibrio desulfuricans</i> (2 %) and <i>Escherichia</i> subgroup (13 %) Other (2 %)	
85-year-old female at autopsy	Caecum	91	11	<i>Bacteroidetes</i> : <i>Bacteroides fragilis</i> (3 %) <i>Firmicutes</i> : <i>Ruminococcus gnavus</i> (2 %), <i>Clostridium lituseburense</i> (2 %), <i>Enterococcus</i> group (35 %) <i>Proteobacteria</i> : <i>Klebsiella</i> subgroup (36 %) <i>Actinobacteria</i> : <i>Bifidobacterium infantis</i> (2 %)	Hayashi <i>et al.</i> , 2005 ⁽¹⁵⁾
	Recto-sigmoid colon	90	27	<i>Firmicutes</i> : <i>Clostridium xylanolyticum</i> (1 %), <i>C. purinolyticum</i> (1 %), <i>C. ramosum</i> (1 %), <i>C. leptum</i> (11 %), <i>Eubacterium cylindroides</i> (1 %), <i>Ruminococcus hansenii</i> (2 %), <i>R. gnavus</i> (1 %), <i>Lactobacillus reuteri</i> (1 %), <i>Enterococcus</i> group (19 %), unclassified (7 %) <i>Proteobacteria</i> <i>Desulfovibrio desulfuricans</i> (1 %), <i>Escherichia</i> subgroup (7 %), <i>Klebsiella</i> subgroup (22 %) <i>Actinobacteria</i> : <i>Bifidobacterium infantis</i> (2 %) Others (19 %)	
87-year-old female at autopsy	Caecum	92	22	<i>Bacteroidetes</i> : <i>Bacteroides fragilis</i> (2 %) <i>Firmicutes</i> : <i>Veillonella parvula</i> (1 %), <i>Clostridium leptum</i> (4 %), <i>Ruminococcus hansenii</i> (1 %), <i>R. gnavus</i> (3 %), unclassified (12 %), <i>Lactobacillus delbrueckii</i> (1 %), <i>L. mali</i> (8 %), <i>Enterococcus</i> group (1 %), <i>Streptococcus salivarius</i> (41 %), <i>S. pneumoniae</i> (16 %) <i>Proteobacteria</i> : <i>Escherichia</i> subgroup (7 %), <i>Klebsiella planticola</i> (1 %)	Hayashi <i>et al.</i> , 2005 ⁽¹⁵⁾
	Recto-sigmoid colon	92	26	<i>Bacteroidetes</i> : <i>Bacteroides fragilis</i> (2 %) <i>Firmicutes</i> : <i>Clostridium xylanolyticum</i> (2 %), <i>C. leptum</i> (1 %), <i>Ruminococcus hansenii</i> (2 %), <i>R. gnavus</i> (5 %), <i>Lactobacillus delbrueckii</i> (7 %), <i>L. reuteri</i> (27 %), <i>L. mali</i> (14 %), <i>Streptococcus salivarius</i> (11 %), <i>S. pneumoniae</i> (1 %) and unclassified (11 %) <i>Proteobacteria</i> : <i>Escherichia</i> subgroup (1 %) <i>Actinobacteria</i> : <i>Actinomyces-Bifidobacterium catenulatum</i> subgroup (9 %), <i>B. bifidum</i> (3 %), <i>B. infantis</i> (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 <i>et al.</i> (1998) ⁽⁹⁾
Lactic acid bacteria	2 males, 2 females	Development and validation of group-specific primers for human studies	Detection of <i>Lactobacillus</i> at $>1 \times 10^5$ cfu (g wet weight faeces) ⁻¹ ; interindividual variation; intraindividual variation over 6 months	Walter <i>et al.</i> (2000) ⁽³⁹⁴⁾
	2 adults on probiotic trial	Monitor changes in LAB population during <i>Lactobacillus</i> 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 <i>et al.</i> (2001) ⁽³⁹⁵⁾
Lactobacilli, leuconostocs and pediococci	12 adults 1 baby boy	<i>Lactobacillus</i> 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 <i>Lactobacillus</i> were below the detection limit, but complexity of fingerprint increased after introduction of solid foods to the diet	Heilig <i>et al.</i> (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 <i>et al.</i> (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 <i>et al.</i> (2005) ⁽³⁴⁾

Target population	Subject	Investigation	Overall results	Reference
<i>Clostridium leptum</i> 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 <i>Clostridium leptum</i> subgroup in human faeces	microbiota 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 <i>et al.</i> (2006) ⁽³⁹⁷⁾
All bacteria <i>Bacteroides fragilis</i> subgroup <i>Clostridium coccooides</i> / <i>Eubacterium rectale</i> group (cluster XIVa) <i>Clostridium lituseburense</i> 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 <i>Bifidobacterium adolescentis</i>)	Vanhoutte <i>et al.</i> (2006) ⁽³⁹⁸⁾

**1 Table 8: Human studies (healthy persons) designed to determine the prebiotic effect of short-chain fructooligosaccharides (scFOS),
2 fructooligosaccharides (FOS), galactooligosaccharides (GOS) and inulin.
3**

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 ⁽⁴⁰⁵⁾
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	Kleessen 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⁾

4

1 **Table 9: The prebiotic effect on immune markers**

2

Subject	Trial design	Groups	N	Duration	Key findings of the prebiotic intervention on immune parameters and effect on microbiota	Reference
Healthy elderly (> 70y)	RPC parallel	(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, IFN γ , 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 6.0x10 ⁹ 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+ (⁴¹⁶) - decrease of % lymphocyte expressing ICAM-1 - decrease of % CD3+ NK+ cells	

		(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, TNF α and IL-1 β production by PBMC	(¹¹⁰)
					- positive correlation between numbers of <i>Bifidobacterium</i> spp., <i>Lactobacillus-Enterococcus</i> spp., and the <i>C. coccoides-E. rectale</i> group with % and total number of phagocytosing cells. - negative correlation between numbers of <i>Bacteroides</i> spp. and <i>E. coli</i> d with % and total number of phagocytosing cells.	
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 levels 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)

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

2

3

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 <i>Clostridium perfringens</i> 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 <i>Lactobacillus spp</i> in diarrhoea predominant IBS Higher number of <i>Veillonella spp</i> in constipation predominant IBS	(7)
IBS subjects (Rome II criteria) Control subjects	Lower number of <i>Bifidobacterium catenulatum</i> and <i>Clostridium coccooides</i> Lower number of <i>Lactobacillus spp</i> , Bifidobacteria and lactate-utilizing bacteria Higher number of Sulphate-reducing bacteria	(8)
IBS subjects (Rome II criteria) (16) Control subjects (16)	Lower proportion of <i>Clostridium coccooides</i> and <i>Eubacterium rectale</i> in constipation predominant IBS	(11)
IBS subjects (Rome II criteria) (24) Control subjects (23)	Lower number of <i>Collinsella</i> ; Lower prevalence of <i>Collinsella aerofaciens</i> ; Lower number of <i>Coprococcus eutactus</i> Lower number of <i>Bifidobacterium catenulatum</i>	(9)
IBS subjects (Rome II criteria) (41) Control subjects (26)	Lower number of Bifidobacteria Lower number of <i>Bifidobacterium catenulatum</i>	(10)

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

2

Subjects	Trial design ¹	Groups	N ²	Duration	Key findings	Reference
Pouchitis (active)	Open label	(a) FOS (1 tablet/d) <i>L. rhamnosus GG</i> (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 <i>B. fragilis</i> Had no effect on bifidobacteria Increased faecal butyrate	Welters et al (2002) ⁽²⁰⁵⁾
UC (active)	DB-RCT	(a) Oligofructose / inulin (12 g/d) <i>B. longum</i> (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-1 α 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, each, <i>P. pentoseceus</i> , <i>L. raffinolactis</i> , <i>L. paracasei</i> , <i>L. plantarum</i>) (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 ¹ DB-RCT, double-blind randomised controlled trial
2 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 <i>et al.</i> , 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 <i>et al.</i> , 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)	Ca	Ca Bioavailability, Bone health, Phytoestrogens bioavailability	(Coxam, 2007)(437)
- Rat	Phytoestrogens 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 <i>et al.</i> , 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	Prebiotics	Ca	Ca absorption	(Griffin & Abrams, 2008) ⁽⁴⁴²⁾
-Dog				
- Human	Prebiotics	Ca	Ca absorption, Bone mineralization	(Hawthorne & Abrams, 2008) ⁽⁴⁴³⁾
- Rat				
- Human	Prebiotics (oligofructose, inulin)	Ca, Mg, Fe, Zn	Mineral metabolism, Bone remodelling, Mechanisms of action	(Kelly, 2009) ⁽⁴⁴⁴⁾
- Human	Prebiotics	Ca	Ca absorption, Osteoporosis	(De Vrese, 2009) ⁽⁴⁴⁵⁾
	Probiotics			

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 <i>et al.</i> , 1995) ⁽⁴⁴⁶⁾
FOS (Meioliigo-P, Japan)	5 60 d	↑ femoral Ca content ↑ bone volume	Growing Wistar rats (16 males) AAS	(Takahara <i>et al.</i> , 2000) ⁽⁴⁴⁷⁾
Oligofructose (Orafti) or Inulin (Orafti)	10 13 weeks	Both ↑ femoral Ca content	Growing Fisher rats (30 males, 4 week-old) ICPMS	(Richardson <i>et al.</i> , 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 <i>et al.</i> , 2002) ⁽⁴²⁵⁾
Ca + FOS (Raftilose P95, Orfati)	0.5 + 2.5 or 0.5 + 5.0 or 0.5 + 10 or 1.0 + 50 or 16 weeks	Ns L1-L4 Ca content ↑ trabecular tibial thickness Ns L1-L4 Ca content ↑ trabecular tibial perimeter ↑ L1-L4 Ca content ↑ trabecular tibial perimeter ↑ L1-L4 Ca content ↑ trabecular number	OVX Fisher 344 rats (96 females, 6 week-old) AAS Histomorphometric method	(Scholz-Ahrens <i>et al.</i> , 2002) ⁽²⁹⁵⁾
-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 <i>et al.</i> , 2003) ⁽²⁹⁶⁾
-Inulin (DP>23)	5	↑ femoral BMD ↑ spine BMC		
-HP Inulin (DP 10-65) + ITF _{MIX} (OF)	4 weeks 5+5	↓ bone resorption Ns tibial Ca content	Growing Wistar rats (10 males, 6 week-old) AAS	(Coudray <i>et al.</i> , 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 <i>et al.</i> , 2004a) ⁽⁴⁴⁹⁾
-Inulin	5	Ns femoral Ca content	Growing Sprague-Dawley rats (48 males, 6 week-old) Ca ⁴⁵ kinetics method AAS	(Zafar <i>et al.</i> , 2004b) ⁽³⁰⁴⁾
- Inulin + IF	5 + 0.8 21 d	↑ femoral bone Ca content vs inulin		
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	(Mathey <i>et al.</i> , 2004) ⁽³⁰²⁾
	20 + 7.5	↑ Femoral BMD vs IF ↑ Femoral failure load ↓ urinary DPD	3-point bending test RIA	
	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	Intact or OVX Sprague-Dawley rats (50 females, 6 week-old) DEXA, 3-point bending test ELISA	(Mitamura & Hara, 2005) ⁽⁴⁵⁰⁾
		In OVX rats ↑ femoral Ca content ↑ distal femoral BMD with 3% DFAIII ↑ Maximum breaking force ↓ urinary DPD in DFAIII groups (trend)		
-Difructose anhydride III (DFAIII) (Nippon Beet sugar Mfg)	1.5 8 weeks	In intact rats Ns femoral Ca content	Intact or OVX Sprague-Dawley rats (64 females, 6 week-old, vitamin D deficient or not) AAS	(Mitamura & Hara, 2006) ⁽⁴⁵¹⁾
- DFAIII + vitamin D-deficient		In OVX rats ↑ femoral Ca content		
-Oligofructose (chicory roots, Cosucra)	5	↑ Femur BMD ↑ cancellous tibia area	Growing Wistar rats (38 males, 6 week-old) DEXA (pQCT) ELISA	(Nzeusseu <i>et al.</i> , 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 <i>et al.</i> , 2006) ⁽⁴⁵³⁾

FOS or IF+FOS	4 months	<p>↑Whole body BMD vs control OVX</p> <p>↑tibial BMC vs control OVX</p> <p>↑lumbar BMD and BMC vs control OVX (no additive effects with IF+FOS)</p> <p>↑tibial microarchitectural properties in IF+FOS (↑trabecular number vs OVX control)</p>	OVX Sprague-Dawley rat (69 females, 9 month -old)	(Devareddy <i>et al.</i> , 2006) ⁽³⁰³⁾
Lc Inulin (Beneo HP, Orafti)	5 8 weeks	<p>Ns BMD</p> <p>↑ femoral BMC</p> <p>Ns bone markers (OC , CTX1)</p>	Growing Sprague-Dawley rats (48 females, 3 week-old)	(Jamieson <i>et al.</i> , 2008) ⁽⁴⁵⁴⁾
-Inulin long – chain (Cosucra) or Inulin short – chain (Cosucra)	7.5	<p>Trend to ↑ diaphysal femoral BMD and BMC</p> <p>Ns bone markers (OC ,DPD)</p>	Growing Wistar rats (40 males, 3 month-old)	(Demigne <i>et al.</i> , 2008) ⁽⁴⁵⁵⁾
-Chicory (Cosucra)	7.5	<p>↑diaphysal femoral BMD and BMC</p> <p>↑ Femoral failure load</p> <p>Ns bone markers (OC , DPD)</p>		
-SO (soybean oil) + ITF _{MIX}	3 months 15 + 10.87	Ns femoral Ca content	Growing Wistar rats (24 males rats, 6 week-old)	(Lobo <i>et al.</i> , 2009) ⁽⁴⁵⁶⁾
- SO + Fish oil + ITF _{MIX}	15 +11.5 + 10.87	<p>↑ femoral Ca content</p> <p>↑ tibial Ca content</p> <p>↑ tibial bone strength</p>	3-point bending test	
IF or FOS or IF + FOS (Meiologo-P, Meiji)	15 d 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)	(Ohta <i>et al.</i> , 2002) ⁽³⁰¹⁾
Inulin (Orafti)	6 weeks 10 2 weeks	↑ Mg bone content	C57B16J mice (24 males, 4 month-old)	(Rondon <i>et al.</i> , 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 <i>et al.</i> , 1993) ⁽²⁹⁴⁾
FOS (Meiologo-P, Meiji)	5 28d	↑ apparent Ca and Mg absorption in intact rats ↑ apparent Mg absorption in cececomized rats	Intact or cececomized rats AAS	(Ohta <i>et al.</i> , 1994a) ⁽²⁹¹⁾
FOS (Meiologo-P, Meiji) (low Mg, High Ca and High P)	1 5	↑ apparent Mg absorption	Mg- deficient rats AAS	(Ohta <i>et al.</i> , 1994b) ⁽³⁰⁶⁾
FOS (Meiologo-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 <i>et al.</i> , 1995a) ⁽³⁰⁷⁾
FOS (Meiologo-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 <i>et al.</i> , 1995b) ⁽²⁵⁶⁾
GOS	20 d	↑ apparent Ca absorption	OVX wistar rats AAS	(Chonan <i>et al.</i> , 1995) ⁽⁴⁴⁶⁾
TOS (Meiologo-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 <i>et al.</i> , 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 <i>et al.</i> , 1997) ⁽⁴⁵⁹⁾ y
FOS (Meiologo-P, Meiji)	10 10d	↑ apparent Ca absorption	Growing gastrectomized Sprague-dawley rats (17 males, 4 week-old) AAS	(Ohta <i>et al.</i> , 1998) ⁽²⁵⁹⁾
FOS (Meiologo-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 <i>et al.</i> , 1998) ⁽⁴⁶⁰⁾
-FOS short – chain (Meiologo-P, Meiji) (normal and Ca deficient diet)	10 10d	↑ CaBP levels Independent of 1,25(OH)2D3 action	Rats (intestinal CaBP levels) AAS	(Takasaki <i>et al.</i> , 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 <i>et al.</i> , 2000) ⁽²⁵⁷⁾
FOS + PA (phytic acid)	10+7 21 d	↑ cecal Ca Ns cecal Ca vs PA		
FOS (Meiologo-P, Meiji)	5 60d	↑ apparent Ca absorption ↑ fractional Ca absorption	Growing Wistar rats (16 males, 6 week-old) AAS	(Takahara <i>et al.</i> , 2000) ⁽⁴⁴⁷⁾
-Inulin (Orafti)	10	↑ apparent Ca absorption ↑Ca retention	Adult Wistar rats (32 males, 8 week-old) AAS	(Younes <i>et al.</i> , 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 <i>et al.</i> , 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 0.5 + 5.0 0.5 + 10 1.0 + 50 (16 weeks)	↓ apparent Ca absorption (after 4 wk) Ns apparent Ca absorption ↑ apparent Ca absorption Vs OVX (wk 8) ↑ apparent Ca absorption Vs OVX (wk 4) Vs OVX (wk 8) Vs OVX (wk 16)	OVX Fisher 344 rats (96 females, 6 week-old) AAS	(Scholz-Ahrens <i>et al.</i> , 2002) ⁽²⁹⁵⁾
-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 <i>et al.</i> , 2003) ⁽²⁹⁷⁾
-HP Inulin (DP 10-65) + Oligofructose	5+5	OF+HP : additive effect		
- HP Inulin (DP 10-65) - ITF _{MIX}	10 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 <i>et al.</i> , 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 <i>et al.</i> , 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 <i>et al.</i> , 2004b) ⁽⁴⁵⁰⁾
- Inulin + IF	5 + 0.8 21d			
-FOS short – chain (Meiologo-P, Meiji)	3 4 weeks	↑ apparent Ca, Mg, Fe absorption	Growing Sprague-Dawley rats (48 males) AAS	(Asvarujanon, 2005) ⁽⁴⁶³⁾
-Four non digestible saccharides (DFAIII, Nippon Beet Sugar MFG)	Measurement after 10-14 days			
-FOS short – chain (Meiologo-P, Meiji)	3	↑ apparent Ca, Mg absorption Higher effect with DFAIII DFAIII ↑ Fe absorption		
-Four non digestible saccharides (DFAIII, Nippon Beet Sugar MFG)	4 weeks Measurement after 24-28 days			
-FOS short – chain (Meiologo-P, Meiji)	3	-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	
-Four non digestible saccharides (DFAIII, Nippon Beet Sugar MFG)	5 weeks			
Difuctose 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) After 13 d	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	↑ apparent Ca absorption higher effect when Ca is low (0.25) or high (0.75)	Growing rats, 10 weeks (10 males wistar) AAS	(Coudray <i>et al.</i> , 2005a) ⁽⁴⁶⁴⁾
		After 40 d ↑ apparent Ca absorption higher effect when Ca is low (0.25)		
Inulin (Raftiline, 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)	(Coudray <i>et al.</i> , 2005b) ⁽⁴⁶⁵⁾
			Ca44 method, AAS ICPMS	
-Difuctose anhydride III (DFAIII) (Nippon Beet Sugar MFG)	3	↑ Fe absorption	Growing Sprague-Dawley rats (18 males, 4 week-old)	(Shiga <i>et al.</i> , 2006) ⁽⁴⁶⁶⁾
-FOS (Meiologo-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 (<i>in vivo, in vitro</i>)	(Kobayashi <i>et al.</i> , 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 <i>et al.</i> , 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 <i>et al.</i> , 2006) ⁽⁴⁵²⁾
-Inulin (chicory roots, Cosucra)	5			
-Difructose anhydride III (DFAIII) (Nippon Beet sugar Mfg)	3 months 1.5	In intact rats Ns apparent Ca absorption	Intact or OVX Sprague-Dawley rats (64 females, 6 week-old, vitamin D deficient or not) AAS	(Mitamura & Hara, 2006) ⁽⁴⁵¹⁾
- DFAIII + vitamin D-deficient	8 weeks	↑ apparent Ca absorption in vitamin D-deficient rats		
Inulin (Raftaline, Orafti)	7.5 3 weeks	In OVX rats ↑ apparent Ca absorption (higher effect in vitamin D-deficient rats) -↑ 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 <i>et al.</i> , 2006) ⁽⁴⁶⁷⁾
-Inulin long – chain (Cosucra) or -Inulin short – chain (Cosucra) - Chicory (Cosucra)	7.5	↑ apparent Ca absorption (1 month) Ns 3 month	Growing Wistar rats (40 males, 3 month-old) AAS	(Demigne <i>et al.</i> , 2008) ⁽⁴⁵⁵⁾
Inulin (Orafti)	3 months 10 2 weeks	↑ Mg absorption	C57B16J mice (24 males, 4 month-old) AAS	(Rondon <i>et al.</i> , 2008) ⁽⁴⁵⁷⁾
-GR inulin (Orafti)	0.1 (0.82g/d human equivalent dose)	-Ns on calcemia level	Growing Sprague-Dawley rats (36 females, 6 week-old) Colorimetric assay	(Azorin-Ortuno, 2009) ⁽⁴⁶⁸⁾
-Artichoke inulin - ITF _{MIX} -Artichoke + P95 oligofructose		-↑ calcemia -Ns on calcemia level -Ns on calcemia level		
-SO (soybean oil) + ITF _{MIX}	75 d 15 + 10.87	↑ apparent Ca absorption	Growing Wistar rats (24 males rats, 6 week-old) AAS	(Lobo <i>et al.</i> , 2009) ⁽⁴⁵⁶⁾
- SO + Fish oil + ITF _{MIX}	15 +11.5 + 10.87 15 d	↑ apparent Ca absorption (higher effect)		
Inulin HPX (Orafti)	2.5 5 d	Ns apparent Ca absorption	Wistar rats (24 males, 6 week-old) AAS	(Klobukowski <i>et al.</i> , 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 <i>et al.</i> , 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) % Ca⁴⁵ absorption: % Ca⁴⁵ oral dose / % Ca⁴⁵ IP dose x 100

Fractional Ca absorption: Ca⁴⁷: Sc⁴⁹ 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: (Ca⁴⁵ Ca⁴⁴) = (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)	R study Formula-fed Infants (6-12 month-old) (36)	(Yap <i>et al.</i> , 2005) ⁽²⁶⁸⁾
Oligofructose (Raftilose P95, Orafti)	15 9 days	(↑ apparent and net Mg retention with 0.75 & 1. 25g/d) ↑ true fractional Ca absorption	AAS R, DB, CO study Male adolescents (24)	(Van den Heuvel <i>et al.</i> , 1999a) ⁽²⁶⁹⁾
Oligofructose (Raftilose P95, Orafti) or Sc-FOS + ITF ^{-MIX}	8 3 weeks	Ns with oligofructose ↑ true Ca absorption with Synergy 1	Kinetic technique (Ca ⁴⁴ , Ca ⁴⁸) ICPMS DB, CO study Young Girls (29)	(Griffin <i>et al.</i> , 2002) ⁽²⁷⁰⁾
Sc-FOS + ITF ^{-MIX}	8 3 weeks	↑ true Ca absorption	Kinetic technique (Ca ⁴⁶ , Ca ⁴²) TIMMS R, CO study Young girls (54)	(Griffin <i>et al.</i> , 2003) ⁽²⁷¹⁾
Sc-FOS + ITF ^{-MIX}	8 1 year	↑ fractional Ca absorption	Kinetic technique (Ca ⁴⁶ , Ca ⁴²) TIMMS DB study Male & female adolescents (48)	(Abrams <i>et al.</i> , 2005b) ⁽²⁷³⁾
Sc-FOS + ITF ^{-MIX}	8 1 year	↑ true fractional Ca absorption (32 responders & 16 non-responders)	Kinetic technique (Ca ⁴⁶ , Ca ⁴²) TIMMS DB, PC, Sex stratification study Male and female adolescents (48)	(Abrams <i>et al.</i> , 2007b) ⁽²⁷⁵⁾
Sc-FOS (Actilight, Beghin Meiji)	10 37 days	Ns true fractional Ca absorption (↑ true Mg absorption)	Kinetic technique (Ca ⁴⁶ , Ca ⁴²) TIMMS R, DB, CO study Adolescent girls (14)	(Van den Heuvel <i>et al.</i> , 2009) ⁽²⁷²⁾
Inulin (Chicory roots)	40 28 days	↑ apparent Ca absorption	Low Ca intake (Ca ⁴⁴ , Ca ⁴⁸) ICPMS 3x3 Latin square Young men (9)	(Coudray <i>et al.</i> , 1997) ⁽²⁷⁶⁾
Inulin (Raftiline ST, Orafti) OF (Raftilose P95, Orafti)	17 3 days	Ns mineral (Ca, Mg, Zn, Fe) excretion because of ileostomy	AAS DB, CO study ileostomised patients (5 men and 5 women)	(Ellegard <i>et al.</i> , 1997) ⁽²⁹²⁾
Inulin, FOS, or GOS (Orafti)	15 21 days	Ns true fractional Ca or iron absorption (Methodologic concern : analysis after 24h urines)	AAS DB, CO study Young men (12)	(Van den Heuvel <i>et al.</i> , 1998) ⁽²⁷⁷⁾
Inulin (Raftiline, Orafti) + Ca (210 mg/d)	15 5 days	Ns urinary Ca excretion (lower iPTH lower →later increase in Ca absorption)	Kinetic technique (Ca ⁴⁴ , Ca ⁴⁸) ICPMS R, DB, CO study Young woman (50)	(Teuri <i>et al.</i> , 1999) ⁽²⁷⁸⁾
			AAS IRMA	

Shoyu polysaccharides (SPS)	0.6 4 weeks	↑ in plasma iron in the SPS group	R, DB, PC parallel study Young woman (45) AAS	(Kobayashi <i>et al.</i> , 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 <i>et al.</i> , 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 <i>et al.</i> , 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 <i>et al.</i> , 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 <i>et al.</i> , 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 <i>et al.</i> , 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 <i>et al.</i> , 2003) ⁽²⁸³⁾
Chicory fructan fiber (Cosucra)	8 3 months	↑ apparent Ca absorption ↑ apparent iron absorption	DB parallel design POM (13) AAS	(Kim <i>et al.</i> , 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 <i>et al.</i> , 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 <i>et al.</i> , 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 <i>et al.</i> , 2009) ⁽²⁸⁶⁾

AAS: Atomic Absorption Spectrometry

Fractional Ca: (Ca⁴⁴, Ca⁴³) ratio ; (Ca⁴⁶, Ca⁴²) 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 probiotic 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 <i>et al.</i> , 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 <i>et al.</i> , 2007b) ⁽²⁷⁵⁾
Sc-FOS (Actilight, Beghin Meiji)	10 37 days	Ns bone resorption (DPD) Ns PTH Ns Vitamin D Ns PTH	R, DB, CO study Adolescent (40) HPLC	(Van den Heuvel <i>et al.</i> , 2009) ⁽²⁷²⁾
Inulin (Raftiline, Orafiti) + Ca (210 mg/d)	15 5 days		R, DB, CO study Young woman (50) IRMA	(Teuri <i>et al.</i> , 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 <i>et al.</i> , 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 <i>et al.</i> , 2004) ⁽²⁸⁷⁾
Sc-FOS + ITF _{-MIX}	10 6 weeks	↑ Bone turnover (OC-DPD)	R, DB, PC, CO design POM (50) IRMA –ELISA	(Holloway <i>et al.</i> , 2007) ⁽²⁸⁸⁾
Isoflavones + probiotics 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 <i>et al.</i> , 2008) ⁽⁴⁷¹⁾
Sc-FOS + ITF _{-MIX}	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 <i>et al.</i> , 2009) ⁽²⁸⁶⁾
ITF _{-MIX} + Ca + CPP + fermented milk				
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 <i>et al.</i> , 2005) ⁽²⁸⁹⁾

MD: Bone Mineral Density, BMC: Bone Mineral Content, PP: Caseinophosphopetide, DPD: Deoxy pyridinoline, 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)	(477)
		↓EAT for FOS and inulin	
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	(478)
		↓EAT	
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	(480)
		↓EAT	
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	(482)
		↓EAT	
Male C57Bl/6J mice fed a HF–HC diet	10% FOS – 4 wk	↓BW gain (NS)	(483)
		EAT =	
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	(485)
		↓EAT, IAT, VAT	
Male C57Bl/6J mice fed a HF–carbohydrate free diet	10% FOS – 14 wk	↓BW gain	(486)
		↓EAT, VAT, SAT	
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	(488)
		↓EAT for fructans from Agave tequilana Gto	
Female Sprague–Dawley rats	5% inulin + 5% cellulose versus 10% cellulose – 4 and 8 wk	↓BW gain (NS)	(489)
		↓whole body fat mass	
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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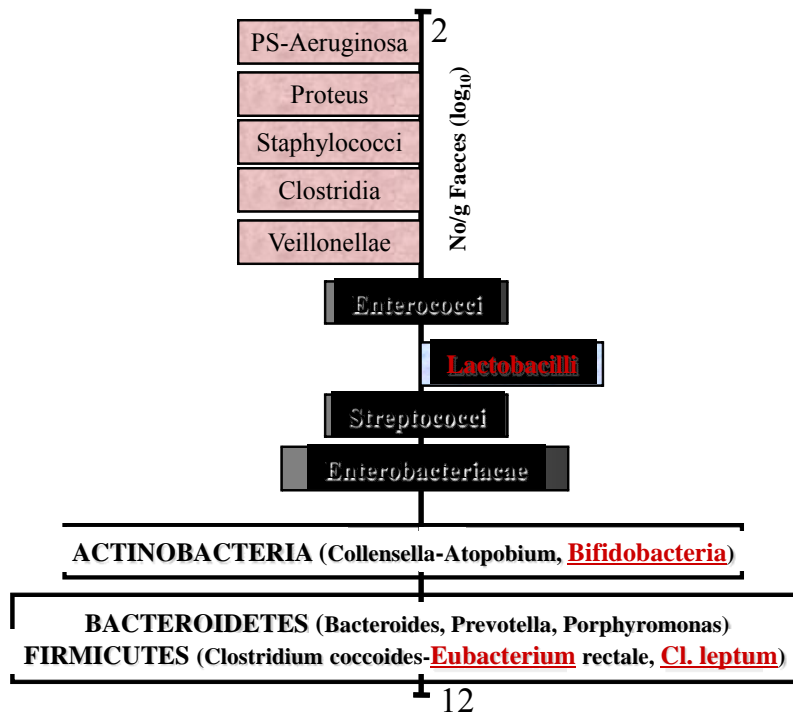
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Figure 1: Schematic representation of gut microbiota

1331 Major phylla and genera are located on a logarithmic scale as N° of CFU/g of faeces. Genera on the left site
 1332 are likely to be potentially harmful whereas those on the right site are potentially beneficial to health. Those
 1333 that sit both on the left site and the right site either contain species that are potentially harmful and species
 1334 that are potentially beneficial to health or contain genera/species that still need to be classified. Indeed many
 1335 of these have only recently been identified in the gut microbiota and their activity(ies) is/are still largely
 1336 unknown.
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