Structure and function of non-digestible carbohydrates in the gut microbiome

Beneficial Microbes, 2022; 13(02): 95-168

Wageningen Academic Publishers

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Received: 30 June 2021 / Accepted: 25 March 2022

REVIEW ARTICLE

Abstract

Together with proteins and fats, carbohydrates are one of the macronutrients in the human diet. Digestible carbohydrates, such as starch, starch-based products, sucrose, lactose, glucose and some sugar alcohols and unusual (and fairly rare) α-linked glucans, directly provide us with energy while other carbohydrates including high molecular weight polysaccharides, mainly from plant cell walls, provide us with dietary fibre. Carbohydrates which are efficiently digested in the small intestine are not available in appreciable quantities to act as substrates for gut bacteria. Some oligo- and polysaccharides, many of which are also dietary fibres, are resistant to digestion in the small intestines and enter the colon where they provide substrates for the complex bacterial ecosystem that resides there. This review will focus on these non-digestible carbohydrates (NDC) and examine their impact on the gut microbiota and their physiological impact. Of particular focus will be the potential of non-digestible carbohydrates to act as prebiotics, but the review will also evaluate direct effects of NDC on human cells and systems.

Keywords: prebiotics, short-chain fatty acids (SCFA), non-digestible carbohydrates

1. Introduction

Carbohydrates in the diet and the gut

Together with proteins and fats, carbohydrates are one of the macronutrients in the human diet. Digestible carbohydrates such as starch, starch-based products, sucrose, lactose, glucose and some sugar alcohols and unusual (and fairly rare) α-linked glucans, directly provide us with energy while other carbohydrates including high molecular weight polysaccharides, mainly from plant cell walls, provide us with dietary fibre. Some oligo- and polysaccharides, many of which are also dietary fibres, are resistant to digestion in the small intestines and enter the colon where they provide substrates for the complex bacterial ecosystem that resides there. This review will focus on these non-digestible carbohydrates (NDC) and examine their impact on the gut microbiota and their physiological impact. Of particular focus will be the potential of non-digestible carbohydrates to act as prebiotics, but the review will also evaluate direct effects of NDC on human cells and systems. The aim of the review is to attempt to uncover the relationships between structure and functional properties in nondigestible, soluble and insoluble fibres. Inevitably the available literature does

not provide even coverage of all dietary fibre components and some fibres have been investigated in greater depth than others. In this review the authors have focussed on literature that illuminates structure-function relationships rather than attempting to provide a comprehensive review of all of the literature in the area. This means that the discussion tends to be driven by structural complexity of the fibre rather than its importance as a prebiotic.

The colon of an adult human contains roughly 500 g of content, of which most is bacteria (Hill, 1995); the daily faecal output is about 100 g. Food takes about 16 h to transit the large intestine. Water and other remaining absorbable nutrients are removed from the food before the indigestible matter arrives in the rectum. In Europe the average dietary fibre (DF) intake is 20.5 g/d, 15-25 g/d for males and 14-21 g/d for females (Stephen *et al.*, 2017). The major components of DFs are non-starch polysaccharides (NSP) and these relate to 20-45% of the dry matter supplied to the colon. Monosaccharides and oligosaccharides represent each an additional 10%, while starch represents less than 8% of the dry matter. Some sugar alcohols are minor dietary substrates for the colonic microbiota (Langkilde *et al.*, 1994), as well as some unusually linked α-glucans (e.g. isomaltooligosaccharides, resistant maltodextrins, reuteran). Both lipids and protein from dietary origins can pass through the intestinal system into the proximal colon if consumption levels are high, and proteins may be fermented while lipids are generally not. In addition, catechins, lignin, polyphenols, tannins and micronutrients may also be available for the colonic microbes. About 2,000 species of bacteria, as well as fungi, protozoa and archaea, have been detected in the large intestine and the diversity of species varies by geography and diet (Almeida *et al.*, 2019). Therefore, the competition for substrates occurring between the colonic microbes has a significant influence on the metabolites generated. Bacterial growth and metabolic activity are greatest in the proximal colon (Topping and Clifton, 2001). The pH increases as stool moves from the proximal to distal colon (from about 5.5 to 7.0). The total amount of short chain fatty acids (SCFAs) is highest in the proximal colon and their production usually declines toward the distal colon. Of the total SCFA produced in the colon, ≥90% is absorbed by the epithelium. The major SCFAs, acetate, propionate and butyrate account for ≥95% of the total SCFAs (molar ratios approximating 60:20:20 (Cook and Sellin, 1998; Hijova and Chmelarova, 2007)). The total concentration of SCFAs increases, depending on the diet from 70 to 140 mM in the proximal colon, to 20 to 70 mM in the distal colon (Topping and Clifton, 2001). *In vivo* studies have been hindered by a lack of methodology to measure SCFA production directly, since observations in humans largely rely on the measurement of SCFA in stool, excluding the detection of absorbed/systemically relevant SCFAs (Morrison and Preston, 2016). SCFAs can be measured in plasma, but this also is not a good indicator of colonic production as much of the absorbed butyrate is metabolised in the colonic epithelium and the propionate is targeted to the liver where it is metabolised. Nevertheless, there is evidence that diet-driven changes in microbiota lead to variations in the stool SCFA profile. The amount and relative abundance of SCFA may be considered as biomarkers of a healthy status (Ríos-Covián *et al.*, 2016).

Evolution of the concept of prebiotics

The concept of prebiotics and also of synbiotics was first proposed by Gibson and Roberfroid (1995) who defined prebiotics as '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' and a synbiotic as 'a mixture of probiotics and prebiotics that beneficially affects the host by improving the survival and implantation of live microbial dietary supplements in the gastrointestinal tract, by selectively stimulating the growth and/or by activating the metabolism of one or a limited number of health-promoting bacteria, and thus improving host welfare'.

The earliest recognition of a dietary factor that influences the gut microbiome, however, is probably the 'Bifidus factor' identified by Györgi in a series of publications in 1954 (Gauhe *et al.*, 1954; Gyorgy *et al.*, 1954a,b,c; Rose *et al.*, 1954). The Bifidus factor was present in human milk but not bovine milk and was essential for growth of '*Lactobacillus bifidus*', now reclassified as *Bifidobacterium*. This factor was dialysable, stable to autoclaving, did not reside in the fat, protein or salt fraction and hydrolysed to acetic acid, D-glucosamine, L-fucose, D-glucose, and D-galactose. It is now clear that this is a human milk oligosaccharide, probably lacto-N-biose I (Kitaoka *et al.*, 2005).

The role of the gut microbiota in health, and the position that diet plays in regulating this relationship, was first described in detail by the pioneering Japanese microbiologist Tomotari Mitsuoka. He noted in 1969 that diet influences the bacteria in the gut, which, in turn, influences health (Figure 1, adapted from Mitsuoka, 2013) citing a Japanese article from 1969. The ideas put forward by Mitsuoka are still largely valid today.

The initial definition of a prebiotic referred to 'one or a limited number of bacteria in the colon' and reading this today, the reader needs to bear in mind the state of knowledge of the human gut in 1995. Figure 2 is adapted from Gibson and Roberfroid (1995) and reveals the understanding that prevailed at that time.

As we have acquired increased knowledge of the microorganisms that inhabit the gut and of the functioning of their complex ecosystem, our understanding of what is

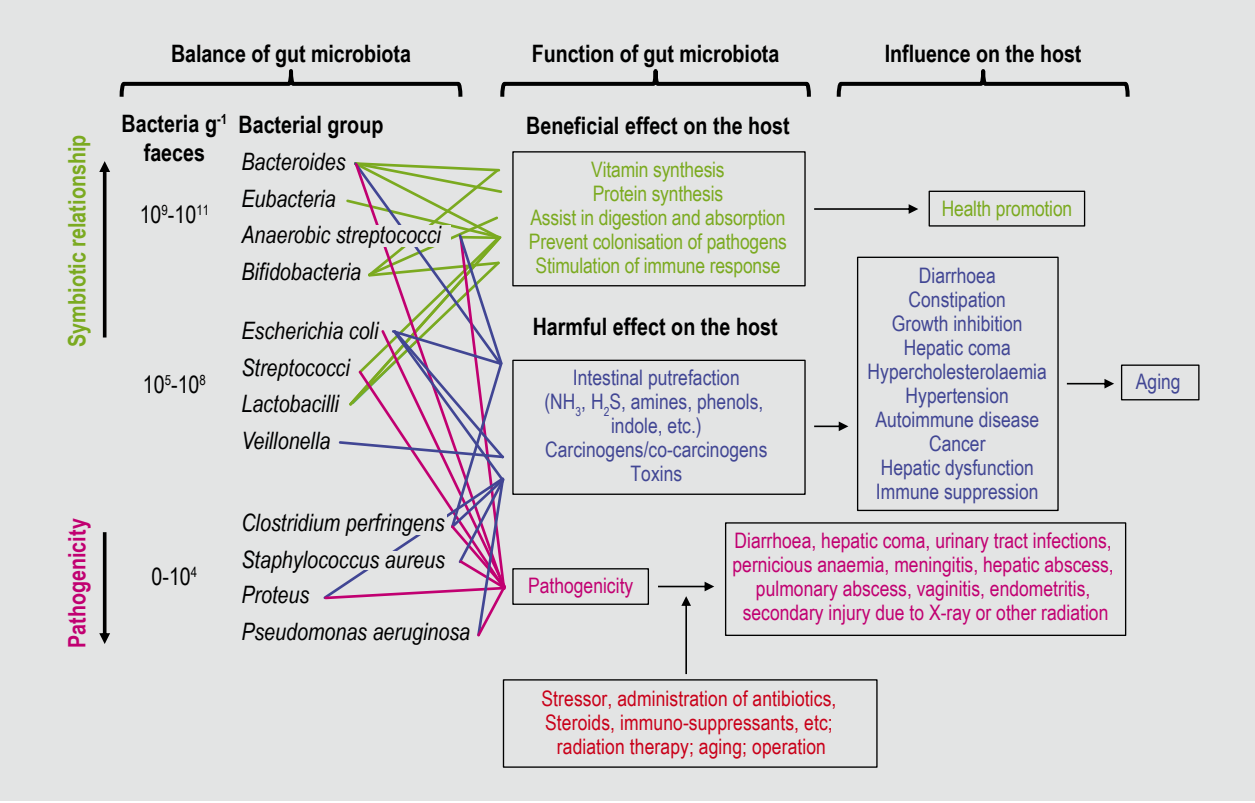

Figure 1. An early functional view of the gut microbiome and its potential impact on health. Modified from Mitsuoka (2013).

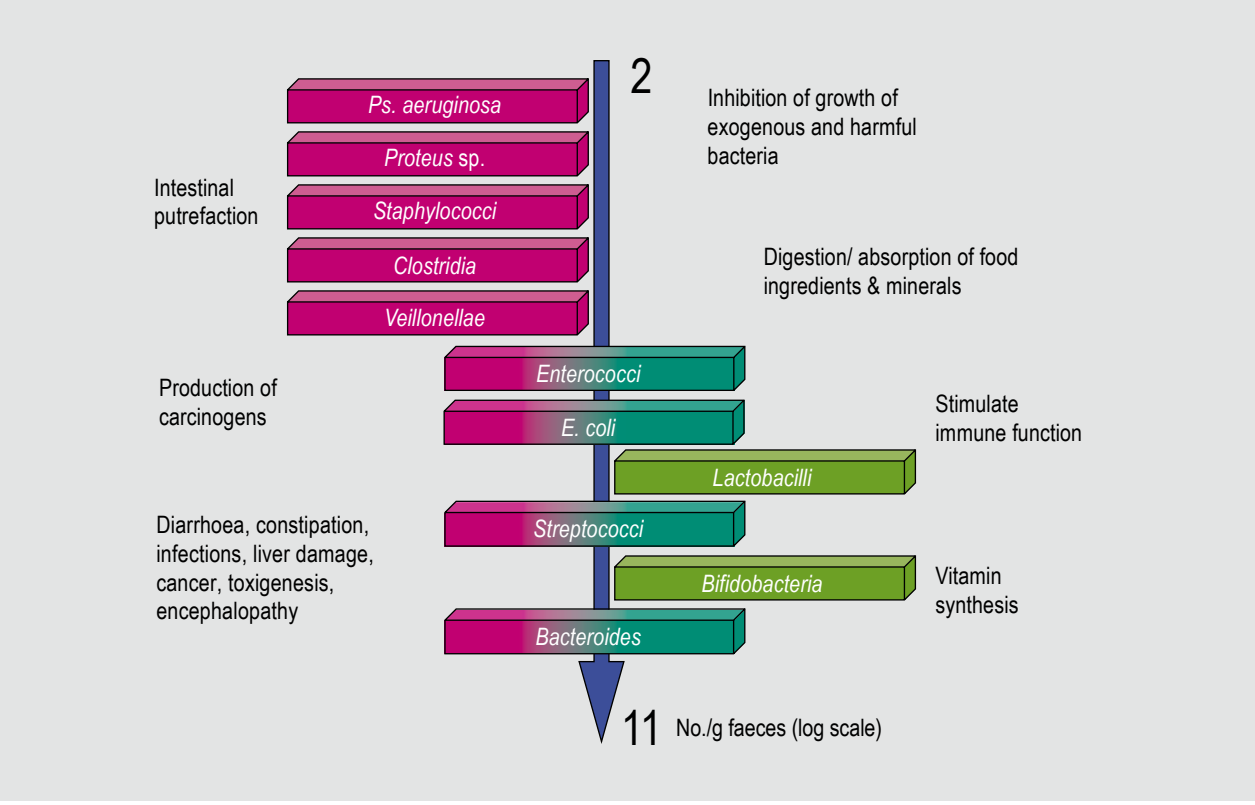

Figure 2. An early concept of prebiotics. Adapted from Gibson and Roberfroid (1995).

meant by a prebiotic has necessarily evolved. The latest definition that was derived by a scientific consensus of experts in the field under the auspices of the International Scientific Association for Probiotics and Prebiotics (ISAPP) is rather more nuanced 'a substrate that is selectively utilised by host microorganisms conferring a health benefit' (Gibson *et al.*, 2017). The commentary published to accompany this definition goes on to clarify what could or could not be considered as a prebiotic (Figure 3).

Although all of the currently recognised prebiotics are carbohydrates (hence the focus of this monograph), it must be made clear that none of the definitions over the years (Gibson *et al.*, 2017) have ever specified the chemical composition of a prebiotic and there is clear potential for other nutrients that enter the large intestine in appreciable quantities to also be considered as prebiotics, notably dietary polyphenols (Del Rio *et al.*, 2013; Dueñas *et al.*, 2015; Gibson *et al.*, 2017; Plamada and Vodnar, 2021; Rodríguez-Daza *et al.*, 2021).

Structure-function relationships of carbohydrates fermented by the gut microbiota

A very large diversity of molecular structures of carbohydrates exists in our diets, that provide food to an equally diverse gut microbial community. There is good evidence that different bacteria and bacterial groups are favoured by different oligo- and polysaccharide structures, and therein lies the potential to create positive shifts in the human gut microbiota through an understanding of their requirements and ability to compete for different substrate structures.

Uncovering the relationship between the structure of non-digestible dietary oligo- and polysaccharides (NDC) and function in the gut microbiota is key to using diet effectively to improve gut health and the numerous chronic diseases associated with dysbiosis. While research linking NDC structure to function in the gut is perhaps still in its infancy, certain relationships are becoming evident and show promise towards identifying fibre structures for predictable microbiota shifts to improve health.

Implicit in the idea of shaping the gut microbiota with NDCs is an understanding of:

1. The carbohydrate requirements of different bacterial groups and, in some cases, even of individual species.
2. How bacteria compete and cooperate in the metabolism of carbohydrates and which one's thrive.
3. How fibres respond in different gut bacterial communities and whether strategies can be developed to gain predictive responses in individuals differing in their gut microbiota.
4. The different roles of chemical and physical fibre structures in support of different bacterial groups.
5. How fibres can be 'matched' with beneficial 'probiotic' bacteria to support their growth.

Additionally, *in vitro* systems to analyse carbohydrate fermentation in the gut, which are necessary to use to understand such structure-function relationships, must be relevant and predictive to the human gut microbiome.

Dietary fibres come in a large variety of structures, and gut bacteria likewise have a range of abilities to utilise them, as well as specificities towards variations in structure.

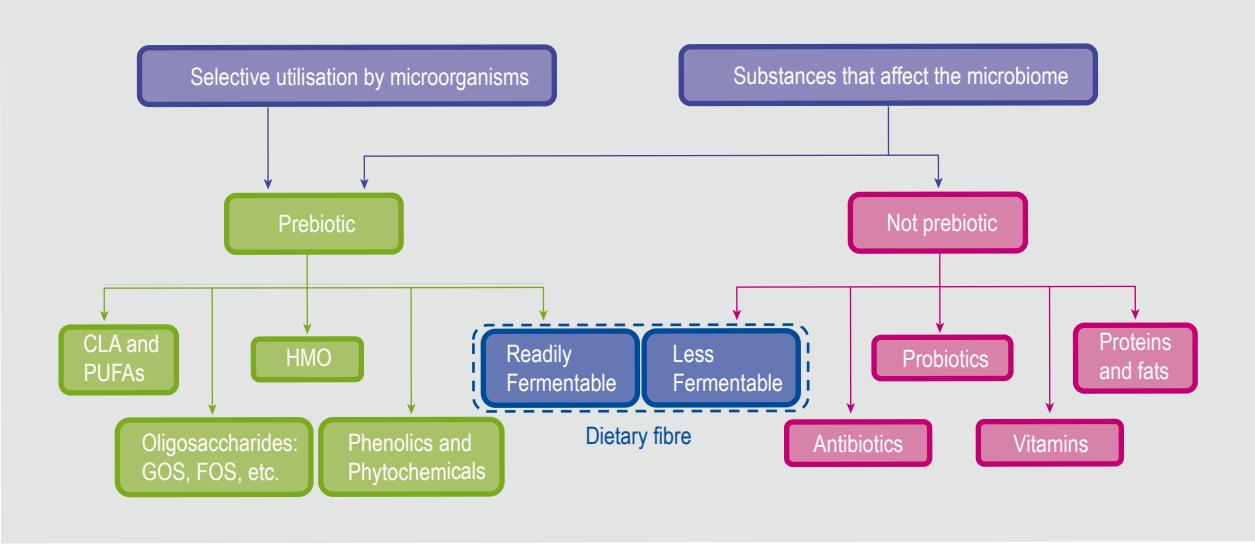

Figure 3. Current thinking on prebiotics and candidate prebiotics. Adapted from Gibson *et al.* (2017). CLA = conjugated linoleic acid; PUFA = polyunsaturated fatty acids; HMO = human milk oligosaccharides; GOS = galacto-oligosaccharides; FOS = fructo-oligosaccharides. Dietary fibres may be considered as prebiotics if they are readily fermentable. Less fermentable fibres are unlikely to mee the definition of a prebiotic.

Generally, a structure-function relationship infers that the carbohydrate is preferentially fermented by certain bacteria and, in turn, they and often other members are supported in the community ecosystem. Non-fermentable carbohydrates are generally attributed to providing bulk and facilitating laxation, though they too may have other roles in terms of microbiota structure and gut health. In our discussions, the term 'structure' refers most often to carbohydrate chemical structure, which refers to the sugar or monosaccharide composition and the linkages that attach the sugars into oligosaccharides and polysaccharides. Size is also a factor affecting bacterial utilisation and competition of carbohydrates. In the smaller oligosaccharide range, studies have clearly shown that size differences of homoglycans support different bacteria (Falony *et al.*, 2009a,b; Kaplan and Hutkins, 2003; Rivière *et al.*, 2018). In larger polysaccharides, the role of size is not as clear. Another type of 'structure' important to our discussion is the physical structure of fibres, which is related to their soluble or insoluble form, with the latter being a fermentable insoluble matrix fibre. Some bacterial groups have particular affinity and ability to utilise fermentable insoluble fibres (Hamaker and Tuncil, 2014).

Non-digestible carbohydrate utilisation by gut bacteria

To understand how NDC, fibres or prebiotics shift gut bacterial communities and generate desirable metabolites, it is useful to first understand some basic aspects of how gut bacteria access carbohydrates as food, and how they compete for them. The gut bacteria receive dietary carbohydrates and break them down to monosaccharides, or sugars, for energy. Generally, nearly all dietary fibres, whether soluble or insoluble, have the potential to be fermented in the gut (Hamaker and Tuncil, 2014). Soluble fibres, because they are easily accessed by bacteria, are usually quickly fermented, though this depends on the complexity of their chemical structure (Hamaker and Tuncil, 2014). Insoluble fibres, such as those in cereal brans and various vegetables and fruits are mostly cell wall matrix-bound fibres. Even though some insoluble fibres may not be fermentable, most are to some degree utilised by gut bacteria that have the encoded genes and machinery to access and degrade them (Luis and Martens, 2018). An example of a difficult-to-ferment fibre is cellulose, which is often wrapped inside other fibre polymers and further is in a semi-crystalline form that makes it hard to access by bacterial enzymes. Yet, there are gut bacteria that are adept at degrading cellulose, and at least a portion of dietary cellulose is likely always to be fermented (Flint *et al.*, 2008).

A healthy gut microbiota contains genes to degrade and utilise most of wide array of dietary fibre structures found in foods and it is important to realise that a mix of fibres is needed to satisfy the food needs of any diverse gut microbiota community. Many bacterial groups can utilise one fibre type. Still, even then, there are differences in specificity to structure that make bacteria more or less competitive to utilising a particular fibre.

Broadly speaking, the Gram-negative bacteria of the *Bacteriodetes* phylum access fibre polymers through assemblies on their outer membrane that physically bind to the carbohydrate, digest it to a small oligosaccharide size, and transport those digested fragments to its periplasmic layer (between the inner and out membranes). Then, other enzymes digest the oligosaccharides to monosaccharides that are transported into the cytoplasm for metabolic processing (Figure 4).

Within the genome of each bacterial strain are encoded polysaccharide-utilisation loci (PUL) that are gene clusters containing all the genetic information to synthesise and construct the outer membrane SUS-like (SUS = starch utilisation system) assemblies (Cockburn and Koropatkin, 2016). Common Gram-negative bacteria include *Bacteroides* and *Prevotella* species that live in the lumen of the gut.

The two other major phyla in the gut, *Firmicutes* and *Actinobacteria*, have a variety of other mechanisms to access and utilise dietary carbohydrates. For instance, the families comprising the *Clostridium* cluster bacteria, which reside more prominently in the mucosal area of the large intestine, have cellulosomes to bind and digest carbohydrates (Artzi *et al.*, 2017; Ben David *et al.*, 2015). Cellulosomes are appendages that are directed outward from the bacterial membrane, which can be interchanged with different carbohydrate-degrading enzymes and binding proteins and presumably can be directed into insoluble fibre matrices for their degradation and fermentation. The *Firmicutes* contain other members that employ different mechanisms to access dietary carbohydrates (e.g. assemblies analogous to SUS of Gram-negative bacteria, and secretion of extracellular enzymes (Martens *et al.*, 2014)). Actinobacteria, another Gram-positive phylum contains *Bifidobacterium* species, which are generally adept at utilising oligosaccharides brought in through membrane transporters (Figure 5). Hence, they are often promoted when provided prebiotic oligosaccharides such as galacto-oligosaccharides (GOS) and fructo-oligosaccharides (FOS).

This preference is exemplified by the metabolism of FOS by bifidobacteria and lactobacilli. The small fructan oligosaccharides, kestoses or kesto-*n*-oses, are trimeric or oligomeric fructans containing one glucose and two or more fructose linked by β(2-1) and/or β(2-6) linkages (Verspreet *et al.*, 2015). Bifidobacteria have an observed preference for shorter chain fructans which could partly be explained by their sucrose (GF) transporter that is also able to transport GF2 and GF3 molecules into the bacterial cell for further usage (Ryan *et al.*, 2005). Indeed, the β-fructofuranosidase activity is predicted to be

intracellular. This is even truer in lactobacilli that mainly have intracellular metabolic enzymes for oligosaccharides and for which transport is the most limiting factor; with poor ability to transport polysaccharides, while tri- and tetrasaccharides are transported and metabolised by pathways available for disaccharides (Gänzle and Follador, 2012). In *Lacticaseibacillus paracasei* for example (Kaplan and Hutkins, 2003) the FOS transport system has greater affinity for chains with lower degree of polymerisation (DP) than for longer ones, DP3 and DP4 are absorbed more rapidly than DP5. A similar situation has been observed with *Lactiplantibacillus plantarum* (Saulnier *et al.*, 2007). In comparison to sucrose, the relative activity of β-fructofuranosidase in the presence of fructans is higher for shorter (DP<10) than longer chain fructans and the presence of a glucose at terminal position also seems to increase the activity (Ryan *et al.*, 2005). In some bacteria, that show a prolonged lag period before growth *in vitro*,

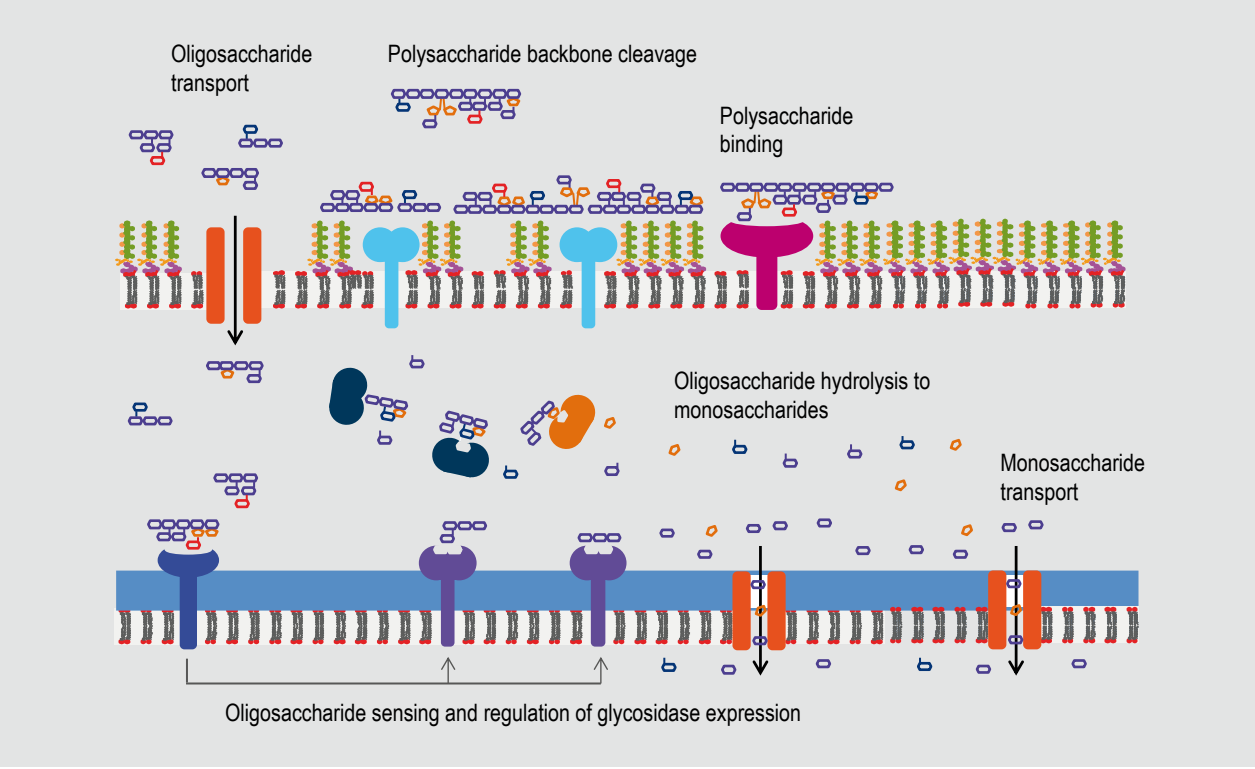

Figure 4. Generic scheme for the metabolism of complex polysaccharides in Gram-negative bacteria, such as *Bacteroides* spp. (based on Hamaker and Tuncil, 2014; Larsbrink *et al.*, 2014; Martens *et al.*, 2014).

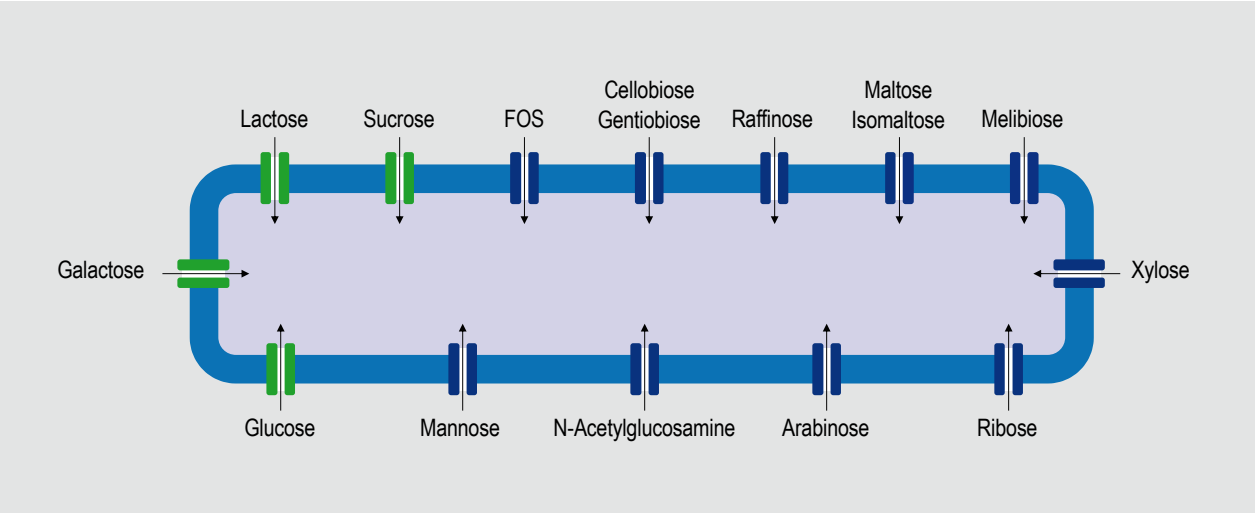

Figure 5. Some known transport systems identified in *Bifidobacterium* spp. Modified from Lee and O'Sullivan (2010).

a switch in gene expression seems a prerequisite to use long-chain inulin (Scott *et al.*, 2014).

Fibre utilisation in gut microbiota of different community types

To understand better how fermentable fibres affect the gut microbiota, fermentation metabolites, and health, one must consider the gut bacteria community composition and structure into which the carbohydrates are received. How the microbiota responds to fermentable fibres in general, and prebiotics in particular is fundamentally related to the gut bacterial community structure and to how fibres are competed for as food sources. In the study by Chen *et al.* (2017a), FOS and two structurally different arabinoxylans (AX) were examined in an *in vitro* human faecal fermentation system for their responses in two microbiotas with different ecologies that fit into the *Prevotella*-dominant and *Bacteroides*-dominant enterotypes. Results demonstrated that fermentable fibres have markedly different effects depending on the initial makeup of the microbiota. In the case of the *Prevotella* enterotype, the three fibres were all consumed by the dominant *Prevotella* operational taxonomic unit (OTU) with an equal enhancement of propionate with FOS being the only fibre to increase butyrate substantially; while in the *Bacteroides*-dominant microbiota, none were propionate enhancers and both FOS and the structurally simple sorghum AX increased butyrate levels (Figure 6). Contrary to the *Prevotella*-dominant enterotype, the main *Bacteroides* OTU showed a mixed response in its ability to compete for the three fibre substrates and other bacteria were also favoured. Thus, microbiota community differences influence which bacteria compete best on different fibres and affect SCFA metabolites.

As an additional note, many of the fibre structure and gut function studies cited in this review are based on *in vitro* human faecal fermentations, and microbiota changes and metabolites produced may be different when tested in the human body/*in vivo*.

2. Effect of non-digestible carbohydrates on the gut microbiota

Galacto-oligosaccharides

Galacto-oligosaccharides (GOS) are an important class of dietary prebiotics that exert beneficial effects on intestinal microbiota and gut barrier function. GOS consisting of 3-10 molecules of galactose and glucose are known to facilitate the growth of desirable intestinal microbiota and are considered as potent prebiotics.

GOS are manufactured by transfer reactions, catalysed by β-galactosidases, using lactose as a donor and acceptor (reviewed by Gänzle, 2012; Torres *et al.*, 2010) called trans-galactosyl oligosaccharides (TOS). They can be composed (Bouhnik *et al.*, 1997) of a complex mixture of structures with varying linkages and degrees of polymerisation). The structural complexity of the product depends on the

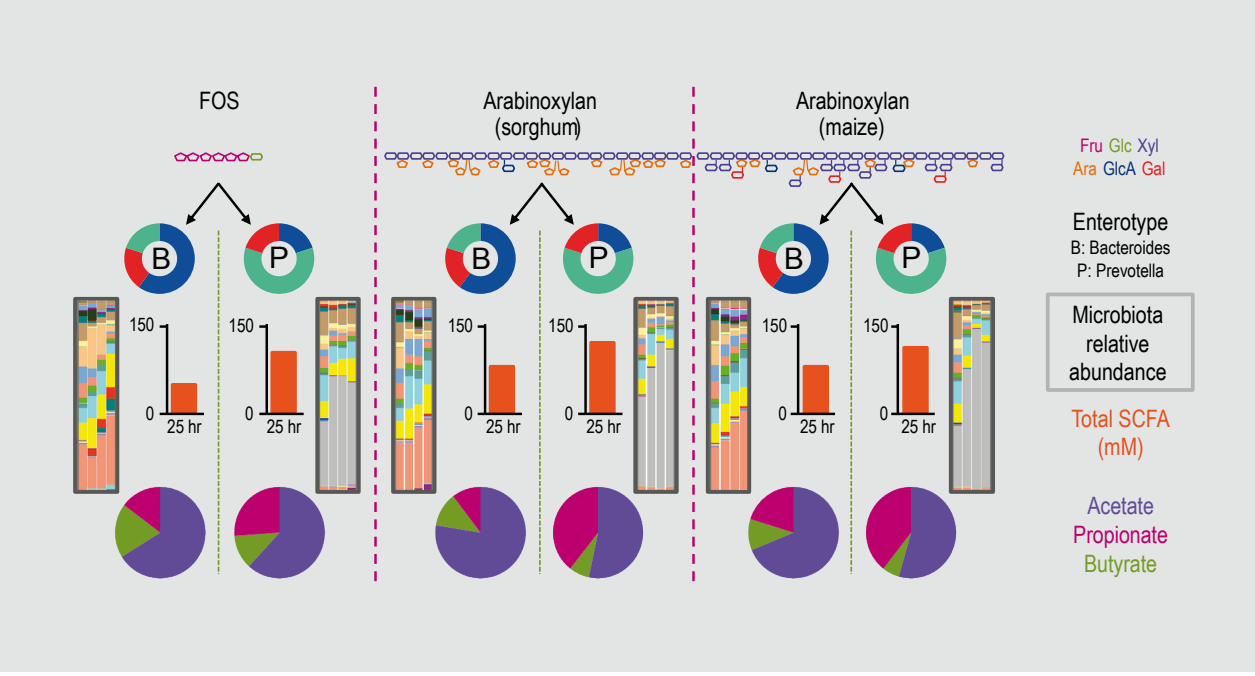

Figure 6. Differences in microbiota relative abundance and short chain fatty acids (SCFA) produced during *in vitro* fermentation with faeces from two healthy adult donors when fed fructooligosaccharides (FOS), sorghum arabinoxylan, and corn arabinoxylan (Adapted from Chen *et al.*, 2017b). Fru = fructose; Glc = glucose, Xyl = xylose, Ara = arabinose, GlcA = glucoronic acid, Gal = galactose.

enzyme(s) used for its manufacture. A recent comparative analysis of commercial GOS products (Van Leeuwen *et al.*, 2016; Figure 7) illustrates the complexity and also makes the point very clearly that not all GOS products are the same; in fact, GOS as a category of prebiotics have a unique degree of structural variation regarding degree of polymerisation, branching and linkage composition (Figure 7). With this in mind it is clearly important to consider the particular GOS product studied when evaluating studies on the properties of GOS.

GOS products are generally either made by enzymes of *Bacillus circulans* (BC-GOS) or by enzymes from *Bifidobacterium bifidum* (BB-GOS). Both forms of GOS have been extensively studied in human volunteer studies and have consistently shown effects on the gut microbiome. A consistent bifidogenic effect can be seen across such studies (Alander *et al.*, 2001; MacFarlane and MacFarlane, 2007; Walton *et al.*, 2012).

GOS have shown a range of health benefits including reduction of allergies and infections in infants when mixed with inulin in a 9:1 ratio (Braegger *et al.*, 2011; Cuello-Garcia *et al.*, 2016), impacts on immune function (Vulevic *et al.*, 2008) and reactive asthma (Williams *et al.*, 2016) metabolic syndrome (Vulevic *et al.*, 2013), traveller's diarrhoea (Drakoularakou *et al.*, 2009), irritable bowel syndrome (IBS) (Silk *et al.*, 2009) and social skills in autistic children (Grimaldi *et al.*, 2018).

Despite the variation seen between different GOS products, there have been very few studies addressing the structure-function relationships among GOS molecules. One such study that is particularly revealing is that of Ladirat *et al.* (2014). These authors found that *Bifidobacterium* populations significantly increased on both DP2 and DP3 GOS fractions with *Bifidobacterium adolescentis*, *Bifidobacterium angulatum*, *Bifidobacterium longum* and *Bifidobacterium thermophilum* being stimulated. High Performance Anion Exchange Chromatography (HPAEC) revealed that GOS components were metabolised at different rates, displaying a size-dependence on rate of metabolism.

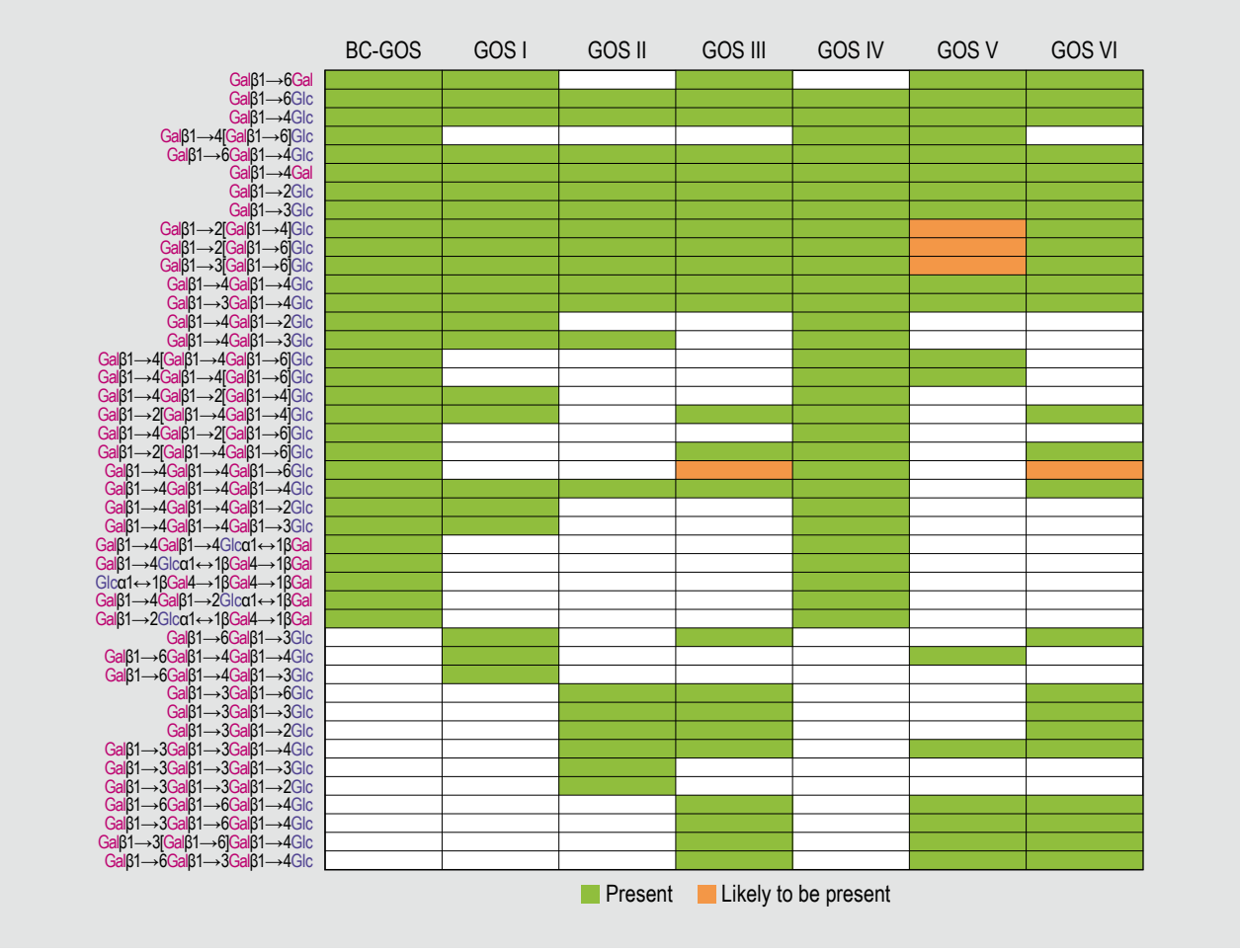

Figure 7. Structural complexity and diversity of commercial galacto-oligosaccharide (GOS) products. BC-GOS is manufactured by *Bacillus circulans*, GOS I-VI are unidentified commercial GOS products. Modified from van Leeuwen *et al.* (2016).

Further analysis revealed structure-based differences in metabolism. Within the disaccharides, the α- or β-(1↔1) linkages were most resistant and β(1→4) and β(1→6) linkages hydrolysed fastest. Curiously, the presence of the β(1↔1) in the trisaccharides resulted in faster metabolism of an adjoining β(1→4) linkage.

It is clear that the degradation of GOS by bifidobacteria and lactobacilli is highly species-specific (Böger *et al.*, 2019). In this study *Bifidobacterium breve* and *Bifidobacterium lactis* preferred GOS as a substrate to glucose while lactobacilli had a limited ability to grow on purified GOS. Most of the strains tested were unable to metabolise branched structures, particularly those of DP 4 and above. Exceptions were *B. lactis* subsp. *infantis*, *B. breve* and *B. adolescentis*. This is consistent with the more diverse carbohydrate metabolic systems present in bifidobacteria (Lee and O'Sullivan, 2010). Lactobacilli possess limited capacity to transport and metabolise oligosaccharides (Gänzle and Follador, 2012).

It is evident, that GOS preparations can have beneficial effects on microbiota. At the present time, however, we do not have sufficient comparative studies to form any conclusions over the extent to which the health benefits are influenced by structure (Ambrogi *et al.*, 2021; Logtenberg *et al.*, 2021; Zhang *et al.*, 2022).

Xylans and xylo-oligosaccharides

Xylo-oligosaccharides (XOS) have been studied as candidate prebiotics since the 1990s. Okazaki *et al.* (1990) employed a mixture of xylose, xylobiose, xylotriose, and other saccharides as a carbon source for *in vitro* fermentations with *B. adolescentis*, *B. longum*, and *Bifidobacterium infantis*. In this study, *B. adolescentis* showed a remarkable ability to use both xylobiose and xylotriose. Hopkins and others (1998) carried out fermentations with commercial XOS (Suntory, Japan; 70% purity and DP2-4) and found that the ability of bifidobacteria for growing on XOS depended on the strain. In an *in vitro* study by Mäkeläinen *et al.* (2010) three different XOS preparations with various sizes of xylose polymers and DP, and a significant increase of *B. lactis* was seen for the XOS DP2-10 and XOS DP2-5.

The preference of bifidobacteria to ferment low-substituted XOS, both *in vitro* and *in vivo*, has been described previously (Campbell *et al.*, 1997; Okazaki *et al.*, 1990). On the other hand, oat XOS were not selective for bifidobacteria exclusively, because *Bacteroides* spp., *Clostridium* spp., *Lactobacillus acidophilus*, and *Klebsiella pneumoniae* also showed moderate growth on these substrates (Jaskari *et al.*, 1998; Van Laere *et al.*, 2000b). Also, the more highly branched wheat AX hydrolysates (singly and doubly substituted arabino-XOS) could only be (partly) fermented by the *Bifidobacterium* spp. and *Bacteroides* spp. tested.

Kabel and others (2002) studied *in vitro* fermentability of differently substituted XOS and concluded that the neutral-XOS, arabino-XOS, acetylated-XOS, and acidic-XOS obtained from hydrothermally treated xylan-rich byproducts were fermented by human faecal inocula. For all fermentations described, a distinction could be made between the 1st stage of the fermentation (0 to 40 h) and the 2nd stage (>40 h). In the 1st stage of the fermentation, the pH decreased, whereas in the 2nd stage the pH remained constant or even increased slightly. In the 1st stage of fermentation of neutral-XOS and arabino-XOS, mainly acetate and lactate were formed. Lactic acid bacteria (LAB) may play an important role in this part of the fermentation, as they do not produce butyrate or propionate, but they do produce acetate and lactate (Verbeke *et al.*, 2015). A high concentration of acids formed might be desirable because, by a decrease in pH, the growth of potentially pathogenic microorganisms and putrefactive bacteria will be inhibited (Gibson and Roberfroid, 1995).

The ability of bifidobacteria to metabolise XOS depends on the efficiency of their xylanolytic enzyme systems. One xylosidase and a few arabinosidases have been purified and characterised from bifidobacteria. They are β-D-xylosidase from *B. breve* K-110 and arabinosidases from *B. adolescentis* DSM20083 (Van Laere *et al.*, 1997, 2000a) and arabinosidase from *B. breve* (Shin *et al.*, 2003).

The human experimental data on XOS are limited and evidence of the prebiotic efficacy of XOS is sparse. Study on the effects of XOS on the faecal content of humans showed that it was maintained within the normal range by daily intake of XOS (Kobayashi *et al.*, 1991). Short-chain fatty acids, especially acetic acid, in the faecal matter of subjects were increased and the increased putrefactive products, such as *p*-cresol, indole, and skatole were decreased by the continuous intake of XOS.

Childs *et al.* (2014) investigated the impact of XOS on the human gut microbiota and markers of immune function either alone or in combination with a probiotic strain of *Bifidobacterium animalis* subsp. *lactis*. XOS increased populations of bifidobacteria and reduced fasting plasma HDL concentrations compared to placebo. XOS also significantly increased the faecal levels of the added *B. lactis* probiotic when fed as a synbiotic combination.

Arabinoxylo-oligosaccharides have also been studied for their impact on the gut microbiota. Three oligosaccharide mixtures (average molecular weights of 354, 278, and 66 kDa respectively), made by AX hydrolysis, have been studied for their fermentation properties (Hughes *et al.*, 2007). In general, AX *in vitro* human faecal fermentation resulted in an increase in bifidobacteria, lactobacilli and eubacteria groups, with the 66 kDa fraction most promotive of lactobacilli. There was an inverse relationship between

AX lower molar mass and bifidobacteria and lactobacilli groups. In further support, a hydrolyzate of average DP 60, described by the (Van den Abbeele *et al.*, 2013) as 'long-chain arabinoxylan', in dynamic *in vitro* fermentation models (SHIME and TIM-2), increased *B. longum* and propionate production and in a simulated lumen and mucosal microbiota M-SHIME experiment, increased bifidobacteria in both locations (Truchado *et al.*, 2017).

Fermentability of arabinoxylooligosaccharides (AXOS) is affected by its degree of feruloylation and is probably due to hindrance to enzyme digestion, but also possibly inhibition by freed ferulic acid (Snelders *et al.*, 2014). Various bacteria have feruloyl esterases that are necessary to break apart cross-linked cell wall matrix-bound AXs as well as to remove individual feruloyl residues. Feruloylation and removal of residues, in addition to other bound phenolic acids, is a relatively understudied area that could be important to understanding gut bacterial utilisation of the AXs.

In a specific examination of *Bifidobacterium* species and their response to AXOS, XOS, and D-xylose and L-arabinose, selective utilisation was observed (Pastell *et al.*, 2009). Different strategies were revealed by species, which emphasises that even closely related species/strains have different requirements as well as ways to access and utilise substrate. For instance, *B. longum* used AXOS by first hydrolysing off arabinose and fermenting it, while *B. adolescentis* removed arabinose and went on to consume the unsubstituted xylan backbone.

An interesting study compared water-unextractable AX, water-extractable AX, and AXOS from wheat on microbiota and SCFAs in rats (Damen *et al.*, 2011). Insoluble AX increased butyrate and the butyrogenic bacteria *Roseburia* species and *Eubacterium rectale*, while water-extractable AX and AXOS selectively promoted bifidobacteria. Combining the different arabinoxylan fractions caused bifidogenic and butyrogenic effects in the colon with lowered pH and proteolytic fermentation metabolites.

In terms of high molecular weight xylans in foods that are delivered to the gut microbiota, two structural groupings can be considered. First, the xylans that are largely linear and unbranched and second, the largest and perhaps most structurally diverse, the arabinoxylan (or 'heteroxylan') class. In their native forms, the polymeric xylans are usually considered as insoluble fibre as they are found in cell wall structures often cross-linked via phenolic compounds. Yet, the arabinoxylans can be solubilised with a simple, though commercially expensive, alkali treatment that de-esterifies the phenolic cross-links. Some native arabinoxylans, such as those found in wheat endosperm cell walls, are naturally soluble (Delcour *et al.*, 1999). The largely unbranched xylans associate tightly into insoluble fibrils and the oligosaccharides, due to their small size, are readily soluble. Regarding their function in the gut, generally when in their soluble form they are well fermented, though chemical structure determines their rate of fermentation. In their insoluble cross-linked or associated forms, they exhibit a range of fermentability from being essentially non-fermented (e.g. the highly cross-linked maize bran arabinoxylan) to being moderately fermented (e.g. wheat bran arabinoxylan).

Although the term 'xylans' is sometimes used to indicate all polysaccharides containing a β-1,4-linked xylan backbone, including the arabinoxylans discussed below, for purposes of this report xylan refers to polymers containing such a backbone with few if any branches. Such xylans are found in agricultural crops (e.g. straw, sugar cane and other stalks, wood processing by-products) and from fruit berries (Cantu-Jungles *et al.*, 2017). For example, in the acai berry, xylan was nearly linear with only 2.8% glucuronic acid branched residues.

AX, or 'heteroxylans', are a common group of polysaccharides found in plant cell walls and are particularly high in cereals. They are distinguished by a β-1,4-linked xylan backbone with either simple arabinosyl branches or more complex combinations of sugars (including galactose, xylose, glucuronic acid) and linkages in the branches. They may have barren stretches of no branches on the backbone (unsubstituted) or may be highly substituted with one or two branches on individual xylosyl units (mono- or disubstituted). Three general types of AX are found in the structure-function literature: (1) insoluble matrix-bound AX, (2) soluble AX, either naturally water-extractable or solubilised with alkali, and (3) oligosaccharides that are fabricated from the polymers to make AXOS. When in their soluble form, AX are well fermented and, in their insoluble cross-linked form, they show a range of fermentability from being poorly fermented (e.g. highly cross-linked maize bran AX) to being moderately fermented (e.g. wheat bran AX).

The most common AX found in diets are those originating from cereal grains, which are concentrated in the bran layer and are in the cell wall remnants in the endosperm and germ. Structural diversity of this class of fibres is high with chemical structures differing in sugars and linkages found in branches, and the density of branches that are placed on the backbone (Rumpagaporn *et al.*, 2015).

In this group of cereal brans, the major AX isolated from wheat bran has a very complex structure with different branch types and an array of linkages connecting sugars. High branch complexity is found in maize AX with a comparably high amount of xylose found in branches. Branch complexity decreases in rice and sorghum bran AX. All of the bran AX shown can be considered as highly substituted.

There are structural differences in arabinoxylans depending on their anatomical location in the cereal grain. The most studied wheat endosperm cell wall arabinoxylan is water-extractable. It is structurally simpler than ones found in bran being high in unsubstituted regions of the xylan backbone and with numerous single arabinose branches.

AX-degrading species have been found in both major human gut phyla of *Bacteroidetes* and *Firmicutes*; however only select species have been shown to degrade AX. Ndeh and Gilbert (2018) noted that this gives the xylan-degraders competitive advantage when the fibre is available and could be used to modulate the gut microbiota. Some *Bifidobacterium* and *Lactobacillus* species utilise the oligosaccharides produced from arabinoxylan degradation by other bacteria (e.g. *Bacteroides* species).

AX in their intact state in plant cell walls have been shown to be variably fermented based on their source. Differences in degree and rate of fermentability are based primarily on differences in physical structure that inhibit bacteria access for digestion and fermentation. For instance, in an *in vitro* human faecal fermentation, Rose *et al.* (2010) showed that maize bran with 69% AX of total dietary fibre (TDF, 70 g/100 g dry matter (DM)) was very poorly fermented in the 24 h incubation period, while wheat with 57% AX of TDF (52 g/100 g DM) was fairly well fermented, and rice bran with 37% AX of TDF (31 g/100 g DM) was as well or better fermented than the alkali-solubilised AX from each. In the intact state the fibres were far more butyrogenic (21, 26, and 20% butyrate of total SCFA at 24 h) than when in the soluble state (10, 16, and 14% butyrate of total SCFA), and were less propiogenic when intact (8, 10, and 14% propionate of total SCFA) than soluble (28, 22, and 19% of total SCFA). Thus, physical accessibility plays a significant role in their utilisation, favouring butyrogenic bacteria in the intact form and propiogenic bacteria in the soluble form. The same report suggested that maize and rice AX are fermented by a debranching mechanism leading to the xylan backbone, and wheat preferentially starting at the backbone. This difference is likely due to the high substitution (branching) of maize and rice bran AX and the higher degree of unsubstituted (barren) backbone of wheat bran AX. Williams *et al.* (2011) showed a similar difference in fermentation kinetics between insoluble and soluble cereal AX, and additionally found that the corresponding monosaccharides were fermented at similar rates to the soluble polysaccharides showing that digestion was not a limiting step to utilisation.

D'hoe *et al.* (2018) examined different arabinoxylan-containing insoluble (total and ultrafine grind, and aleurone) and soluble wheat bran fractions in faecal fermentations of six individuals, showing a reduction of Proteobacteria, an increase in *Dorea* and butyrogenic *Roseburia* for aleurone, and increase in bifidobacteria for ultrafine and soluble bran fractions.

With slow rate of fermentation, SCFA metabolites are generated throughout the length of the large intestine. It was proposed that early man consumed high levels of plant cell wall polysaccharides, even up to 80 to 120 g/day (Leach, 2007), which at low levels of fermentation would have still provided significant SCFA production in proximal and distal locations.

AX are generally more fermentable when in the soluble rather than cross-linked insoluble forms. When water-soluble wheat AX was cross-linked by a treatment with a combination of horseradish peroxidase and hydrogen peroxide, it became less fermentable as measured by SCFA output (Hopkins *et al.*, 2003). As noted above, Rose *et al.* (2010) showed mostly a large increase in fermentability when cereal AXs were solubilised by alkali treatment of brans.

Once in the soluble form, AX fermentability is determined by chemical composition rather than physical state. *In vitro* rate of fermentation was dependent on chemical structure and rate varied considerably from a simpler branched, though highly substituted, structure of sorghum AX, which was as fast fermenting as the fructooligosaccharide (FOS) control, to slow fermentation of more complex branched structures found in corn bran and a fraction of wheat bran (Rumpagaporn *et al.*, 2015). Structures that had more terminal xylose in the branched structures, rather than just comprising the β-1,4-linked backbone, as well as a few other sugar and linkage combinations, were slow fermenting (Figure 8). While fermentation response is sensitive to structure of the soluble AX even at the genotypic level, growing environment was not found to affect structure or response (Zhang *et al.*, 2021). On a practical note, animal chow diets containing different AX sources are likely to lead to somewhat different gut microbiota compositions.

AX structural differences also determine which xylan-degrading bacteria are promoted. An artificial human faecal-based community comprised of 47 bacterial species when fermented on five different plant xylan sources revealed differential response on *Bacteroides* species, indicating their specificity to competition on structures (Centanni *et al.*, 2017). In another study, Chen *et al.* (2017b) showed maize and sorghum alkali-extracted soluble AXs promoted different bacteria in a *Bacteroides*-dominant enterotype versus a *Prevotella*-dominant enterotype.

In high-fat feeding of mice, long-chain AX restored important gut bacterial groups of *Bacteriodes-Prevotella* species, *Roseburia* species, and bifidobacteria (Neyrinck *et al.*, 2011). This was with a concomitant improvement in gut barrier function and lowering of an inflammation factor. In

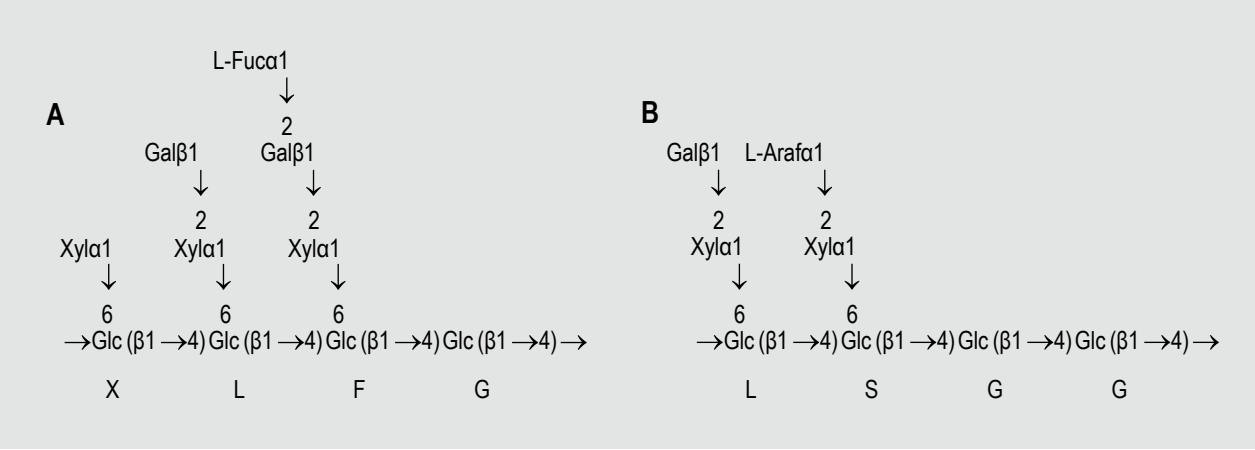

Figure 8. Representative structures of XXXG- and XXGG-type XyGs. (A) XXXG-type XyGs, comprising a Glc4Xyl3 repeating motif with variable branch extensions (bold residues). Tamarind seed XyG and primary cell wall XyGs (for example, from lettuce leaves) are distinguished by the absence of fucose in the former. (B) XXGG-type XyGs, comprising a Glc4Xyl2 repeating motif. These XyGs are common to solanaceous species (for example, tomato) and are typified by branches extended with arabinofuranosyl residues. Standard single-letter abbreviations for designating backbone decorations are shown.' Adapted from Larsbrink *et al.* (2014).

a rat study, six-week feeding of long-chain AX increased caecal *Verrucomicrobia* and *Firmicutes* with lower level of *Bacteroidetes* (Van den Abbeele *et al.*, 2011). The AX and inulin groups showed promotion of butyrogenic bacteria including *Roseburia intestinalis*, *Eubacterium retale*, and *Anaerostipes caccae*, as well as bifidobacteria. Along with an increase in mucin levels, a lower caecal abundance of the mucin-degrader *Akkermansia muciniphila* was observed (Pereira *et al.*, 2021; Schupfer *et al.*, 2021).

Xyloglucans

Although perhaps just as commonly found in diets as the cereal arabinoxylans, the primarily dicot cell wall xyloglucans are far less studied in terms of their effect on the gut microbiota.

Xyloglucans make up to 25% of the dry weight of dicot cell walls, such as in lettuce, onions and tomatoes; and are also present in cereal cell walls, though in much smaller amounts. Tamarind is a commercial source of xyloglucan and is used in some parts of the world as a food additive (Hartemink, 1996). They are generally water-extractable, though this is dependent on the nature of the cell wall matrix. They also vary in structure and presumably this is matched by specificity of gut bacterial strains to utilise and compete on them. Xyloglucans are comprised of a backbone glucan structure of β-1,4-linked glucosyl units with α-1,6-linked xylosyl units, some with further linked galactose or fucose (Figure 8) (Larsbrink *et al.*, 2014).

An early report on the fermentability of xyloglucans by human gut microbiota showed that only a selected group of gut bacteria can utilise this class of dietary fibre polysaccharides (Hartemink, 1996). They analysed 138 gut bacteria strains from 75 species and found only 9 strains able to degrade the tamarind xyloglucan; consisting mostly of *Clostridium* spp., and one each of *Bifidobacterium* sp. and *Bacteroides* sp. This contrasts with the work by Larsbrink *et al.* (2014), showing that 70 strains from 6 species from an overall screen of 292 Bacteroidetes strains from 29 species to be xyloglucan degraders. Specificity to xyloglucan structure was revealed in that tamarind xyloglucan supported the growth of all but one of the 25 *Bacteroides ovatus* strains, while none of the *Bacteroides xylanisolvens* strains grew on it. In the case of bifidobacteria, Hartemink *et al.* (1996) found that clostridia were the primary degraders of the polysaccharide. While xyloglucan utilisation is relegated to relatively few phylogenetic diverse strains, which can be considered as niche species, evaluation of metagenomes of many individuals shows the ability for its degradation exists in all.

Pectins

Pectins are a group of complex structural hetero-polysaccharides occurring ubiquitously in plants. Their chemical structures are heterogeneous, depending on the origin, location in the plant and the extraction method used (Bagherian *et al.*, 2011; Srivastava and Malviya, 2011). These polysaccharides are mainly composed of a backbone of galacturonic acid units (GalA) connected by α(1,4) links. Varying proportions of the acid groups as methoxyl esters and neutral sugars can constitute side chains (Kertesz, 1951). The pectin network is generally formed with three block copolymers: namely, homogalacturonan (HG), rhamnogalacturonan (RG-I) and the substituted galacturonan (SG) rhamnogalacturonan-II (RG-II) (Yapo,

2011b). In addition, the SGs xylogalacturonan (XGA), apiogalacturonan-1 (ApGA), galactogalacturonan (GGA), and arabinogalacturonan (ArGA) have also been purified from plant cell wall material (Ovodova *et al.*, 2006; Yapo, 2009; 2011b).

HG is the most abundant pectic polysaccharide, constituting approximately 65% of total pectin, this structure is also referred to as the 'smooth region' of the pectin molecule (Wolf *et al.*, 2009). HG consists of a linear α-1,4-linked GalA homopolymer with a typical degree of polymerisation of ~100; shorter regions have been also detected (Nakamura *et al.*, 2002; Wolf *et al.*, 2009). HG can be partially methyl-esterified at the C-6 carboxyl and also be O-acetylated at O-2 or O-3 (MacKinnon *et al.*, 2002; O'Neill *et al.*, 1990). The degree of methylation and in some cases also acetylation (DM/degree of esterification (DE) and degree of amidation (DA), respectively) vary according to the origin and the developmental stage of the plant (Voragen *et al.*, 2009). Methylation can occur randomly or in blocks of methylated residues (Celus *et al.*, 2018; Tanhatan-Nasseri *et al.*, 2011). Varying patterns of the smooth (HG) regions and the 'hairy' regions of rhamnogalacturonan (RG-I) occur in different pectins (Cornuault *et al.*, 2018; Fan *et al.*, 2018).

The RG-I backbone is composed of the repeating diglycosyl [→2)-α-L-Rhap-(1→4)-α-D-GalpA-(1→] and is believed to be branched at O-4/O-3 positions by 4 different side chain types, viz. (1→5)-α-L-arabinan, (1→4)-β-D-galactan, arabinogalactan-I, and with arabinogalactan-II (Mikshina *et al.*, 2015). The functional diversity of RG-I has been reviewed by Mikshina *et al.* (2015), emphasising the diversity of RG-I types related to plant origin, specific plant tissues, and even for different developmental stages of the tissue, reflecting a sophisticated diversity of plant glycobiology and molecular diversity (Mikshina *et al.*, 2015).

A distinct and highly conserved region of rhamnogalacturonan is designated as RG-II, consisting of GalA units, with complex branches of peculiar monosaccharides, apiose, fucose, xylose, aceric acid, 3deoxy-lyxo-2-heptulosaric acid (DHA), and 3-deoxy-manno-2-octulosonic acid (KDO) (Doco *et al.*, 1997). The pectic polysaccharide RG-II is a quantitatively minor component of the plant cell wall, constituting around 0.1-8.0% (W/W) of plant cell walls (Yapo, 2011a). The fine structure of RGs-II remains somewhat enigmatic as emphasised and reviewed by Yapo, who highlighted 'the freak structural diversity and the functional versatility of RG-II' (Yapo, 2011a).

Due to its proportionally small quantities within the pectin molecules especially little is known about RG-II with regards to its different effects on the gut microbiota and its interaction with the gut and mucosa associated lymphoid tissue (GALT and MALT) during its passage through the gastrointestinal tract (GIT). RG-II is greatly reduced or absent in commercial pectin due to the extraction and purification procedures used (Liu *et al.*, 2007). To assess how different RG-II structures function, the development of methods for their preparation is required. Due to its structural diversity, it is likely to have diverse biological activities. Recently Wu *et al.* (2018) have reported that the fungus *Penicillium oxalicum* could efficiently lead to the recovery of RG-II domains by degrading all other pectic domains (Wu *et al.*, 2018). Such novel preparation protocols will aid to characterise and investigate the function of rare RG-II pectin derived structures.

Several studies have reported the modulation of gut microbial communities by RG-I (Gulfi *et al.*, 2007; Khodaei *et al.*, 2016; Strube *et al.*, 2015a,b; Thomassen *et al.*, 2011). Gulfi *et al.* have shown that the hairy regions from ripe apples revealed to be a fermentable substrate for human colonic bacteria, showing a substantial impact on pH and SCFA production (Gulfi *et al.*, 2007). High molecular weight galacto-rhamnogalacturonan isolated from potato pulp, has been also reported to have prebiotic effects and lead to an increase in the levels of *Bifidobacterium* and *Lactobacillus* spp. using *in vitro* fermentation of human faeces (Thomassen *et al.*, 2011). Khodaei *et al.* (2016) produced galactose-rich oligosaccharides/oligomers (oligo-RGI) from potato rhamnogalacturonan-I (RG-I) and investigated their digestibility and fermentability *in vitro*. Most of RG-I and its oligomers remained unhydrolyzed under intestinal conditions. Investigations of prebiotic properties in a continuous culture system inoculated with immobilised faecal microbiota revealed stimulation of *Bifidobacterium* spp. and *Lactobacillus* spp. by both RG-I and oligo-RG-I, with oligo-RG-I being more selective. The amounts of SCFA were higher in oligo-RG I fermentations and FOS (Khodaei *et al.*, 2016). This research emphasises the potential of knowledge still to be gathered on the function of oligomer structures derived from RG-I molecules. Baggio *et al.* (2022) reported that RG-l enhances intestinal epithelial barrier function in a microbiota-independent manner through activation of TLR4 and PKC signalling pathways. The authors suggested that RG-I may be implemented in dietary approaches to enhance mucosal healing in inflammatory bowel diseases).

Recently, a carrot-derived pectic polysaccharide, enriched in rhamnogalacturonan-I (RG-I) was classified as a potential prebiotic ingredient selectively promoting gut microbial members and supporting gut barrier integrity (Van den Abbeele *et al.*, 2020). A depolymerised RG-I-enriched citrus pectin selectively modulated gut microbiota, increased SCFA production, and promoted the growth of *Bifidobacterium* spp., *Lactobacillus* spp. and *Faecalibaculum* spp. (Mao *et al.*, 2019).

One important characteristic of pectin is it´s DE, pectin is characterised as high methoxylated (HM) pectin (DE >50%) and low methoxylated (LM) pectin (DE <50%). The effects of the DE on pectin utilisation by the gut microbiota has been investigated in several studies (Dongowski and Lorenz, 1998; Dongowski *et al.*, 2000, 2002; Larsen *et al.*, 2018; Tian *et al.*, 2017). Dongowski and Lorenz (1998) have investigated the degradation of pectins with a DE of 95, 66, 34 and 0%, by human faecal microbiota *in vitro* (pH 7.8), they have found a faster depolymerisation and fermentation of LM pectins (LMP) compared to HM pectins (HMP). HMP has been reported to lead to the production of high levels of acetate, then propionate and then butyrate (Cantu-Jungles *et al.*, 2017; Jonathan *et al.*, 2012). Though pectin has been reportedly associated with proportionally high acetate production (Gulfi *et al.*, 2005; Jonathan *et al.*, 2012), it has to be considered that acetate can be converted into butyrate (Duncan *et al.*, 2002, 2004). Recently, Cantu-Jungles *et al.* (2017) have investigated a pectic polysaccharide (PW-AP) with a DE of 70% from peach palm fruits (*Bactris gasipaes*) and its fermentation profile by the human gut microbiota *in vitro.* They have reported a similar total amount of SCFA as FOS controls, whereby higher acetate and propionate and lower butyrate were produced in the PW-AP fermentations. Moreover, the pectin fraction led to a significantly lower gas production than FOS, as well as a lower production of branched chain fatty acids (Cantu-Jungles *et al.*, 2017).

There is a lack of studies investigating the effect of different LMP and HMP molecules on the whole human microbiome with sequencing technologies *in vivo*. Nevertheless, recently Tian *et al.* (2017) investigated LMP and HMP digestion in piglets. In the piglet ileum, LMP was more efficiently fermented by the microbiota than HMP, which was mainly fermented by the microbiota in the proximal colon.

Min *et al.* (2015) have investigated the impact of DE of three pectin substrates: 76.0% HMP, 21.2% sugar beet pectin and 22.8% soy pectin, and compared their fermentation to the fermentation of FOS by human faecal microbiota in anaerobic fermentations at 37 °C. The average DP of HMP, sugar beet pectin and soy pectin was 492, 3,729, and 1,510, respectively. They have found higher total SCFA production for the pectins compared to FOS and detected gut microbiota modulatory effects by denaturing gradient gel electrophoresis (Min *et al.*, 2015).

A recent study by Larsen *et al.* (2019) aimed at providing detailed information on the pectin substrates implemented, unravelling some of the structure to function effects of 9 different pectins in the TIM-2 model *in vitro*. Four of the pectins were high methoxyl pectins with highest DE of 70.0 and 74.7%; others were LM pectins with DE between 11.4 and 35.6%. Larsen *et al.* (2019) included an analysis of correlation between bacterial abundances and the structural properties of pectins, i.e. content of monosaccharides, GalA, RG fraction, DE and the degree of branching (DBr). The analysis revealed that the levels of *Oscillospira*, *Blautia*, *Dorea*, *Ruminococcus*, *Coprococcus*, *Ruminococcus torques*, *Lachnospiraceae* and *Clostridiales* within phylum *Firmicutes*, and *Paraprevotella*, *Bacteroides uniformis*, *B. ovatus*, *Parabacteroides distasonis* and *Prevotella* within phylum *Bacteroidetes*, correlated significantly or showed a tendency to increase with higher fractions of simple sugars (Gal, Rha, Xyl, and Glc) and lower content of GalA and DE values.

The species *Faecalibacterium prausnitzii* and family *Ruminococcaceae* showed positive correlation with the sugar residues, GalA and DE. *F. prausnitzii*, is commonly referred to as a marker for intestinal health, exhibiting anti-inflammatory effects in the gut (Walters *et al.*, 2014).

Abundance of *Prevotella copri* was reported to correlate positively with Ara and DE and negatively with DBr. Whereby, the genera *Coprococcus* and *Lachnospira* correlated positively with DBr and negatively with DE and Ara (Larsen *et al.*, 2019). *P. copri* is associated with induced insulin resistance in mice and rheumatoid arthritis in humans (Pedersen *et al.*, 2016; Pianta *et al.*, 2017); and an increase in the levels of *Coprococcus* has been linked to reduced severity of IBS in humans (Tap *et al.*, 2017). Studies as the study by Larsen *et al.* (2018) indicate that pectins might be indeed valuable substrates to promote the growth of next generation probiotics as well as endogenous beneficial bacteria.

Further studies are needed to better understand the fermentation patterns of pectins with different DEs by the human faecal microbiota, using current methods for whole microbiome analysis. In the future, besides the DE the studies should provide more information on the molecular size of the pectin, as well as the degree of acetylation, where applicable to evaluate the sole effect of the DE. Moreover, information on the esterification pattern (randomly methylated or in blocks of methylated residues) will further elevate the quality and interpretation of the studies performed, the DE pattern (blocky vs random) is likely to impact the bioavailability of sites for enzymatic cleavage and degradation by microbial enzymatic machineries.

The plethora of potential pectic oligosaccharide (POS) preparations extrapolates the molecular diversity of pectin derived substrates. POS are oligosaccharides which can be obtained by partial hydrolysis of pectins, and their structure and function are being increasingly investigated (Gómez *et al.*, 2016; Míguez *et al.*, 2016), their production on a larger scale has not yet been realised efficiently.

Even though pectins essentially contain the same repeating elements, these vary in amounts and chemical

fine structures. In addition, protein and ferulic acid may be found in some pectins, e.g. sugar beet derived pectin contains ferulic acid residues in the arabinan side chains (Oosterveld *et al.*, 1996). The full structural complexity of pectin is not yet fully explored and only points at the potential of pectin as a prebiotic and gut microbial community modulator with potential physiological benefits. To fully explore the potential of pectin derived molecules and their effects on health via the direct or indirect effects on the gastrointestinal tract and its residing gut microbiota, it is necessary to apply and expand state of the art technologies for substrate preparation and analysis.

Some recent advances in the application of -omics technologies and gut microbial *in vitro* fermentation tools facilitate advanced insights into the prebiotic potential of pectic substrates. The structure-function relationship of novel and rare pectic structures available only in small amounts can be now investigated in miniature *in vitro* colon models operating at a low fermentation volume, such as e.g. the CoMiniGut model recently developed by (Wiese *et al.*, 2018). When pectinolytic enzyme preparations are implemented to process fruits and vegetables, e.g. apple, tomato and carrot, RG-II is released as a main soluble polysaccharide fraction, while other pectic polysaccharides are heavily degraded (Doco *et al.*, 1997). RG-II has been found highly concentrated in processed beverages, such as wine (Pellerin *et al.*, 1996). In the past no evidence has been reported on the metabolism of RG-II by a microorganism and it was assumed that it would require microbial consortia to metabolise the complex glycan (Ndeh and Gilbert, 2018). In the past decade, Martens *et al.* (2011) have for the first time shown that two closely related human gut *Bacteroides*, *Bacteroides thetaiotaomicron* and *B. ovatus*, can utilise RG II (Martens *et al.*, 2011). Their studies were recently extended by Ndeh *et al.* (2017) who have revealed that around two thirds of the 30 human gut microbiome (HGM) *Bacteroidetes* grew on the pectic glycan. Ndeh *et al.* (2017) reported the RG-II degradome of *B. thetaiotaomicron* which can utilise RG-II from wine and apple pectin; cleaving all but one of its 21 distinct glycosidic linkages (Ndeh *et al.*, 2017). The revision of the current structural model of RG-II, highlighted how individual gut bacteria orchestrate manifold enzymes to metabolise the most challenging glycans in the human diet. Currently, it appears that RG-II utilisation in the human gut microbiota is restricted to the *Bacteroidetes*. This finding suggests a valuable possibility for the selective manipulation of the human gut microbiota by RG-II (Ndeh and Gilbert, 2018). The potential degradation of RG-I and RG-II by human gut microbiota has been recently reviewed in Ndeh and Gilbert (2018).

Although POS have been suggested as emerging prebiotics, the International Scientific Association for Probiotics and Prebiotics (ISAPP) did not include POS within the list of prebiotic candidates in their last expert consensus about prebiotics. It is hence necessary that further research is conducted to evaluate the effects of POS on the composition and metabolic activity of gut microbiota (Míguez *et al.*, 2020). The development of prebiotics capable of modulating the gut microbiota in the elderly has become an area of great interest due to the particular vulnerability and frailty of this population. The prebiotic effects of POS obtained from lemon peel were studied on the microbiota from elderly donors using an *in vitro* continuous colon model (TIM-2). The study identified increments in beneficial species such as *F. prausnitzii* and larger alpha diversity values were observed with POS in comparison with FOS and in some cases with standard ileal efflux medium (Miguez *et al.*, 2020).

β-glucans

Cereal β-glucans are repeating linear polymers of two β-(1,4) glucosyl units alternated with β-(1,3) glucosyl units. Fungi and yeast contain β-glucans that are branched. All are soluble polysaccharides.

Several studies examined whether β-glucans increased bifidobacteria and lactobacilli. In *in vitro* human faecal fermentations, generally these two bacteria groups were notably increased when β-glucans had been enzymatically reduced to oligosaccharides before treatment (Hughes *et al.*, 2008; Jaskari *et al.*, 1998), showing again that they are well suited to take up and utilise small hydrolysates and not polymers. Hughes *et al.* (2008) examined barley and oat hydrolysed β-glucan fractions differing in molecular mass and showed a promotion of the *Bacteroides-Prevotella* group, but not others. When size was reduced further to DP3-4, the *Lactobacillus-Enterococcus* group was increased, though not as much as in the inulin control. The β-glucan hydrolysates were propiogenic. In a similar way, some lactobacilli strains were promoted with barley β-glucan hydrolyzates DP3-4 (Snart *et al.*, 2006).

Long-term human studies have been done with β-glucan supplementation showing both no effect and positive changes in the gut microbiota community. Mitsou *et al.* (2010) supplemented 52 subjects (mean age 49) with a low level of barley β-glucan (0.75 g/day) for 30 days. In the older subject group (>50 years) there was a significant increase in both bifidobacteria and *Bacteroides*, with no undesirable side effects.

Fructans

Fructans are carbohydrates mainly consisting of fructose units linked or not to one glucose unit. Different forms exist depending on the type of fructose-fructosyl linkages [β(2-1) or β(2-6)] and on their core molecular structure (linear, branched or cyclic). Inulin-type fructans are characterised by β(2-1) linkage, they are mostly linear but can also have a very small degree of branching. They will vary in their DP

(from 2 to 200 in plants) and by the presence or not of a glucose at the terminal position. Polymeric linear fructans are mostly extracted from chicory roots giving rise to inulin (DP2-60). Oligomeric linear fructans obtained by hydrolysis of inulin are typically called oligofructose (or FOS); FOS (or scFOS) are also manufactured by enzymatic synthesis from sucrose with fungal β-fructofuranosidase using sucrose as glycosyl donor and acceptor. Inulin and oligofructose/FOS are established prebiotics (Gibson *et al.*, 2017). Such FOS is mainly composed of three oligosaccharides, 1-kestose (DP3), 1-nystose (DP4) and 1F-β-fructofuranosylnystose (DP5) but can also contain higher oligomers. Laevan-type fructans mostly have the β(2-6) linkage and are rather present in polymeric form in bacteria, their DP can reach up to 100,000. Phlein-type fructans basically have the same structure as laevan but the term is rather used for plant-derived fructans that are also generally shorter polymers. Graminan-fructans are branched fructans that present both β(2-1) and β(2-6) linkages and again a glucose is possible at terminal position but not necessarily present (Roberfroid, 2007; Verspreet *et al.*, 2015).

The inulin-type fructans of different chain length originating from chicory roots or produced from beet sugar have been extensively studied for their effects on the microbiota as well as their associated benefits and are well known prebiotic fibres for many years (Roberfroid *et al.*, 2010). Historically most human studies have focused on the effect of fructans on the gut populations of bifidobacteria and lactobacilli evaluated by cultural methods. Clinical studies with daily dose of fructans between 5 and 20g and with more modern technics (16S rRNA-based for example) to evaluate composition of faecal microbiota have confirmed the effect on bifidobacteria and showed variable responses on *Anaerostipes*, lactobacilli and *Faecalibacterium prausnitizii* for examples (Swanson *et al.*, 2020). The induced modulation of the gut microbiota composition and activity is likely linked to health benefits in the gut and beyond. Several recent systematic reviews and meta-analysis have highlighted that via their specific modulation of the gut microbiota, inulin-type fructans can improve: bowel function by increasing the number of bowel movements (De Vries *et al.*, 2019); glycaemic control as evaluated by fasting blood glucose or insulin, glycolysated hemoglobin (HbA1c) and homeostasis model assessment of insulin resistance (HOMA-IR) and more particularly in prediabetes and type 2 diabetes (Wang *et al.*, 2019) associated with obesity (Rao *et al.*, 2019); regulation of blood lipids, for instance low-density lipoprotein cholesterol in all type of populations but also high-density lipoprotein in type 2 diabetes (Liu *et al.*, 2016); immune function and again especially in those who are particularly susceptible to modifications of their immune response (Lomax and Calder, 2008). There is also increasing interest in supplementing diet early in life to support establishment of a well diverse and resilient gut microbiota when breastfeeding is not possible (Firmansyah *et al.*, 2016).

So far, most studies evaluating structure function relationships are *in vitro* fermentation studies using human faecal slurry or pure bacterial strains. Some studies compared the effects of different DP of inulin-type fructans on the modulation of intestinal microbial composition and activities (Van de Wiele *et al.*, 2007). The presence or not of a glucose molecule at terminal position has also been investigated (Boger *et al.*, 2018; Ryan *et al.*, 2005; Saulnier *et al.*, 2007). Much less information is available on laevan-type fructans or branched fructans but some *in vitro* fermentation studies have been conducted on fructans from the following origins: agave, DP3-16 (Allsopp *et al.*, 2013; Mueller *et al.*, 2016; Velázquez-Martínez *et al.*, 2014); stevia, DP3-8 (Sanches Lopes *et al.*, 2016); dahlia; wheat and barley grain, DP <20 (Jenkins *et al.*, 2011). Most of these studies compared different sizes of chain-length on the stimulation of bacterial growth and some studies have even compared branched to linear fructans of comparable degree of polymerisation (Jenkins *et al.*, 2011; Mueller *et al.*, 2016).

Historically, most studies on fructans were conducted with a focus on bifidobacteria and lactobacilli groups. Bifidobacteria appear to hydrolyse different varieties of fructans: linear β(2-1)- but also β(2-6)-based fructans (Marx *et al.*, 2000) and branched fructans (Allsopp *et al.*, 2013; Velázquez-Martínez *et al.*, 2014) but are rather sensitive to the degree of polymerisation. They will generally ferment more easily molecules with lowest degree of polymerisation (<DP5) and the lowest they preferred (Mueller *et al.*, 2016; Scott *et al.*, 2014; Valdés-Varela *et al.*, 2017; Velázquez-Martínez *et al.*, 2014) but some individuals are also able to use more complex structures (Rivière *et al.*, 2018). Lactobacilli are also well equipped to metabolise oligosaccharides and the identification of two or more metabolic pathways to degrade FOS indicate that they are among preferred substrates (Gänzle and Follador, 2012). Dependency of growth promotion is highly strain specific. Like bifidobacteria, some strains of lactobacilli will use only low DP fructans (DP3-DP5), for instance *Ligilactobacillus salivarius* W57 specifically uses GF2 (DP3) and *L. acidophilus* W37 only uses short-chain compounds of FF and GF types. Some other strains will grow on all DP ranges with a faster utilisation of low DP and some other will grow indifferently on all DP ranges (De Vuyst *et al.*, 2014; Gänzle and Follador, 2012). Strains like *Lactocaseibacillus paracasei* subsp. *paracasei* W20 can use a large range of GF types (at least DP2-40) as well as FF types (at least DP2-14) thanks to the excretion of an exoenzyme from the family of GH32 β-fructosidase (Boger *et al.*, 2018).

The effect of DP is seen for both linear- (e.g inulin) and branched-type (e.g. agave) fructans but in fructans with

comparable DP, especially above 5, branched-fructans seem to be used faster than inulin. It may be that a mixed-type structure that leads to a more compact conformation and better solubility, thus greatest accessibility of substrate to the bacteria (Mueller *et al.*, 2016). High branching will also lead to a high number of non-reducing terminal fructosyl residues, substrates for β-fructosidases.

More recently the capacity to use fructans has been investigated in other dominant gut bacterial groups. *In vitro* studies with human faecal slurry demonstrate higher fibre (e.g. fructans) utilising capacity in *Prevotella*-dominant enterotype than in *Bacteroidetes*-dominant enterotype (Chen *et al.*, 2017b). *Bifidobacterium*, *Anaerostipes* and *Prevotella* seem to be the three most enriched bacterial genera when human faecal slurry is used for *in vitro* fermentation in presence of FOS or inulin. Two microbial groups, *Clostridium* cluster *XVIII* and *Streptococcus* were also representative of FOS fermentation whereas *Ruminococcus* was representative of inulin fermentation but in general the effect on the composition of the microbiota was similar between FOS and inulin (Fehlbaum *et al.*, 2018). Fructans can also be fermented by bacteria producing butyrate: *E. rectale, Ruminococcus inulinovorans, F. prausnitzii, Eubacterium hallii* (Scott *et al.*, 2014). While all of them were able to grow on linear β(2-1)-fructans of different chain length with similar maximum specific growth rate, *F. prausnitzii, E. hallii* were not able to grow on dahlia long chain inulin (DP around 25). It is quite clear that chain length of fructans may differently modulate the growth of some bacteria, but it may be rather strain- than group-dependent, for bifidobacteria and lactobacilli at least (Boger *et al.*, 2018; Selak *et al.*, 2016). *In vivo*, more complex microbial interactions will take place to indirectly stimulate or inhibit the growth of bacteria and modify the composition of the gut microbiota. For example, some cross-feeding phenomenon can also happen and interfere with the direct effect of the prebiotic fibre (Belenguer *et al.*, 2006). Bacteria secreting exoenzyme, such as *L. paracasei* subsp. *paracasei* W20 as cited above appear as keystone strains to stimulate cross-feeding as it induces temporary accumulation of short-chain compounds available for other bacteria (Boger *et al.*, 2018). Also other bacterial groups, such as *Anaerostipes,* can therefore be indirectly stimulated by dietary supplementation with fructans by consuming the monosaccharides or SCFA (i.e. acetate) produced by their fermentation (Vandeputte *et al.*, 2017). This cross-feeding may explain why in human studies we do not always observe the same effects of inulin-type fructans on gut microbiota (Swanson *et al.*, 2020) because there is a strong influence of its initial composition itself dependent on usual dietary fibres intake among other factors (Healey *et al.*, 2018). Inhibition of potential pathogens with prebiotics is generally mediated by selective stimulation of bacterial strains that will develop at the expense of organisms that are not able to use them. In this case also, chain length of β-fructans also seems to influence ability of bifidobacteria (e.g. *B. longum* PLA20022 or *B. breve* IPLA20006) to reduce toxicity of *Clostridioides difficile* when co-cultured. Short-chain β-fructans and mixtures of short and long chains are more efficient than longer chain ones alone (Valdés-Varela *et al.*, 2017). In another *in vitro* study, effective growth inhibition of both *Escherichia coli* O157 or O86E strains was induced when *B. longum* was grown on two types of short-chains fructans (DP2-5 and DP2-9) but *Limosilactobacillus fermentum* could only inhibit growth of the two pathogens when grown on the shortest chains. This result was correlated with the different abilities of the tested fructans to stimulate the growth of the probiotic strains, the higher the maximum growth rate, the better inhibition of the pathogens (Likotrafiti *et al.*, 2013).

Higher SCFA production is a marker of higher fibre utilisation capacity by gut microbiota. Fructans generally induce higher production of acetate and butyrate and less propionate in comparison to other types of fibres based on *in vitro* studies (Chen *et al.*, 2017b). In humans, direct comparison with other fibres is scarce but it is confirmed that inulin would favour acetate and butyrate production over propionate production (Boets *et al.*, 2015). When they induce similar growth rate of one bacterial strain (e.g. of bifidobacteria group), inulin-type fructans of different chain length induce also similar decrease of pH in culture-media, suggesting similar level of bacterial fermentative activity (Valdés-Varela *et al.*, 2017). This seems also valid when a complete human gut microbiota is used as inoculum of fermentation, total production and relative proportion of SCFA are similar between FOS and inulin from chicory used at the same level (Fehlbaum *et al.*, 2018). Other types of fructans (e.g. branched fructans from agave) also induce production of SCFA when they are fermented (Allsopp *et al.*, 2013). Also, after 24 h of *in vitro* fermentation, production of SCFA via fermentation is similar among inulin-type fructans and cereal fructans from barley grain and wheat stem; longer molecules (max DP12) induced greater SCFA concentrations than shorter ones (average DP3-6), but no intermediate measure is available to illustrate more precisely the kinetic (Jenkins *et al.*, 2011). Again *in vivo,* more interactions will influence the overall fermentation process and metabolites production. Metabolic cross-feeding of bacteria will influence the quantity and relative proportions of the different SCFA (Belenguer *et al.*, 2006) and can also alter physiological parameters as for instance intestinal transit time (Rivière *et al.*, 2018).

In terms of structure-function relationships the degree of polymerisation, then type of structure and presence of glucose at terminal end of fructans can influence the composition and activities of the gut microbiota but the difference is seen at level of strains rather than bacterial groups, as illustrated by studies evaluating bifidobacteria and lactobacilli groups. *In vivo* the interactions among

bacterial groups need to be considered as they will modify the metabolic output from fermentation. However, this potential different effects on the gut microbiota may also be reflected at physiological level. In a systematic review and meta-analysis on fructans and bowel function, while overall dietary supplementation with inulin-type fructans increase the number of bowel movements, the effect seems greater with fructan derived oligosaccharides than with longer chain (De Vries *et al.*, 2019). On the contrary another systematic review and meta-analysis on benefits of inulin-type fructans and glycaemic control, effects appear more significant with inulin than with other fructans derived ingredients (i.e. oligosaccharides) (Wang *et al.*, 2019).

Resistant starch

Bacteria in the human lower intestine may be exposed to as much as 20 g of resistant starch per day (Topping and Clifton, 2001). The fermentation products of resistant starch by gut bacteria include gases (methane, hydrogen, carbon dioxide) and SCFAs (acetate, propionate, butyrate, and valerate). Lesser amounts of other organic acids (lactate, succinate, and formate), branched SCFAs (isobutyrate and isovalerate), and alcohols (methanol and ethanol) are also produced. Starch degradation is a cooperative process in the lower gut, generalised as (1) degradation of starch polymers into glucose; (2) glycolysis with SCFA or other organic acids as end products; and (3) methane production by methanogenic *Archeae* spp. from formate, hydrogen gas, and carbon dioxide products of bacterial metabolism of resistant starch. The common fermentation outcomes of resistant starch can be summarised as increased faecal bulking, decreased faecal pH, increased faecal SCFA production and decreased faecal secondary bile acids levels (Hylla *et al.*, 1998; Jenkins *et al.*, 1998; Phillips *et al.*, 1995).

Human studies have not revealed any clear structure-function relationships with respect to type of resistant starch and proportions of individual SCFA. *In vitro* studies, however, seem to indicate that high proportions of butyrate are a characteristic of resistant starch fermentation (MacFarlane and MacFarlane, 1993).

Martínez *et al.* (2010a) used pyrosequencing to characterise the impact of resistant starches on the composition of faecal microbiota in humans. Study participants consumed either RS2 (granular form of high-amylose corn starch) or RS4 (chemically modified by phosphate cross-linking) and were compared with control digestible starch in the form of crackers. Both forms of resistant starch increased representatives of the *Actinobacteria* and *Bacteroidetes* phyla and decreased *Firmicutes*. The two forms of resistant starch differed in their ability to change species. RS2 increased the abundance of *Ruminococcus bromii* and *E. rectale*, which was consistent with prior results from *in vitro* studies on starch fermentation in the large intestine (Kovatcheva-Datchary *et al.*, 2009a; Leitch *et al.*, 2007). In contrast, RS4 was associated with increased *B. adolescentis* and *P. distasonis* (Martínez *et al.*, 2010b). Why the 2 different resistant starches change the composition of the microbiota remains unclear, because multiple genera are capable to degrade starches (Bird and Topping, 2008; Louis *et al.*, 2007; Vital *et al.*, 2018). It was suggested that the differential ability for individual bacterial species to degrade starches may represent differences in substrate binding (Martínez *et al.*, 2010b). The degradation of RS2 is largely linked to *Firmicutes*, with *R. bromii* initiating a cascade of reactions leading to microbial alterations with increased levels of fermentation end products and especially butyrate (Vital *et al.*, 2018).

3. Effects of carbohydrate fermentation in the gut

Effect of fermented carbohydrates on the immune system

Fermentable carbohydrates clearly have an impact on bacteria that utilise them and either, due to the higher number of such bacteria and an according change in microbial composition, stimulate the immune system differently and/or, by the fermentation of the carbohydrate, produce short chain fatty acids (SCFA) and other metabolites that *per se* may hold immunomodulatory effects (Hamer *et al.*, 2008). Fibres that support the growth and metabolism of specific bacteria exerting beneficial effects on the host health are called prebiotics as indicated in the introduction section. Many of the endpoints associated with beneficial effects of prebiotics are associated with diseases such as inflammatory bowel diseases, allergies, type II diabetes and cardiovascular diseases (Ouwehand *et al.*, 2005), which are all recognised to be influenced by imbalances in the immune system, e.g. a chronic inflammatory state sometimes combined with a hyperreactive local or systemic immune system. Common for these diseases is that a dysbiotic gut microbiota is believed to play a major role in the development and, in some cases, in the 'maintenance' of the disease.

The full nature of prebiotic effects can only be addressed in *in vivo* models even though one may gain some information about the potential prebiotic properties of a fibre by *in vitro* growth of specific bacteria or the production of SCFA by bacteria growing on a substrate containing the fibre as the only carbohydrate source. Whether an increase in specific bacteria in the GIT will give rise to specific beneficial effects on the immune system may depend on the composition of the microbiota, the location in the gut, and specific immunological and physiological conditions in the gut.

Effects of prebiotics by selectively increasing the populations of certain bacterial groups within the microbiota

Prebiotics can have effects on different bodily locations: even though the definition originally states that the effect of the prebiotics should affect bacterial growth and metabolism in the colon, increasing awareness that also the growth and metabolisms of microorganisms present in the small intestine may be affected by some prebiotics emerges. Even though the concentration of microorganisms here is several orders of magnitude lower than in the colon, and the flow through this part of the gastrointestinal tract is much faster than in the colon, thereby limiting the time available for the fermentation process, the upper part of the GIT may be of major importance regarding the interaction of the bacteria with the immune system. Alternatively, the dietary carbohydrates themselves may interact with the epithelial and immune cells (see Section 3). Throughout the small intestine the mucosa is scattered with isolated lymphoid follicles that together with Peyer's Patches (PP) serve as gate keepers, allowing a limited number of microorganisms and macromolecules to get access to the immune cells underlying the mucosal epithelium, interacting with various pattern recognition receptors, such as Toll like receptors (TLRs), C-type lectin receptors, etc. (Brandtzaeg, 2013). This is an important mechanism for the immune system to establish a protective barrier which prevents invasion of pathogens. Despite the fact that direct interactions of prebiotics with the epithelial layer have been shown *in vitro* (Vogt *et al.*, 2014), it is highly likely that through modifying the microbiota composition in the upper GIT, a community which is much more prone to compositional changes, prebiotics have an indirect effect on the immune systems as well, since these microbes interact with the cells of the underlying GALT.

Effects on gut infections

The microbial composition in the GIT may affect the virulence of pathogenic bacteria by providing conditions that either support or inhibit growth and secondary metabolite/toxin production of the pathogen or by affecting the physical environment of the microbiota, e.g. changes in the production of mucus by the host. Hence, addition of a new source of nutrient, the prebiotic, that can only be exploited by some bacteria, creates changes in the microbial composition which in turn may affect how the microbiota affects the virulence of pathogens in the gut. *Salmonella* in mice fed various prebiotics showed that certain of the prebiotics (FOS and XOS) led to an increased translocation of salmonella in the gut (Petersen *et al.*, 2009). A similar study showed increase *Salmonella* translocation with FOS and lactulose in rats (Bovee-Oudenhoven, 2003; Ten Bruggencate *et al.*, 2003). A more recent study showed improved effects of fructo-oligosaccharides on piglet health against *Salmonella* infection (Lepine *et al.*, 2019). In a guinea pig model for *Listeria* infection, other prebiotics had a similar effect (Ebersbach *et al.*, 2010). This effect is perhaps associated with the ability of the pathogen to grow on the prebiotic, or, more likely, due to a sudden increase in SCFA production (due to the swift fermentation of the prebiotic), which induced irritation of the gut epithelium (Bovee-Oudenhoven, 2003; Ten Bruggencate *et al.*, 2003). Other changes include production and secretion of immunoglobulin A (IgA), production of antimicrobial peptides, and increased production of mucus (Liévin-Le Moal and Servin, 2006). Such properties of the epithelial layer represent key effector parts of the mucosal immune system, which may be modulated by the microbiota (Brandtzaeg, 2013). Anti-adhesive effects to epithelial cells and functioning as decoy-receptors by fibres is discussed in Section 5 on immune effects of prebiotics through increased production of microbial metabolites.

Effects on production of antimicrobial peptides from enterocytes, neutrophils and Paneth cells

Antimicrobial peptides comprise a diverse group of low molecular proteins which by interaction with the bacterial membrane kill Gram-positive or Gram-negative bacteria. Production of some antimicrobial peptides takes place in the enterocytes, but most of the secreted antimicrobial peptides originates from the Paneth cells, granule-rich epithelial cells located at the base of small intestinal crypts (Bevins and Salzman, 2011). The Paneth cells are mainly confined to the small intestine in healthy humans, reflecting the importance of protecting this large area against microbial invasion. Some bacteria have been demonstrated to induce enhanced production of antimicrobial peptides from enterocytes *in vitro*. Bacteria shown to enhance production of antimicrobial peptides include bifidobacteria, lactobacilli and the probiotic *E. coli* Nissle 1917 strain, but not several other (commensal) *E. coli* strains (Schlee *et al.*, 2008; Wehkamp *et al.*, 2004). Notably, Paneth cells do not alter the secretion of antimicrobial peptides due to direct microbial stimulation. Rather, their secretion is affected by cytokines, in particular interleukin (IL)-22, the induction of which is determined by the gut microbiota (Hooper and MacPherson, 2010).

Hence, as the microbiota, through its presence as well as the number of cells and the specific microbiota composition in the crypt of the epithelium of small intestine, may play a key role in the secretion of antimicrobial peptides, carbohydrate fibres that stimulate the growth of specific bacteria may represent an important means to maintain/establish a strong barrier towards invasive pathogens.

Microorganisms in the gastrointestinal tract may induce increased secretion of mucus

Mucus consists of several heavily glycosylated proteins called mucins of which mucin 1, 3, and 4 are membrane proteins present on the apical surface of the enterocytes. Mucin 2 (MUC2) is secreted from the goblet cells located in the villi, and by interaction with the other mucins, MUC2 forms a thick viscous gel/layer on top of the epithelial cells that separates microorganisms from the mucosa and accordingly protects against invasion of pathogens through the epithelial surface (Bergstrom *et al.*, 2010). Goblet cells are secretory cells derived from the stem cells located in the crypt bottom. They contain granula with densely packed MUC2 polymers that are released into the lumen upon stimulation. The importance of MUC2 in protection against pathogenic invasion can be demonstrated by the MUC2 knock out mouse or mice defective in the inflammasome proteins NLRP6 (NOD-like receptor family pyrin domain containing 6), ASC (apoptosis-associated speck-like protein containing a C-terminal caspase recruitment domain) or caspase-1. Such mice have a dysfunctional gut microbiota and are prone to intestinal infection by pathogens, such as *Prevotellaceae* and TM7 bacteria (e.g. *Citrobacter rodentium* (Gill *et al.*, 2011; Van der Sluis *et al.*, 2006, 2008; Wlodarska *et al.*, 2011)). Goblet cells of germ-free mice are fewer in number and smaller in size than in conventionally raised mice. This signifies a role of the microbiota in the development and maintenance of goblet cells and MUC2 production. For several microorganisms it has been shown that they increase the secretion of MUC2 and or MUC3, e.g. *Clostridium tyrobutyricum* DSM 2637 (Hudcovic *et al.*, 2012), *L. plantarum* 299v (Dykstra *et al.*, 2011; Mack *et al.*, 1999), *Lacticaseibacillus rhamnosus* R0011, and *B. bifidum* R0071 (Dykstra *et al.*, 2011). Some of these strains are used as probiotics. Selective stimulation of these probiotic strains by prebiotics would likely enhance their functionality.

Effects on the specificity and amount of IgA secreted

Dimerized IgA molecules with specificity towards the microorganisms present in the gastrointestinal tract are secreted from the mucus into the lumen of the gut (Van der Waaij *et al.*, 1996). Here the IgA binds to the bacteria and thereby facilitates their exclusion from the epithelial surface and accordingly, their translocation and access into the circulation. The amount of IgA secreted into the gut lumen strongly depends on the presence and diversity of the microbiota in the gut. Germ free mice and new-born babies are almost devoid of IgA in the faeces and the IgA concentration increases along with the postnatal colonisation of the gut. Recently, several prebiotics/fibres have been shown to increase IgA, such as XOS (increased in saliva, but very likely then also in the gut, although this was not tested (Childs *et al.*, 2014)), galactooligosaccharides (Vulevic *et al.*, 2013), a galactooligosaccharide/inulin mixture (Gourbeyre *et al.*, 2013), FOS from yacon flour (Velez *et al.*, 2012) and β-1,4-mannobiose (Kovacs-Nolan *et al.*, 2013). The GOS/inulin mix also increased IgA in mother's milk of mice (Gourbeyre *et al.*, 2012), protecting the suckling offspring against pathogens. However, there were also several other prebiotics that showed no effect on faecal IgA (Lecerf *et al.*, 2012; Lomax *et al.*, 2012; Scalabrin *et al.*, 2012), despite showing effects on microbiota composition.

Effect on homeostasis in the gut – anti-inflammatory effects

The gut microbiota plays an important role for homeostasis of the gut mucosal immune system and establishment of tolerance towards the commensal bacteria and food proteins. This has become evident from studies with germ free mice where the number of leucocytes in the lamina propria and epithelium is low as compared to conventionally reared mice and in which tolerance towards food proteins cannot become established. The mechanisms involved in homeostasis and tolerance establishment are complex and only beginning to become understood. However, it may be noted that dendritic cells and macrophages present in Peyer's patches and lamina propria acquires specific phenotypes that stimulate the development of regulatory T-cells and IgA producing B-cells that is dependent on the presence of the microbiota (Rescigno, 2014). 'Inhibit' type macrophages (called M1) can rapidly kill pathogens, and are thus the primary host defence, while 'Heal' type macrophages (M2) routinely repair and maintain tissue integrity (Mills and Ley, 2014). Intestinal macrophages undergo activation in response to environmental signals, including microbial products, and develop a M1 or M2 phenotype depending on the activation signals. It has been shown that activation of dectin-1 induces M1 macrophages (Loures *et al.*, 2014).

Fibres that can be exploited as nutrients by a subgroup of the gut microbiota, may increase this group in proportion, which in turn may affect both the mucosal defence mechanisms outlined above as well as the maintenance of homeostasis in the gut. Depending on the specific structure of the fibre, different bacteria may thrive on it. Hence the mucosal barrier may be strengthened or weakened according to such changes. For instance, a multistrain probiotic product induced a mixed proinflammatory and anti-inflammatory phenotype in polarised and unpolarised macrophages (Isidro, 2014), depending on the initial phenotype of the macrophages. At present it is unclear which probiotic component(s) in the mixture is/are responsible for this activity, but if this/these strain(s) could be selectively induced or repressed by feeding prebiotics, the polarisation of macrophages could perhaps be steered. That this is feasible is exemplified by the fact that acidic oligosaccharides derived from pectin stimulated M1 macrophage activation through modulation of the microbiota (Bernard *et al.*, 2014).

Immune effects of prebiotics through increased production of microbial metabolites

Apart from the direct action/interaction of the microorganisms with the mucosa, which may influence the innate barrier functions as well as the mucosal homeostasis, microorganisms that grow on complex carbohydrate structures are recognised to produce various low-weight molecular compounds, such as short chain fatty acids (SCFA; mainly acetate, propionate, and butyrate). It is very difficult to indicate which SCFA are produced from which carbohydrates and thus determine a structure-function relationship, because all members of the gut microbiota produced at least one of the SCFA, and there is enormous functional redundancy in this microbial activity. Particular classes of carbohydrates have been linked to increased ratios of certain SCFA, e.g. resistant starch is known to increase butyrate particularly (DeMartino and Cockburn, 2020). However, despite a clear structure-function relationship, due the importance of SCFA in physiology, the topic is covered below.

These microbially produced SCFA may affect the microbial composition simply by changing the pH in the gut. But perhaps most importantly, they may influence signalling in the host. Two major SCFA signalling mechanisms have been identified, inhibition of histone deacetylases (HDACs) and activation of G-protein-coupled receptors (GPCRs) (Tan *et al.*, 2014).

The SCFA produced by the gut microbiota act in the intestine as well as at other sites in the body. SCFA bind to two G-protein coupled receptors, the GPR41 (a.k.a. FFAR3) and GPR43 (FFAR2), with different affinities for the individual SCFA for both receptors. Binding of the ligand induces a signal transduction in the cell which, depending on the cell type, induces various responses. The GPR41 is primarily expressed in adipose cells, but also in the intestine, pancreas, lung and spleen (Brown *et al.*, 2003). The GPR43 is highly expressed in bone marrow and in white adipose tissue (Cox *et al.*, 2009; Kimura *et al.*, 2013). The high GPR43 expression in bone marrow reflects the expression in granulocytes and monocytes (Cox *et al.*, 2009). The preferred energy substrate for the gut epithelial cells is butyrate, and little butyrate escapes to the circulation (Hamer *et al.*, 2008). Instead, acetate and propionate are to a large extent absorbed into the circulation and may affect systemic cells expressing the SCFA receptors, such as adipose tissue and blood neutrophils. The overlap between in receptor affinity for the SCFA and the lack of synthetic small molecule ligands that display selectivity between these two receptors has, until recently, hindered efforts to resolve their individual functions. However, the importance of the two receptors and SCFA has been demonstrated using knock-out mice. It has been shown that SCFA through GPR1-signaling are involved in the secretion of satiety hormones, glucagon-like peptide 1 (GLP-1) and peptide tyrosine tyrosine (PYY). Both GPR41 and GPR43 are involved in energy metabolism and homeostasis. The SCFA have been shown to have an anti-inflammatory effect through ligation with GPR41, GPR43 or both (Maslowski *et al.*, 2009; Masui *et al.*, 2013; Voltolini *et al.*, 2012), in addition to induction of regulatory T cells (T-regs) in a GPR43-dependent manner in mice (Smith *et al.*, 2013).

Another mechanism through which SCFA, and in particular butyrate, modulate gene expression is through inhibition of HDACs (Licciardi *et al.*, 2011; Steliou *et al.*, 2012; Vinolo *et al.*, 2011). Regulation of HDAC activity may be critical for establishing immune-suppressive responses through T-reg cell activation, but also for controlling intestinal epithelial cells homeostatic responses to the luminal environment (Asselin and Gendron, 2014).

In deciphering the mechanism of action of SCFA, individual SCFA or combinations thereof have been shown to be beneficial when given as enemas in inflammatory bowel disease (IBD), although not in all studies. Sometimes only butyrate was tested, sometimes mixtures of SCFA (Hamer *et al.*, 2008). The inconsistent results could be explained since the use of enemas is often hampered by a low compliance rate and a short and discontinuous exposure of the colon mucosa to the SCFA. Alternatively, the inflammatory state of the volunteer might have clouded the effect of the SCFA. It has been shown for instance that butyrate enemas have a beneficial effect on pain perception during colonic extension in healthy individuals, but not in irritable bowel syndrome individuals, that have a low-grade inflammation (Vanhoutvin *et al.*, 2009). Similarly, effects of butyrate enemas on inflammation and antioxidant status in the colonic mucosa of patients with ulcerative colitis in remission depended on the level of inflammation (Hamer *et al.*, 2010).

In should be mentioned that conversion of prebiotics into SCFA can also have adverse effects, especially in the case of prebiotics that are fermented fast (such as lactulose, short-chain FOS, or lactose in lactose-maldigesters) or are given at high doses. Fast fermentation leads to (transient) accumulation of SCFA and lactate in the proximal colon. This has been shown in rats to lead to increased translocation of *Salmonella* (Bovee-Oudenhoven, 2003). The supposed mechanism pertains to epithelial stress or perhaps even damage due to the high concentrations of lactate and SCFA (accompanied with a low pH), allowing the pathogen to translocate. A similar mechanism is thought to be involved in lactose-intolerance where the colonic microbiota plays a role (He *et al.*, 2008). The imbalance between the removal and production rate of osmotic-active components (lactose, SCFA and intermediate metabolites, e.g. lactate, succinate, etc.) (e.g. due to reduced uptake by the epithelium) is thought to cause diarrhoea in these individuals. Not only lactose causes diarrhoea at high doses,

this is also true for lactulose and FOS. Strikingly, for all these three carbohydrates it has been shown that a gradual increase in intake over the course of several weeks may adapt the microbiota and/or the colonic epithelium to cope with the higher doses of substrate and and/or metabolites (He *et al.*, 2008).

Too rapid production of SCFA may also negatively impact other processes in the body, although currently it is unclear what exactly too rapid is. Of note is that animal studies have shown that a high concentration of propionate in the brain leads to repetitive behaviour similar as that observed in autism (MacFabe *et al.*, 2011). Since the liver is generally very well capable of clearing the SCFA from the portal vein (Bloemen *et al.*, 2009, 2010), other metabolic disorders, e.g. linked to mitochondrial metabolism may be affected as well in autistic spectrum disorders (MacFabe *et al.*, 2011). Although the animal model with a single injection of propionate in the brain is not reflective of the *in vivo* situation, in humans there is continuous production of SCFA, including propionate, and hence the brain may be chronically exposed to this metabolite. When production is rapid, this exposure may even be enhanced.

4. Potential for manufacture of second-generation prebiotics

The main categories of oligosaccharides presently available or in development as functional food ingredients include carbohydrates in which the monosaccharide units are mainly fructose, galactose and/or glucose. However, other less common monosaccharide units such as xylose, galacturonic acid, rhamnose, fucose or *N*-acetyl-glucosamine are also used for the production of some particular oligosaccharides (Table 1).

Although various types of functional oligosaccharides can be found as natural components in many common foodstuffs (i.e. fructo- and xylo-oligosaccharides in fruits and vegetables, kojibiose, isomaltulose and erlose in honey, soybean oligosaccharides, etc.), they are usually present in low amounts. On the other hand, it often seems to be the case that although polysaccharides are fermented by the colonic microbiota, the selectivity for health-promoting bacterial groups is increased by partial hydrolysis. For this reason, plant polysaccharides such as inulin, starch, and xylan can be considered as sources of prebiotics (Rastall, 2010). Therefore, different production methods for these and other non-naturally occurring oligosaccharides have been developed. Strategies include:

1. extraction from natural sources;

Table 1. Main bioactive food-grade oligosaccharides commercially available.

Name (abbreviation)	Molecular structure[1]	Main linkages
Lactulose	Gal-β(1,4)-Fru	
Isomaltulose / palatinose	Glc-α(1,6)-Fru	
Lactosucrose	Gal-β(1,4)-Glc-α(1,2)β-Fru	
Glycosylsucrose / erlose	Glc-α(1,4)-Glc-α(1,2)β-Fru	
Soybean-oligosaccharides (SOS)	$(Gal\text{-}\alpha(1,6))_n$-Glc-α(1,2)β-Fru	
Oligofructose (OFr)	$(Fru)_n$	β(2,1)
Fructo-oligosaccharides (FOS)	$(Fru)_n$-Glc	β(2,1); β(2,6)
Galacto-oligosaccharides (GOS)	$(Gal)_n$-Glc	β(1,3), β(1,4); β(1,6)
Gentio-oligosaccharides (GEOS)	$(Glc)_n$	β(1,6)
Koji-oligosaccharides[2] (KOS)	$(Glc)_n$	α(1,2)
Nigero-oligosaccharides[2] (NOS)	$(Glc)_n$	α(1,3)
Malto-oligosaccharides (MOS)	$(Glc)_n$	α(1,4)
Isomalto-oligosaccharides (IMOS)	$(Glc)_n$	α(1,6)
Xylo-oligosaccharides (XOS)	$(Xyl)_n$	β(1,4)
Pectic-oligosaccharides (POS)	$(GalA)_n$ and/or $(GalA\text{-}Rha)_n$ [3]	α(1,4); α(1,2)
2'-Fucosyllactose (2'-FL)	Fuc-α(1,2)-Gal-Glc	
Lacto-*N*-neotetraose (LNnT)	Gal-β(1,4)-GlcNAc-β(1,3)-Gal-β(1,4)-Glc	

[1] Gal = galactose; Fru = fructose; Glc = glucose; Xyl = xylose; GalA = galacturonic acid; Rha = rhamnose; Fuc = fucose; GlcNAc = N-acetyl-glucosamine.
[2] Found in commercial IMOS syrups (Goffin *et al.*, 2011).
[3] GalA residues can be partially esterified and Rha units ramified.

2. hydrolysis using physical, chemical or enzymatic methods (controlled depolymerisation of polysaccharides, which in turn are often obtained by extraction);
3. synthesis using chemical or enzymatic engineering.

Examples of functional oligosaccharides produced by these strategies are shown in Table 2 and their production, grouped by raw material and manufacturing approach is schematically represented in Figure 9.

Enzymatic synthesis of prebiotics

In most cases, the lack of efficient processes for the production of biologically active oligosaccharides is the main drawback limiting their applications. In consequence, the development of oligosaccharide engineering strategies represents a challenge. This is most easily accomplished by the use of enzymatic procedures. The high substrate specificity together with the stereo- and regio-selectivity of enzyme catalysis is a key advantage. Polysaccharides can be depolymerised to variable extents in a more controlled way, oriented to a specific product, compared to acid-catalysed degradation (Barreteau *et al.*, 2005). Moreover, a wide variety of oligosaccharides have been obtained by enzymatic synthesis. Oligosaccharides are normally built up from simple sugars (mono- and/or disaccharides) that participate in glycosyl transfer reactions catalysed by specific enzymes such as glycosidases or glycosyltransferases (Díez-Municio *et al.*, 2014). In addition, oligosaccharides can be enzymatically rearranged as is the case in isomaltulose production (Table 2 and Figure 9). For their application in foodstuffs, both the initial substrates and enzymes must have a 'safe' status, reasonable cost and be easily available.

Enzymatic synthesis can achieve high product yields from a wide range of abundant raw materials, using food-grade, commercial, cheap enzymes operating under mild reaction conditions. This makes their industrial production economically viable. Buffered solutions of lactose, sucrose and maltose, as well as industrial by-products containing these disaccharides, are suitable and cost-effective starting materials for the synthesis of oligosaccharides (Crittenden and Playne, 1996) (Figure 9). These enzymatic processes usually produce a range of oligosaccharides differing in

Table 2. Examples of functional food-grade oligosaccharides produced by different strategies.

Extraction from natural sources

- Raffinose: extracted from sugar beet.
- Soybean oligosaccharides, SOS (raffinose, stachyose and verbascose): extracted from soybean.

Hydrolysis (controlled polysaccharide depolymerisation using physical, chemical or enzymatic methods)

- Oligofructose, OFr: hydrolysis of inulin, often extracted from chicory roots.
- Malto-oligosaccharides, MOS: hydrolysis of starch, often extracted from edible tubers.
- Xylo-oligosaccharides, XOS: hydrolysis of xylan, often extracted from corn cobs.
- Pectic-oligosaccharides, POS: hydrolysis of pectin, often extracted from sugar beet pulp, citrus or apple.

Chemical synthesis

- Lactulose: isomerization of lactose in alkaline media, catalysed mainly by borates or aluminates.
- 2'-fucosyllactose, 2'-FL: synthesis of the intermediate benzyl-2'-FL from fucose and lactose and subsequent hydrogenation step.
- Lacto-*N*-neotetraose (LNnT): synthesis of the intermediate benzyl-LNnT from *N*-acetyl-lactosamine and lactose and subsequent hydrogenation step.

Enzymatic synthesis

- Galacto-oligosaccharides, GOS: transgalactosylation from lactose by β-galactosidases (EC 3.2.1.23).
- Fructo-oligosaccharides, FOS: transfructosylation of sucrose by β-fructofuranosidases (EC 3.2.1.26), inulosucrases (EC 2.4.1.9) or levansucrases (EC 2.4.1.10).
- Isomalto-oligosaccharides, IMOS: transfructosylation of maltose by α-glucosidase (EC 3.2.1.20); maltose is often obtained by degrading starch with α-amylase (EC 3.2.1.1).
- Lactosucrose: transfructosylation of lactose by β-fructofuranosidases (EC 3.2.1.26) or levansucrases (EC 2.4.1.10), or by transgalactosylation of sucrose by β-galactosidases (EC 3.2.1.23).
- Glycosylsucrose (erlose): transglucosylation of sucrose by cyclomaltodextrin glucanotransferase (EC 2.4.1.19) or by transfructosylation of maltose by levansucrases (EC 2.4.1.10).
- Palatinose (isomaltulose): rearrangement of the glycosidic linkage from sucrose: α1,2- to α1,6-fructoside.

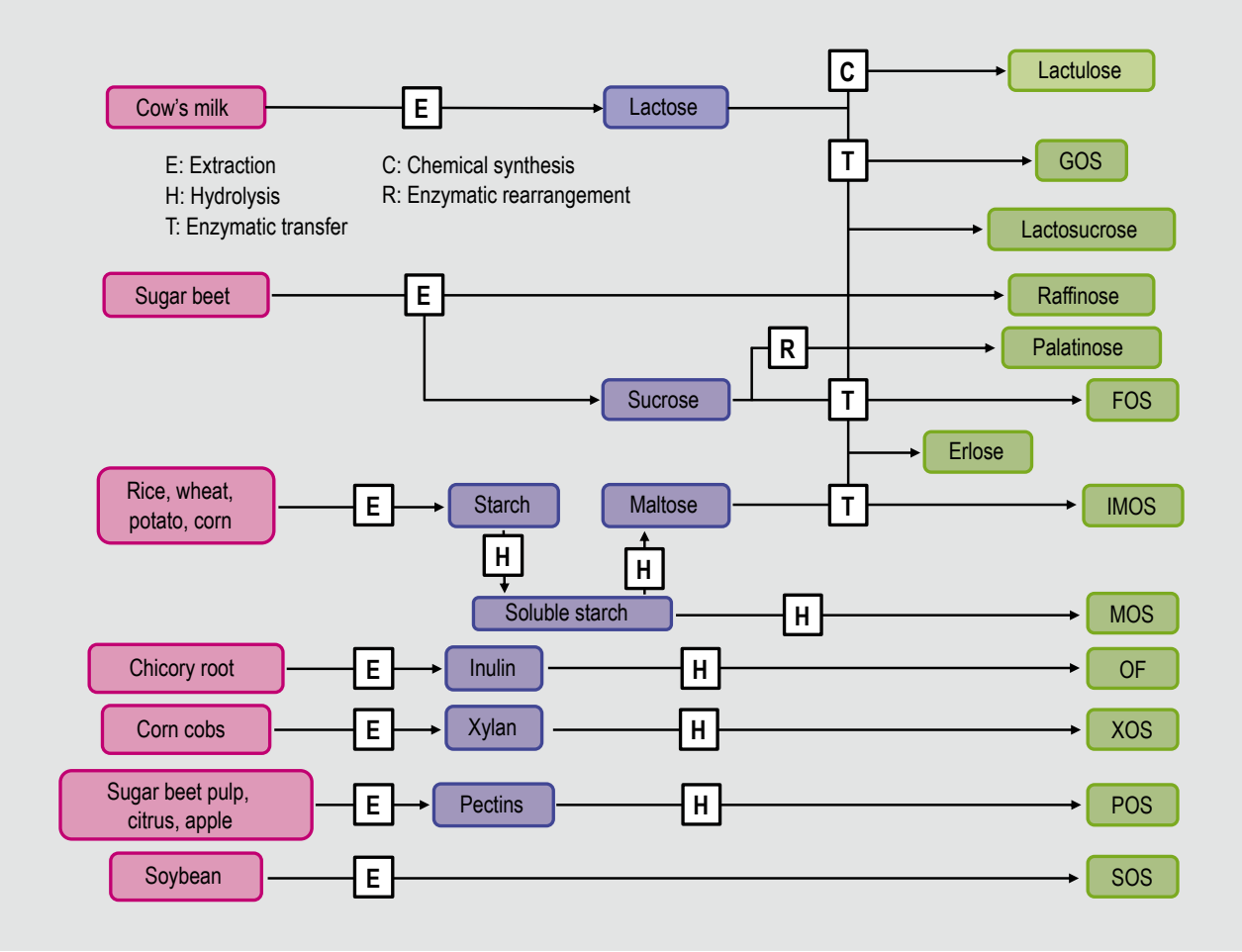

Figure 9. Overview of typically food-grade oligosaccharides manufacture methods, including the different strategies: extraction (E), hydrolysis (H), and synthesis both chemical (C) and enzymatic by transglycosylation (T) reaction or intramolecular rearrangement (R). GOS = galacto-oligosaccharides; FOS = fructo-oligosaccharides; IMOS = isomalto-oligosaccharides; MOS = malto-oligosaccharides; OFr = oligofructose; XOS = xylo-oligosaccharides; POS = pectic-oligosaccharides; SOS = soybean-oligosaccharide. (Based on and updated from Sako *et al.*, 1999).

their DP and sometimes in the position of the glycosidic linkages. Unreacted substrates and monosaccharides may also be present after enzymatic oligosaccharide formation, but they can be removed by physicochemical purification or fractionation processes, leading to a higher purity grade of the oligosaccharide of interest (Moreno *et al.*, 2014b; Mussatto and Mancilha, 2007). This is not, however, always necessary.

Enzymes suitable for the industrial production of oligosaccharides are normally from microbial sources. Microorganisms are preferred to plants and animals as sources of enzymes because they exhibit a number of important advantages (Filice and Marciello, 2013; Gurung *et al.*, 2013; Panesar *et al.*, 2006) such as:

- economic feasibility (they are generally cheaper to produce);
- easier handling (their enzyme contents are more predictable and controllable);
- higher multiplication rate;
- higher production yields (greater catalytic activity);
- genes encoding for microbial enzymes are efficiently translated and expressed as active proteins into well-known microorganisms, such as *E. coli*;
- enhanced versatility on acceptor substrates;
- better stability;
- regular supply due to absence of seasonal fluctuations.

Most industrial microorganisms have been genetically modified to overproduce the desired activity and not to produce undesirable side-activities. Biotechnology can transfer genes from a plant, animal, or microorganism, from which commercial scale enzyme production is not adequate, to a microorganism that has a safe history of enzyme production for food use. Additionally, microbial enzymes can be extracted and purified before they are used in food manufacturing.

As shown in Table 2, important oligosaccharides with well-recognised prebiotic status such as GOS or FOS are commercially produced by enzymatic synthesis. GOS are produced from lactose by the action of a wide variability of microbial β-galactosidases which have transgalactosylation activity, whereas FOS are obtained by transfructosylation of sucrose by β-fructofuranosidases, inulosucrases or levansucrases. According to the enzyme employed, four major classes of FOS can be distinguished: inulin-type fructans (β(2,1) linkages), levans (β(2,6) linkages), mixed levans (composed of both β(2,1) and β(2,6)-linked fructose units) and the neoseries consisting of linear β(2,1)-linked β-D-fructofuranosyl units bound to C6 of the glucose moiety of sucrose (Monsan and Ouarné, 2009). Fructan-type oligosaccharides are obtained most commonly by the hydrolysis of inulin (Table 2), and this is the manufacturing route for the principal providers of oligofructose products. Some other food-grade oligosaccharides (i.e. the trisaccharides lactosucrose and glycosylsucrose, also referred to as maltosylfructose, coupling sugar or erlose) are enzymatically synthesised from a mixture of two simple sugars (Figure 9 and Table 2; Crittenden and Playne, 1996).

New sources for prebiotics

Use of renewable and/or alternative natural sources such as seaweeds and marine microalgae

These sources can provide polysaccharides or oligosaccharides with unique biochemical and fermenting properties (Charoensiddhi *et al.*, 2020; De Jesus Raposo *et al.*, 2015; Zaporozhets *et al.*, 2014). Nevertheless, given the structural complexity of these compounds, their structural elucidation is challenging and, therefore, the structure-function relationship to be stablished may be unclear.

Synthesis of new tailored prebiotics with enhanced target-specific functional properties

A more rational design of next generation prebiotics with high predictive value targeted at particular species of beneficial gut bacteria will result in high added value both from an academic or industrial perspective.

Current research is oriented towards the search for new prebiotics that stimulate not only the growth of *Bifidobacterium* and *Lactobacillus* but also other beneficial bacterial species in the human colon as butyrate producers (e.g. *Roseburia*/*E. rectale* group) (Rivière *et al.*, 2016), as well as other bacterial targets associated with health, such as *A. muciniphila* (Cani and Van Hul, 2015), *F. prausnitzii* (O'Toole *et al.*, 2017), or *R. bromii* (Scott *et al.*, 2015), among others.

On the one hand, sequencing of individual microbial genomes has generated numerous reference blueprints for human gut bacteria (Human Microbiome Jumpstart Reference Strains Consortium, 2010), which can be analysed using bioinformatics approaches to predict functionality. Recently, the generation of genome-scale metabolic models derived from human gut metagenomics data, based on literature-derived experimental data and comparative genomics, has shown to be a promising tool to investigate host-microbiome interactions and the factors involved in the cultivation of fastidious bacteria (Magnúsdóttir *et al.*, 2016). On the other hand, in addition to side-directed mutagenesis, recent developments in the combined use of computational protein design and molecular engineering have led to novel and efficient enzymes. The design of enzymes with specific features aimed at new acceptor substrates and new regioselectivities offers an excellent opportunity to establish new synthesis procedures and to enhance the glycodiversification of products (Daudé *et al.*, 2014; Devlamynck *et al.*, 2016; Schmid *et al.*, 2016). Nevertheless, protein engineering currently requires significant research efforts in each case because the structure-function relationship of many enzymes with glycosidase activity is poorly understood due to the paucity of studies on their three-dimensional structure determination (Moreno *et al.*, 2017).

5. Non-microbiota mediated effects of fermentable carbohydrates on gut physiology

The effect of fermentable carbohydrate fibres on the gut microbiota was discussed in Section 2. However, there is evidence that such carbohydrates can have more direct, non-microbiota mediated effects on cells of the immune system and these are discussed in this section (Figure 10). The direct effects of the carbohydrates on the immune system can be divided into two aspects, the effects on the mucosal immune system described by the acronym MALT (mucosa associated lymphoid tissue) and its component the GALT (gut associated lymphoid tissue). The mucosa-associated lymphoid tissues, which are lymphoid structures in the mucosal tissues, form the first line of defence against pathogens that enter the body. Fundamentally, the mucosal immune system has evolved to tolerate commensal microbes, while responding quickly and effectively to pathogenic challenges (Nochi *et al.*, 2018). Most infections occur at the mucosal site and the interaction of fibre with the mucosa and MALT are hence important factors to be considered. There is also evidence *in vitro* that certain carbohydrates can interfere with the initial adhesion step of pathogenic bacteria and their toxins with host receptor oligosaccharides. If such a mechanism acts in the gut then this too might inhibit microbial infection of the gut.

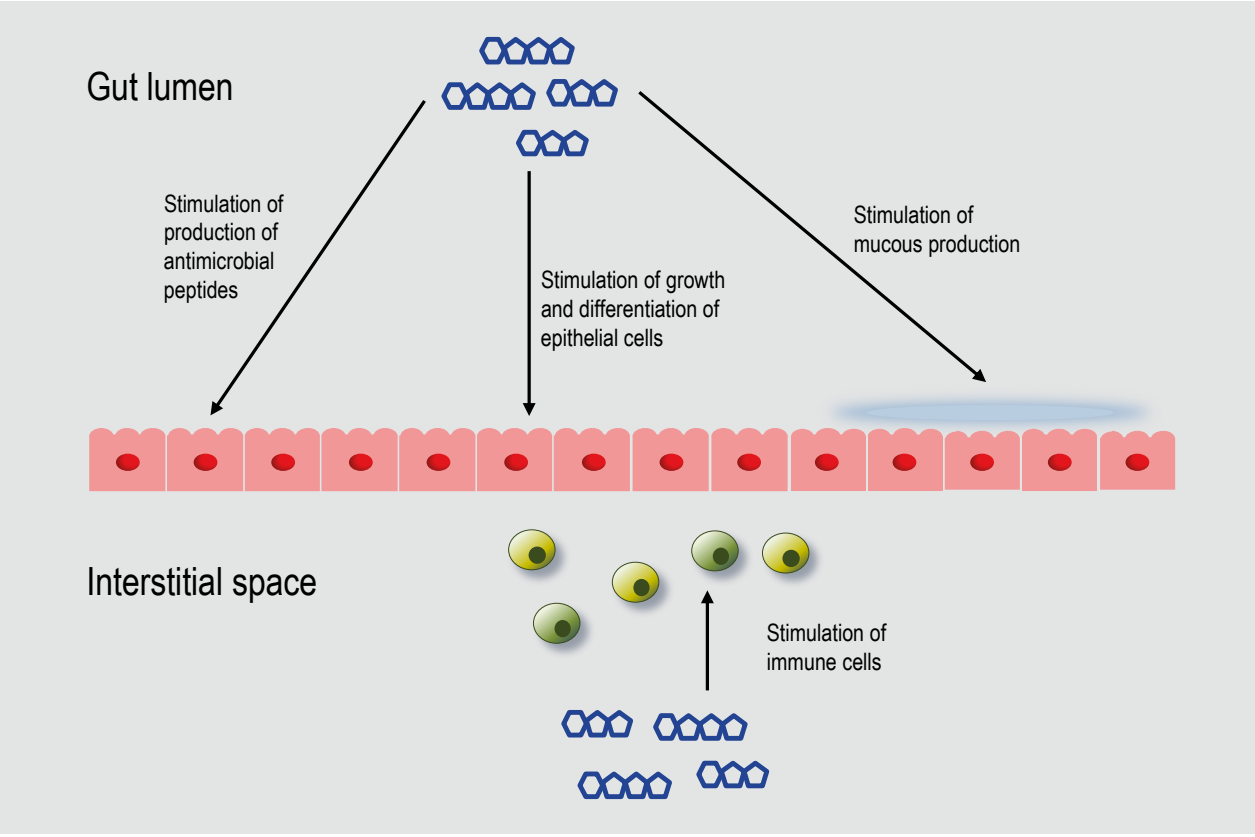

Figure 10. Schematic overview of putative direct mechanisms of prebiotics involved in beneficial as well as adverse effect on the intestinal epithelium: prebiotics themselves may stimulate growth and differentiation of epithelial cells, induce mucus and antimicrobial peptide production, and stimulate immune cells. In addition, some prebiotics are suggested to act directly on the intestinal cells by binding to specific receptors or to act as irritants on the epithelial cells, which might lead to a higher permeability and risk of bacterial translocation from the intestine.

Direct effects of carbohydrates on the gut mucosa

The interaction of dietary carbohydrates with the gut mucosa and the muco-adhesive properties of carbohydrate fibres are relevant as irritations or pathological changes of the mucous are often associated with disease states of the GIT. The mucin layer is an efficient system for protecting the epithelium from bacteria by promoting their clearance and separating them from the epithelial cells, thereby inhibiting inflammation and infection.

Several studies have investigated the muco-adhesive properties of fibres, for instance pectins (Schmidgall and Hensel, 2002; Thirawong *et al.*, 2007, 2008). In an *ex vivo* system based on porcine colonic tissue various neutral and acidic polysaccharides were tested by Schmidgall and Hensel (2002) concerning their bioadhesive potential to form artificial mucin layers on colon epithelial membranes (Schmidgall and Hensel, 2002). Rhamnogalacturonans with a low degree of esterification and linear oligogalacturonides derived from pectin showed significant bio-adhesion against colonic mucosal membranes, whereas HM pectins and neutral polysaccharides were ineffective. Within a linear structure-activity relationship, strongly acidic homogalacturonides were shown to be most adhesive agents. Esterification, branching or non-linear backbone structures were reported to reduce the adhesive properties. The 'artificial mucin layers' are thought to provide protective effects on colonic mucous membranes against toxic agents as shown by incubation of the tissue with TritonX-100 (Schmidgall and Hensel, 2002).

Thirawong *et al.* (2007) examined the muco-adhesive performance of various pectins with different degrees of esterification and molecular weights with porcine gastrointestinal mucosa, i.e. buccal, stomach, small intestine and large intestine, using a texture analyser equipped with a muco-adhesive platform (Thirawong *et al.*, 2007). Pectins showed a stronger muco-adhesion on large intestinal mucosa than on small intestinal mucosa. The muco-adhesive properties of pectins on gastric mucosa depended on pH of the medium; a higher maximum detachment force (F_{max}) and work of adhesion (W_{ad}) in a pH 4.8 medium than a pH 1.2 medium was revealed. The results also demonstrated that the mucoadhesive performance of pectins largely depended on their characteristics, i.e. higher degree of

esterification and molecular weight gave a stronger muco-adhesion. These findings are in contradiction with the findings of Schmidgall and Hensel (2002) with regards to the DE. Nevertheless, all investigations suggest that pectin can interact with the mucosal layer (Schmidgall and Hensel, 2002; Thirawong *et al.*, 2007). Furthermore, Thirawong *et al.* (2008) investigated the rheological characteristics of aqueous dispersion of four types of pectin, they reported the force of bio-adhesion to be dependent on the molecular weight of pectin, its initial viscosity and environmental pH. Liu *et al.* (2005) reported that pectin with higher net electrical charges, both positive and negative, showed a higher degree of muco-adhesion with porcine colonic tissues than the less charged ones.

In recent years, the focus has shifted from dietary fibres to effects of (potential) prebiotics on the gut mucosa. Vogt *et al.* (2014) showed that FOS prevented the increase in trans-epithelial electrical resistance (a measure for barrier function) in T84 human intestinal epithelial cell monolayers in a chain-length dependent manner. The largest effect was observed with short-chain FOS (DP <10), whereas inulin with a DP >10 did not have an effect. This is in contrast to the results obtained by Chen *et al.* (2017a), who showed that long-chain inulin (DP between 10 and 60) showed barrier-protective effects, by increasing the expression of barrier reinforcing tight junction proteins (occludin and claudin-2) and antimicrobial peptides (β-defensin-1 and cathelin-related anti-microbial peptide). In this mouse-model, short-chain FOS (DP between 2 and 25) did not show an effect. Similarly, He *et al.* (2017) showed that inulin, but not short-chain FOS restored acute-pancreatitis-associated intestinal barrier dysfunction by upregulating colonic tight junction modulatory proteins, antimicrobial peptides, and improved general colonic histology in a mouse model for acute pancreatitis. Also, the effect of the short-chain FOS observed with T84 cells, could not be recapitulated in a human clinical trial. In the trial, where individuals were vaccinated against hepatitis B, the long-chain inulin was more effective: it stimulated a Th1-like cytokine response and elicited higher antibody titers against the vaccine than short-chain FOS (Vogt *et al.*, 2017). Therefore, it seemed that *in vivo* inulin showed better effects, compared to the *in vitro* effects on T84 cells. This may indicate that apart from a direct effect of the FOS or inulin on epithelial cells, other (synergistic) effects may occur, e.g. through modulation of the gut microbiota composition or production of SCFA.

Direct effects of dietary carbohydrates on immune cells

Mammalian cells, in particular cells of the immune system, express various receptors on their surface that recognise different carbohydrate structures, that bind to carbohydrate structures on the surface of (pathogenic) microorganisms and it this manner elicit an immune response. Some of these receptors can also bind to the carbohydrate structures of fibres (recently reviewed by (Sun and De Vos, 2019)). Phagocytotic cells such as neutrophils, macrophages and dendritic cells in particular display a number of carbohydrate receptors with different and overlapping carbohydrate specificity (Table 3).

Essentially, all of the carbohydrate receptors recognise carbohydrate structures present on the surface of various microorganisms, including bacteria, viruses, yeasts and fungi, but the same or similar structures can be found in algae, plants, mushrooms and mosses, the most used sources for dietary fibres. This means that the individual receptor may bind to various fibre types albeit not always with the same affinity. Most of the receptors belong to the C-type lectin receptor (CLR) family defined as proteins that have one or more domains with conserved residue motifs that are homologous to carbohydrate recognition domains. As the definition is based on the primary protein structure and not the functionality of the receptor, some CLRs contain domains that are not able to bind carbohydrate

Table 3. Carbohydrate binding receptors in the immune system, their presence on different immune cells and their specificity.[1]

	Receptor	Specificity	Cell types
Ca^{2+}-dependent	mannose receptor (MR)	mannose, mannan, chitin, fucose	macrophages, dendritic cells, nonvascular endothelium
	DC-SiGN	mannose, mannan, β-glucans, fucose	dendritic cells, macrophages
	macrophage galactose specific lectin (MGL)	galactose	macrophages
	langerin	mannose	dendritic cells, Langerhans cells
	mincle	mannose	macrophages
Ca^{2+}-independent	dectin 2	α-glucans, mannose, mannan	neutrophils, monocytes, macrophages, dendritic cells
	dectin 1	β-glucans	neutrophils, monocytes, macrophages, dendritic cells
	siglecs	sialic acid containing glycans	macrophages, b cells, monocytes, neutrophils, NK cells, eosinophils

[1] Based on Chen and Seviour (2007), Drummond *et al.* (2011), Geijtenbeek and Gringhuis (2009), Plato *et al.* (2013), and Macauley *et al.* (2014).

structures (Zelensky and Gready, 2004). All the receptors described here, however, are functional carbohydrate-binding receptors and can be divided into two groups according to whether they display calcium-dependent binding or not. The mannose receptor (MR), dendritic cell-specific intercellular adhesion molecule-3-grabbing non-integrin (DC-SiGN), macrophage galactose specific lectin (MGL) and langerin all represent C-type lectins displaying calcium-dependent binding (Blixt *et al.*, 2004; Largent *et al.*, 1984), while DC-associated C-type lectin 1 (dectin-1) shows calcium-independent carbohydrate binding (Brown, 2005). The complement receptor 3 (CR3) has carbohydrate binding activity also. CR3 plays a key role in phagocytosis of complement coated microorganisms (opsonisation) but may also bind directly to β-glucan structures (Ross *et al.*, 1987). Scavenger receptors, which comprise a group of promiscuous receptors binding a vast variety of macromolecules of both endogenous and microbial origin, have also been shown to bind carbohydrate structures present in fibres (Canton *et al.*, 2013). Still new carbohydrate receptors are identified, however, here only a number of carbohydrate receptors which have been related to carbohydrates found in fibres will be discussed.

The total mixture of human milk oligosaccharides (HMOS) with less than 200 structures identified so far, deliver a huge variety of molecules which direct interact with immune cells (Xiao *et al.*, 2019). The specific structure and function-relation needs to be investigated in further studies. The current evidence on dietary carbohydrate interaction and immune cells and the effects observed are described in the next sections.

Carbohydrate binding receptors and their binding specificity

These carbohydrate binding receptors expressed by cells of the immune system, in particular the myeloid cells, are especially adapted to recognise microbial carbohydrate structures, such as β-glucans within the yeast cell wall and mannosylated cell wall components on bacteria and yeast. The action and specificity of the carbohydrate receptors are described in several reviews (Geijtenbeek and Gringhuis, 2009; Sancho and Reis e Sousa, 2012) and only the most fibre relevant receptors will be described in detail here. One important example of a fibre relevant receptor is the dectin-1 receptor, which binds to various β-glucans that contain a (1-3)-β-D-linked backbone, but with several fold higher affinity to β-(1-3),(1-6)β-glucans (laminarin) as compared to β-(1-3),(1-4)β-glucans (Adams *et al.*, 2008; Brown and Gordon, 2005). Whereas the former structure is present in cell walls from brown algae (Allaby, 2006), the latter structures are found in plant fibres and barley and oat are particularly rich sources (Aaman and Graham, 1987). The dependency on the structure for a proper binding to the dectin-1 receptor is reflected in the immune modulating effect of the β-glucans as demonstrated by their capacity to modulate the lipopolysaccharide (LPS) stimulated cytokine response in dendritic cells (Wismar *et al.*, 2011). Although the structure as such is important for the immune modulatory effect, the length of the polymers may represent the major determinant of the immune modulatory activity, due to the fact that the longer the polymers the less soluble they are (Adams *et al.*, 2008; Mikkelsen *et al.*, 2013). Such polymers may also attain different aggregate/gel-like structures in solution that in turn may affect the cells differently. This is exemplified in a study by Sahasrabudhe *et al.* (2016a), who show that particulate β-glucans activates dectin-1 (and synergistically also TLR-4) in human dendritic cells. Interestingly, in a follow-up study, the same team shows that wheat arabinoxylan also activates dectin-1, to the same (low) extent as soluble β-glucans, and much lower than particulate β-glucans. However, similar to soluble β-glucans, arabinoxylan inhibit the particulate β-glucan-induced activation of dectin-1 in human dendritic cells, which demonstrated reduced production of IL-10 and tumour necrosis factor-alpha (TNF-α). The production of the antifungal cytokines IL-4 and IL-23 were increased in dendritic cells stimulated with arabinoxylan and particulate β-glucan (Sahasrabudhe *et al.*, 2016b).

Other receptors than dectin-1 may also bind β-glucan. These include the integrin receptor CR3, scavenger receptors and lactosylceramide (Brown and Gordon, 2003), but none of these other receptors have been clearly demonstrated to play a major role in mediation of the biological response to β-glucans. Common for the β-glucan binding receptors are their presence on different types of myeloid leucocytes, including neutrophils, monocytes, macrophages and dendritic cells. These cells have in common that they belong to the innate immune system, hold phagocytotic activity, and recognise conserved structures on microorganisms, so called microbial associated molecular patterns (MAMPs). Microbially derived carbohydrates comprise one group of such MAMPs.

A number of MAMPs contains mannose in their structure, either as terminal carbohydrate units or as multiple mannose units linked together (Gringhuis *et al.*, 2009; Martinez-Pomares, 2012). The mannose receptor recognises mannose, both as a terminal sugar in complex carbohydrates and as mannose multimers, such as mannan (Martinez-Pomares, 2012; Taylor *et al.*, 2005). The ligands are present on a range of bacteria, fungi, virus-infected cells, and parasites. Other receptors that recognise mannose residues include langerin and dectin-2. Dectin-2 and the mannose receptor also have affinity for α-glucans and chitin, respectively (Levitz, 2010).

Prebiotics have also been shown to interact with receptors on immune cells. Earlier we described the TLR-2 dependent effect of inulin-type fructans (FOS and inulin) on epithelial

cells, but these fibres also interact with TLR-2 on immune cells (Vogt *et al.*, 2013). In this *in vitro* study, where human peripheral mononuclear cells were incubated with different chain-length inulin-type fructans, cytokine production was chain length-dependent. Strikingly, short chain enriched FOS induced a regulatory cytokine balance compared to long chain enriched inulin. Reporter cell line experiments indicated that TLR-2 was primarily activated, with minor effects on other TLR-receptors (Vogt *et al.*, 2013). As already alluded to above in the section on epithelial interaction, the same team showed opposite effects *in vivo*, where the long-chain inulin was effective, and the short-chain FOS was not (Vogt *et al.*, 2017). In a germ-free mouse model, both short-chain FOS and long-chain inulin enhanced T-helper 1 cells in Peyer's patches, whereas only short-chain FOS increased regulatory T cells and a subclass of dendritic cells (DCs) in mesenteric lymph nodes (Fransen *et al.*, 2017). Moreover, resistant starches have been shown to be immunomodulatory by direct interaction with immune cells. High-maize 260 (RS type 2) primarily bound to TLR-2, while Novelose 330 (RS type 3) bound to TLR-2 an TLR-5. When High-maize 260 was interacted *in vitro* with co-cultures of dendritic cells (DCs), epithelial cells and or T-cells, the final effect was driving T-cells activity into a more regulatory status, via skewing towards Th1 cytokine production, attenuating Th2 cytokines and by stimulating regulatory T-cell cytokine production (Bermudez-Brito *et al.*, 2015a). In another study, the same team in the same *in vitro* co-culture model set-up, showed that GOS, inulin, arabinoxylans from wheat, and beta-glucan from barley led to changes in the production of the Th1 cytokines, while inulin, and beta-glucan reduced the Th2 cytokine IL-6. The regulatory T-cell-promoting cytokine IL-10 was induced by GOS whereas inulin decreased IL-10 production (Bermudez-Brito *et al.*, 2015b). Of note is that when DCs were incubated with the fibres in the absence of epithelial cells and T-cells, different cytokine profiles were observed, and in both papers the authors advocated to investigated the cross-talk between intestinal epithelial cells, DCs, and T-cells when interpreting effects of dietary fibres on immunomodulation.

A number of recent studies show also the direct interaction of pectin with immune cells. Lemon pectins of different degrees of methyl esterification (DM) (30, 56, and 74%) were studied for immune receptor activating potential and epithelial barrier protection. The 30 and 74% DM pectins, but not the 56% DM pectin, induced strong protection of the epithelial barrier measured by transepithelial electrical resistance in T84 cells. The lemon pectins mainly interacted with TLR-2, which increased with increasing pectin DM (Vogt *et al.*, 2016). In contrast, in another study, five lemons pectins with different DM (DM7, DM22, DM45, DM60, and DM75) were shown to bind and inhibit TLR-2 and specifically inhibit the proinflammatory TLR-2/TLR-1 pathway, leaving the tolerogenic TLR-2/TLR-6 pathway unaffected. Here, the effect was most pronounced with pectins having a low degree of methyl esterification. Using mutant TLR-2 molecules, the interaction was shown to occur between non-esterified galacturonic acids on the pectin and positive charges on TLR-2 (Sahasrabudhe *et al.*, 2018). The binding of low DM pectin to TLR-2 presumably prevented ileitis in a mouse-model in a TLR-2 dependent manner. It is unclear what (structural) differences between the pectins in the two studies caused high-DM pectins to be more active towards TLR-2 in one study, and low-DM being more active in the other. Perhaps this has to do with the degree of blockiness (DB), or the presence or absence of calcium during incubations. Regarding the latter, the same team showed that calcium reduces inhibition of TLR-2 by low (DM8) and intermediate (DM52) pectins, but calcium has lower impact on TLR-2 inhibition by high DM pectins (DM69). In the absence of calcium, especially DM18 and DM52 strongly inhibited and bound to TLR-2. Addition of 1 or 10 mM calcium to these pectins reduced TLR-2 inhibition and binding (Beukema *et al.*, 2020). The DB is defined as distribution of methyl-esters over the galacturonic-acid backbone of pectin (Beukema *et al.*, 2021). When comparing pectins with similar DM, high DB pectins have a more blockwise distribution of non-esterified galacturocin acid residues compared to low DB pectins. This in contrast to LB pectins, which have a more random distribution of non-esterified galacturocin acid residues. In this study pectins from lemon origin with DM18, DM19, DM33, DM43, DM49, DM52, DM84 and DM86 were used. *In vitro* pectins mainly inhibit TLR-2/TLR-1, as shown previously, but the magnitude depended on both DM and DB. Low DM pectins (DM18/19) with DB86 and DB94 strongly inhibit TLR-2/TLR-1. However, pectins with intermediate DM (DM43/DM49) and high DB (DB60), but not with low DB (DB33), inhibited TLR-2/TLR-1 as strongly as low DM. High DM pectins (DM84/88) with DB71 and DB91 do not inhibit TLR-2/TLR-1 strongly. In human macrophages, low DM and intermediate DM pectins with high DB inhibited TLR-2/TLR-1-induced IL-6 secretion. The authors concluded that both high number (DM) and blockwise distribution of non-esterified galacturonic acid residues (DB) in pectins are responsible for the anti-inflammatory effects observed via inhibition of TLR-2/TLR-1 (Beukema *et al.*, 2021).

The receptors hold different binding specificities, but they also give rise to distinct effects in the antigen presenting cells (Feriotti *et al.*, 2013). One important effect of the carbohydrate-receptor interaction is induction of phagocytosis of microorganisms by binding to multiple carbohydrate receptors at the same time. This has been demonstrated for many of the receptors including the mannose receptor, dectin-1, dectin-2 and DC-SIGN (Kerrigan and Brown, 2009). Phagocytosis is a requirement for induction of a strong cellular immune response for efficient clearance of bacteria, fungi and viruses. Depending

on the specific preparation, plant carbohydrates may hold single or multiple ligands that can ligate to one or many receptors on an antigen presenting cell, respectively, in turn giving rise to different effects in the cell. Thus, depending on the structure, such plant-derived carbohydrates may inhibit the binding of bacterial structures to the pattern recognition receptors (PRRs) and thus, modulate the immune response. Multiple receptors have been implicated in recognition of e.g. yeasts or zymosan. These include CR3 and dectin-1, which bind cell wall β-glucans; mannose receptor (MR), DC-SIGN, TLRs, and galectin-3, which bind mannans or other mannose-containing compounds (Józefowski *et al.*, 2011). However, involvement of some of these receptors in yeast recognition remains controversial. For instance, Brown *et al.* (2002) and Taylor *et al.* (2002) reported that opsonin-independent zymosan binding was unaffected by genetic CR3 deficiency or a blocking monoclonal antibody against CR3, suggesting that CR3 was not the receptor for β-glucan recognition. Also, mannose receptor-deficient macrophages showed normal phagocytic uptake of *Candida albicans*. Moreover, resistance to *C. albicans* infection was unimpaired in mannose receptor-deficient mice (Lee *et al.*, 2003). Another class of C-type lectins that recognise microbial components are scavenger receptors (Plüddemann *et al.*, 2006). There are 8 classes and not all have been characterised in full detail. They bind to modified glucans (acetylated, phosphorylated) as well as to unmodified molecules. Apart from being involved in direct recognition and phagocytic clearance of bacteria, different scavenger receptors collaborate with other classes of PRRs and either increase the repertoire of innate immune recognition or modulate the biological responses of host cells to bacteria (Plüddemann *et al.*, 2006).

When binding to carbohydrate receptors on the surface of cells, fibres may initiate signal transduction to initiate either an immune stimulatory signal or an inhibiting signal. The induced intracellular signalling processes are complex and not fully understood. Binding of fibres to some of these receptors including dectin-2, initiates signalling pathways through immunoreceptor tyrosine-based activation motif (ITAM)-containing adaptor molecules, such as Fc receptor g-chain (FcRg) or Src kinases and SYK, leading to induction of pro-inflammatory cytokines such as IL-6 and TNF-α (Sato *et al.*, 2006; Yamasaki *et al.*, 2009). Conversely, binding of fibres to dectin-1, DC-SIGN and several other receptors induces signalling through protein kinases or phosphatases, but has not hitherto been shown to induce pro-inflammatory gene expression by the binding. Rather the binding of fibres to these inhibitors initiates a signal transduction that may affect signalling through TLRs, thus modulating the TLR-ligand initiated inflammatory signal. There are many speculations regarding the intracellular pathways involved and also partly elucidated intracellular pathways, that to varying degree support the suggested interactions between carbohydrate receptor and TLR signalling (Geijtenbeek and Gringhuis, 2009). A popular theory regarding the modulating activity of fibres is that the fibre binds simultaneously with the binding of a microorganism or just a single TLR-ligand of that microorganisms, and that the simultaneous binding leads to intracellular interaction of the pathways leading to immune modulating of the TLR induced response. For instance, it has been shown that several structurally different non-starch polysaccharides can modulate LPS-induced cytokine production in dendritic cells in a structure-dependent manner (Wismar *et al.*, 2011).

Also, as most experiments are performed with carbohydrate macromolecules isolated from microorganisms the possibility exists that such fibre preparations contain impurities of microbial origin, which may hold TLR-stimulating activities or by other means may interfere with signalling. Hence, whether a fibre holds immune stimulatory or inhibitory properties should be determined by extremely pure, preferably synthetic fibres.

Importantly, most if not all carbohydrate receptors seem to be involved in phagocytosis which is in good accordance with their presence on phagocytotic cells such as dendritic cells, monocytes, macrophages and neutrophils. Which receptors are present on the different phagocytotic cell types, is not described very comprehensively. This hampers our understanding of how complex carbohydrates interact with and affect the different cell types. Many fibres from fungi, including mushrooms and yeast contain mannose units, either as terminal sugars or as a series of many α-linked mannose units (Kéry *et al.*, 1992). Such fibres may bind to different mannose specific receptors including DC-SIGN, dectin-2 and MR, and accordingly the signal induced may be determined by the specific mannose-binding receptors present on a given cell. β-Glucan stimulation through dectin-1 and mannan stimulation through dectin-2 have both been shown to induce nuclear factor κB (NF-κB) through Syk kinase and Caspase recruitment domain-containing protein 9 (CARD9) signalling (Drummond *et al.*, 2011). Interestingly, while dectin-1 has an ITAM-motif in its cytoplasmic domain, dectin-2 and the other receptor of the dectin-2 family, Mincle receptor, have no known intracellular signalling regions, but may associate with the ITAM motif of the FcRg chain and thus, indirectly induce Syk activation (Sato *et al.*, 2006).

Apart from binding mannose, the mannose receptor is also capable of binding terminating fucose and N-acetyl glucosamine residues in a calcium-dependent manner (Taylor *et al.*, 2005). The MR lacks a potential signalling motif in the cytoplasmic tail and hence, requires collaboration with other PRRs to induce, e.g. NF-κB activation (Gazi and Martinez-Pomares, 2009). The dependency for collaboration with other PRRs was nicely demonstrated by Tachado *et al.* (2007). Using non-

phagocytic human embryonic kidney cells transfected with cDNA encoding for human TLR-2 or human MR cDNA alone, no cytokine production was measured in response to the fungus *Pneumocystis*. Conversely, when co-transfecting the two receptors, cytokine production was induced (Tachado *et al.*, 2007).

DC-SIGN also recognises mannose, but with strong preference for high-mannose oligosaccharides, in a calcium-dependent manner. Moreover, as MR, it also recognises fucose-containing ligands (Feinberg, 2001). Upon mannan ligation, DC-SIGN signalling involves the RAF proto-oncogene serine/threonine-proteins kinase (Raf-1; Gringhuis *et al.*, 2007). Phosphorylation of Raf-1 results in modulation of the phosphorylation and acetylation of NF-kB, which in turn induces transcription of the genes encoding proinflammatory cytokines such as IL-12 and IL-6. Upon fucose stimulation, however, the signalling was found to be independent of the Raf-1 signalling cascade and to suppress pro-inflammatory cytokine production, while increasing the IL-10 production (Gringhuis *et al.*, 2009). Whether this discrepancy is due to dual signalling pathways that are initiated by distinct ligands or alternatively, the different responses are caused by different experimental approaches or different sources of ligands, remains to be clarified.

As one would expect from the paragraphs above, knockout gene studies indicate some degree of redundancy of the different carbohydrate receptors. Whether this is an immunological safety net or a means to increase the capacity to distinguish between different carbohydrate structures, and hence microorganisms, by the collaboration of two or more receptors with distinct specificity, is not clear. Collaboration between different PRRs has been described. One example is the collaboration between dectin-1 and TLR-2 (Bauer *et al.*, 2009; Goodridge and Underhill, 2008; O'Neill, 2008). Stimulation with dectin-1 and TLR-2 agonists has synergistic effect on TNF, IL-6 and IL-23 production. Of note, such synergistic effects have also been shown for dectin-1 in collaboration with TLR-1, TLR-4, TLR-5, TLR-7 and TLR-9 (Bonfim *et al.*, 2009; Dennehy *et al.*, 2008). While these studies demonstrate the collaboration between different receptors, they also underline an obstacle for elucidating specific mechanisms behind the action of specific structures; the fact that by far most of the fibres investigated originated from microorganisms and accordingly the likelihood of the presence of contaminants representing other carbohydrate structures as well as other ligands for TLRs exits. The presence of such PRR ligands may result in induction of other cellular pathways initiating other cytokines and accordingly other immune responses, clouding the effects of the fibre alone. As an example, curdlan, a (1-3)-β-glucan from the Gram-negative soil bacterium *Alcaligenes faecalis*, is often used to assess the immunomodulating effect of 1-3-β-glucans. However, many preparations of curdlan are not pure and contain varying amounts of LPS, which may induce additional stimulatory pathways in the immune cells. A preparation of commercially available curdlan has been shown to contain an appreciable amount of LPS (Wismar *et al.*, 2010). Likewise, some of the reported work on dectin-1 is at least partly based on the use of zymosan from yeast (Kerrigan and Brown, 2009). Again, apart from an abundance of β-glucans, zymosan has been shown to contain mannan and TLR-2 ligands, which may give rise to confusing results (Ikeda *et al.*, 2008; Jawhara *et al.*, 2012). *Vice versa*, mannan has been shown to be contaminated with β-glucans (Brown *et al.*, 2002). While the use of knock out mouse strains has clearly demonstrated the indispensability of dectin-1 for induction of Th1 and Th17 responses (LeibundGut-Landmann *et al.*, 2007, 2008; Osorio *et al.*, 2008), and that β-glucan is the ligand for dectin-1 was shown by transfected cell lines (Brown *et al.*, 2001), it remains to be demonstrated that β-glucan *per se* is capable of this induction.

Keeping in mind that the fibres, by binding to carbohydrate receptors, may induce phagocytosis, it might also be this induced phagocytosis that modulates the TLR response, e.g. by enhancing the uptake of microorganisms or microbial compounds. It has been shown for some TLRs, that signalling from endosomes instead of from the plasma membrane may change the signalling and accordingly which cytokines that are being produced through TLR signalling. This compartmentalised segregation in signalling was demonstrated for TLR-4 showing that ligand binding to and signalling through TLR-4 present in the plasma membrane of antigen presenting cells induces signalling through the adaptor protein MyD88, while when signalling through TLR-4 takes place from endosomal compartments, signalling takes place through the adaptor protein TRIF. The employment of different adaptor proteins according to the localisation of the receptor results in activation of distinct signalling pathways and accordingly different cytokine are produced (Barton and Kagan, 2009). Even though this compartment-dependent signalling has not been demonstrated for other TLRs or PRRs, it cannot be excluded that some fibres due to their capacity to induce phagocytosis act by facilitating the endocytosis of TLR ligands and thus might redirect the TLR signalling to other pathways.

Even preparations of plant derived fibres are likely to contain other macromolecules with putative immune stimulatory/modulatory properties. For instance, they may contain LPS (from Gram-negatives) or LTA (from Gram-positives) or other immunostimulatory compounds of microbial origin simply because the fibres may serve as nutrients for the microorganisms. To be completely sure that any immunomodulatory effect demonstrated can be ascribed to the fibres it is advisable to test for endotoxin

content and if possible, for other contaminants. Out of 28 non-starch polysaccharides (NSP) used to assess their immunomodulatory effects in dendritic cells, several contained endotoxins on testing (Wismar *et al.*, 2011). Due to the nature of different NSP it was not possible to purify for unknown microbial TLR-ligand contaminants. Therefore, addition of a high amount of LPS to all stimuli was carried out to level out these differences in contamination. A diverse group of NSP was capable of suppressing LPS-induced IL-12p70 production; among those, the group, comprising differently structured β-glucans and the galactomannan guar gum, was able to augment the LPS-induced IL-10 production. Only curdlan gave rise to detectable amounts of IL-12p70 in the absence of LPS-stimulation (Wismar *et al.*, 2011).

When assessing the immunomodulating properties of fibres it is important to relate to several factors that may influence the experimental outcome. These comprise physico-chemical parameters, such as molecular mass, molecular fine structure, solubility, polymer conformation in solution, the presence of fibre particles and the experimental system used to assess the immunomodulating effect. The molecular fine structure, mass, solubility and polymer conformation are highly interrelated parameters, and this makes the structure-function relationships difficult to establish (Böhm and Kulicke, 1999; Tosh, 2004). As an example, solubilised β-glucan has been shown to comprise a quite heterogeneous group of polysaccharide conformations, due to variation in degree of polymerisation that affects the aggregation of the β-glucan molecules, which in turn may affects the solubility of the overall β-glucan preparation (Håkansson *et al.*, 2012). The soluble versus particulate nature of a β-glucan preparation may also be a determinant for an immunomodulatory property. Even inert particles that are not being recognised by phagocytes may induce phagocytotic activity. Accordingly, immunomodulating fibres may attain phagocytosis inducing activity if they form insoluble particles in solution, independently of their receptor specific immunomodulating activity. This was demonstrated by comparison of a variety of dried β-glucan samples prepared from barley that were either fully solubilised or just suspended in an aqueous solution. When added together with the probiotic bacterium *L. acidophilus* NCFM to dendritic cells, the two different preparations exhibited opposite immunomodulating properties: while the fully dissolved preparations increased the *L. acidophilus* NCFM-induced production of the Th1 inducing cytokine IL-12, the dispersed preparation diminished the induced production of IL-12 but increased the production of the anti-inflammatory cytokine IL-10 (Mikkelsen *et al.*, 2014). Also, only dispersed β-glucan preparations induced a cytokine response (IL-10) response in the dendritic cells when added alone. These disparate effects of two preparations of the same sample highlight the complexity of the fibre structures and functionality and indicate that solubility is a key determinant of the immunomodulatory properties of carbohydrate fibres.

Finally, the experimental system used may determine the outcome of the testing. When testing the immunomodulating properties of a series of β-glucan molecules, this was done by adding the β-glucan samples alone or together with either LPS from *E. coli* or whole Gram-positive *L. acidophilus* NCFM bacteria to stimulate dendritic cells (Mikkelsen *et al.*, 2014). These two stimuli are known to induce distinct intracellular pathways in the cells, despite both giving rise to IL-12 production of importance for NK cell activation and activation of a cellular immunity (Abbas *et al.*, 2012). While dissolved β-glucans samples inhibited the IL-12 production in LPS stimulated cells, the same samples enhanced the IL-12 induced production in *L. acidophilus* NCFM stimulated cells. Hence, the (microbiological) context of the interaction of fibres with immune cells is very important: it matters what is present in the close environment of the fibres and immune cells.

Taken together, the best documented role of the receptors binding fibre carbohydrates is their role in phagocytosis. The receptors are present on myeloid leucocytes, such as monocytes, neutrophils and dendritic cells. Their cooperative or antagonising action when recognising fibre precludes a clear mode of action from being established. Similarly, due to numerous small changes in structure (e.g. in β-glucans) a clear-cut structure function relationship is still years if not decades away. The work is hampered by numerous factors such as solubility, length and heterogeneity of fibre preparations as well as overlapping specificity of many fibres and receptors.

The bioavailability of dietary carbohydrates for immunomodulation

One intrusive question that emerges in relation to whether orally administered fibres hold the capacity to directly act as immunomodulators is if, or to which extent, such carbohydrate molecules will contact cells of the immune system. To contact most leucocytes in the body, the carbohydrates have to traverse the epithelial barrier, either to get into the circulation or make contact with the vast number of leucocytes present just below the epithelial barrier. Perhaps due to the lack of simple, specific and sensitive assays for quantification of the carbohydrate structures when studying the bioavailability of orally ingested fibres, only few studies have assessed the absorption of fibres from the gastrointestinal tract. In most of the reported studies of fibre absorption, the methods to detect the presence of polysaccharides in tissue and blood have involved labelling of the carbohydrate with isotopes or fluorescence (Rice *et al.*, 2005; Sandvik *et al.*, 2007; Vetvicka *et al.*, 2007; Zhu *et al.*, 2011), but also detection

with specific antibodies was used (Hoshi *et al.*, 2008; Sakurai *et al.*, 1998). Using covalent labelling of the carbohydrate fibres, a 0.5-5% bioavailability in rats was estimated. As the methods used do not distinguish between intact or degraded macromolecules it cannot be concluded as to which extend the fibres had retained their bioactivity. In a study using ELISA based on antibodies specific to α-D-glucan separated from the edible mushroom *Tricholoma matsutake* a peak concentration in blood on 160 ng/ml was determined 24 h after administration of a single dose of 450 mg/kg (roughly corresponding to 13 mg) to mice (Hoshi *et al.*, 2008). In addition, the available antibodies allowed detection of the fibre molecules in various tissue by immunostaining and showed the presence of the fibre in the gut mucosal layer, gut associated lymph node as well as the spleen during a 32 h period after administration of the fibre. Even though the documentation of absorption of significant amounts of fibres upon oral intake is still not very solid, the existing studies indicate some absorption and hence, a possible *in vivo* direct immunomodulating effect of fibres showing such effects in *in vitro* assays is thus not unlikely.

GOS, long-chain fructo (lcFOS)-oligosaccharides, acidic oligosaccharides from human and cows' milk, and specific pectin-derived acidic oligosaccharide hydrolysate (pAOS) have also been shown to be able to cross the epithelium (Eiwegger *et al.*, 2010).

Other examples of dietary carbohydrates that could interact with the immune system

Several dietary polysaccharides/fibres have been studied in *in vitro* assays, animal experiments and human volunteers for their immunomodulatory capacity (Ramberg *et al.*, 2010). It goes beyond the scope of this chapter to discuss these in all detail, but a selection of studies is briefly addressed. Apart from studies with glucans (both β and α; Ramberg *et al.*, 2010), different pectins and/or their structural components have been studied as well, for their anti-inflammatory and cancer repressing properties.

Several studies have reported anti-tumour and antimetastatic properties of rhamnogalacturonan-II, as well the potential to increase interleukin (IL-6, IL-12) production of peritoneal macrophages and stimulation of neutral killer cell activity against tumour cells (Lee *et al.*, 2014; Park *et al.*, 2017a,b).

As reviewed by Smestad Paulsen and Barsett (2005), RG-II has been also described to have intestinal immune modulating activity (Taguchi *et al.*, 2004; Yu *et al.*, 1998, 2001), antiulcer activity and effects on the complement system (Guo *et al.*, 2000; Sakurai *et al.*, 1996, 1998; Yamada *et al.*, 1991). Studies exist also on the direct effects of rhamnogalacturonan I (RG-I) on human health, such as the inhibitory effects and mechanisms of RG-I from potato on HT-29 colon cancer cell proliferation and cell cycle progression (Cheng *et al.*, 2012).

Citrus pectins have also been studied for their anti-tumour properties. Citrus pectin was either treated with heat or pH-treated. The pH treatment at both high and low pH results in the degradation of the pectin into smaller fragments and the cleavage of the side chains from the backbones. Heat treatment leads to degradation of smaller fragments only. Heat-treated citrus pectin was shown to have anti-tumour activity *in vitro* and in a mouse model, whereas pH treated pectin was inactive. This indicates that the (degree of) methylation of pectin may be important for its activity. The anti-tumour activity of heat-treated pectin was not correlated with its immunological activities, or its inhibitory effect on galectin-3 (Hao *et al.*, 2013). In the light of the discovery that some pectic polysaccharides could bind to galectin-3, a galectin-3-targeting anti-tumour mechanism has been proposed. Several other pectins (swallow root pectin, *Hemidesmus* pectin, black cumin pectin, *Andrographis* pectin and okra pectin) have been shown to interact with galectin-3 and inhibit galectin-3 mediated cancer cell processes (Hao *et al.*, 2013). The pH-treated pectin had higher immunomodulating activities than the heat-treated pectin, in addition to over 4,000-fold higher galectin-3-inhibiting activities. It is noteworthy that the pH-treated pectin had no anti-tumour activity. Thus, anti-tumour activity of pectin on the one side and immunomodulation and galectin-3 inhibition on the other are likely brought about by different structural features on the pectin molecule.

Furthermore, Grønhaug *et al.* (2011) demonstrated the immunomodulating activities of pectins from *Biophytum petersianum* and reported the hairy region to be the most important region for immunomodulating activity on Peyer's patches and macrophages.

A recent study by Vogt *et al.* (2016) has shown that the DE of lemon pectin (30, 56, and 74% DE) is impacting the activation of immune receptors and the epithelial barrier protection (Vogt *et al.*, 2016). Similarly, Sun *et al.* (2017) have shown that LMP supplementation restored acute pancreatitis-associated disruption of intestinal barrier integrity as evidenced by upregulation of tight junction modulatory proteins occludin, zonula occludens (ZO)-1, antimicrobial peptides β-defensin-1 (DEFB1) and cathelicidin-related antimicrobial peptide (CRAMP) as well as increase in SCFA production. Low-methoxyl pectin (LMP)-supplemented mice with acute pancreatitis exhibited suppressed intestinal inflammation as shown by decreased ileal and colon cytokine production compared with caerulien group (Sun *et al.*, 2017). A recent study by Yamada *et al.* (2018) investigated the effects of LMP and HMP, on colonic anastomosis healing in rats, their results

demonstrated that LMP promotes the healing of colonic anastomosis more effectively than HMP (Yamada *et al.*, 2018).

Using HEK293 cells transfected with individual toll-like receptor, THP-1 cells and human PBMCs it was shown that FOS stimulate the immune system through TLRs, primarily TLR-2 (Vogt *et al.*, 2013). The effect was dependent on the chain-length (or DP), assessed by cytokine production in humans. β(2-1)-Fructans with a low DP induced a regulatory cytokine balance compared to those with a higher DP as measured by IL-10/IL-12 ratios. Interestingly, the same FOS also showed a DP-dependent effect on barrier function (transepithelial electrical resistance) in T84 monolayers, with again the shorter DP showing highest activity (Vogt *et al.*, 2014). Also, this effect was TLR2-dependent as blocking of TLR-2 led to abrogation of the activity of the FOS.

In addition, reports have described effects of galactomannan (guar gum), glucomanan and various heteroglycans (Ramberg *et al.*, 2010), as well as a mixture of GOS and FOS (Van de Pol *et al.*, 2011; Van der Aa *et al.*, 2011). In human, only β-glucans (Kirmaz *et al.*, 2005; Koray *et al.*, 2009), fucoidan (a sulphated polysaccharide from brown algae (Irhimeh *et al.*, 2007), and arabinogalactans (Riede *et al.*, 2013; Udani, 2013; Udani *et al.*, 2010) have been studied for their immunostimulatory effects.

Anti-adhesive activities of dietary carbohydrates

The direct immune modulating/stimulating activity of fibres has been speculated to impact the resistance towards microbial infections. As discussed briefly above, the fibres may act as decoys for the epithelial receptors, resulting in adhesion of microbes to the fibre molecules rather than to the epithelial cells, as recently reviewed (Monteagudo-Mera *et al.*, 2019). In this manner, the fibres preclude interaction with the host. This has been described for instance for mannan-derived oligosaccharides (MOS) and enterotoxic *E. coli* (ETEC) strains in pigs, for instance (Castillo *et al.*, 2008). There has been a long debate about whether or not MOS would be active *in vivo*, as the *E. coli* receptor for binding to mannan may not be expressed at 39 °C, the body-temperature of pigs. But as evidenced by amongst others Castillo *et al.* (2008), MOS is efficacious in reducing ETEC infection *in vivo*. However, not every MOS preparation may be equally effective.

Five structurally different citrus pectic samples, differing in molecular weight and degree of esterification, were anti-adhesive for *E. coli* O157:H7 binding to human HT29 cells. Lower molecular weight and lower degree of esterification enhanced the anti-adhesive activity (Di *et al.*, 2017). A novel xyloglucan isolated from cranberry, determined to be a branched, three hexose, four pentose oligosaccharide consistent with an arabino-xyloglucan structure, inhibited the adhesion of *E. coli* O157:H7 to HT29 human colonic epithelial cells (Hotchkiss *et al.*, 2015).

GOS have also been found to potentially have non-microbiome mediated effects. The fact that GOS terminate with a β-linked galactosyl residue has led to the suggestion that they may mimic at least some mammalian oligosaccharide pathogen receptors and inhibit adhesion of such organisms. Shoaf *et al.* (2006) found that GOS purified from Oligomate 55 were more effective than inulin, FOS, lactulose and raffinose at reducing adhesion of enteropathogenic *E. coli* to Hep-2 tissue culture cells. They found a dose-dependent inhibition with a maximum of 50% inhibition of adhesion relative to controls. Searle *et al.* (2009), working with BB-GOS could reduce adhesion and infection in mouse models of *Salmonella enterica* serovar Typhimurium.

Direct effects on cell lines in culture have also been demonstrated. Incubation of tissue culture cells with GOS has been shown to have enhance barrier function in LS174T cell monolayers (Bhatia *et al.*, 2015), increase resistance to heat stress in Caco-2 cells (Varasteh *et al.*, 2015), reduce induced inflammatory responses in Caco-2 cells (Zenhom *et al.*, 2011). At the present time, however, we have no data on such effects *in vivo* and the role of these effects in the gut is far from clear.

Prebiotics, including fructans, can also have more direct effect and prevent attachment of intestinal pathogens to the host cell receptor sites by mimicking these structures. Here again the effect seems clearly linked to the chain length of β-fructans with longer chains (DP >20) being more effective antiadhesives than short chains (DP 2-10) (Shoaf *et al.*, 2006).

Anti-tumour activity of dietary carbohydrates

Perhaps the most investigated indirect immune modulatory activity of fibres hitherto is the growth inhibiting effects on tumours. In particular the β-glucans have been investigated for the antitumour activity (reviewed in Ramberg *et al.*, 2010), but also κ-carageenan has been investigated. Whereas β-glucans have been shown to have a beneficial effect, carrageenans have been associated with induction of ulcerative colitis and colon cancer in animal models (Bhattacharyya *et al.*, 2017). Carrageenans are seaweed extracts comprising high molecular weight sulphated polygalactosides. They are used in foods as thickening and gelling agents. When degraded to lower molecular weight forms, they have been shown to induce ulcerative colitis and colon cancer in laboratory animals (Pintauro and Gilbert, 1990; Tobacman, 2001). The sulphate groups are hypothesised to play a role in their detrimental effects.

The effects of β-glucans on tumour growth and survival depend on the type of tumour and have shown most efficient when the fibre treatment is combined with injection of antibodies against the tumour cells (e.g. Modak *et al.*, 2005). Although the mechanism of action is not known, different mechanisms of action have been suggested. Among those are that the fibres promote antibody dependent cellular cytotoxicity, which facilitates the elimination of antibody coated cancer cells by macrophages and other phagocytotic cells (Ross *et al.*, 1999) and that the fibres stimulate hematopoiesis hereby leading to a faster renewal of leucocytes during chemotherapy and radiation treatment.

Effects of fibres on immune cell activity and cytokine production

Apart from the immune-mediated effects in cancer, fibres also stimulate an increase in number and in activity of immune cells. For instance, lentinan has been shown to increase T cell numbers, and the ratio of helper cells over suppressor cells (Hanaue *et al.*, 1989). Also, laminarin has been shown to increase dectin-1 expression on macrophages in the gut-associated lymphoid tissue (Rice *et al.*, 2005). In terms of activity of immune cells, β-glucans have been shown to increase plasma immunoglobulins (Chan *et al.*, 2007; Yun *et al.*, 2003), neutrophil respiratory burst (Murphy *et al.*, 2007), and cytokine production (Oh *et al.*, 2008; Yap and Ng, 2003). Other fibres such as pectins, have also been shown to affect cytokine levels in plasma (Lim *et al.*, 2003). The references provided are just a few examples of numerous reports published in this field.

A few studies in humans have also been carried out. Some with clear results, others with non-significant trends. For instance, arabinogalactan resulted in a trend towards increased white blood cells and oxidative burst activity (Nantz *et al.*, 2001), but showed a clear increase in IgG subtypes (Ramberg *et al.*, 2010). Other examples with β-glucans have been discussed above.

Conclusions and future perspectives

Fibres are interesting dietary ingredients with various functionalities. These range from normalising stool frequencies to immunomodulatory and anti-tumour activities. Some microbial and dietary fibres have been studied in detail already, such as fungal or grain-derived β-glucans and various pectins. However, due to great structural diversity amongst both the β-glucans and the various pectins, structure-function relationships are still (eagerly) awaited. Unravelling these structure-function relationships will perhaps be only half a decade away with the development of reporter cells that carry specific carbohydrate-recognition receptors (e.g. Vogt *et al.*, 2013), and the possibility to carefully modify existing bioactive polymers to decipher required oligosaccharide moieties and/or glycosidic linkages. Yet, complex carbohydrates, such as pectins, may carry many different structures that interact with multiple different lectins. Therefore, cells with single receptors are unlikely to tell the whole picture. Here, the ability to create knock out lines of rodents will help in our understanding of the role of specific receptors. This together should lead to a full understanding of the bioactivity of these important dietary ingredients. In this chapter, the focus has been on the direct and indirect immunomodulatory effects of dietary carbohydrates. Research so far has established that fibres may indeed affect the immune system both directly and indirectly. However, more knowledge is required regarding, e.g. absorption of fibres from the GIT and the relative impact of direct and indirect action, particularly regarding *in vivo* effects, to obtain a full understanding of how fibres affect our immune system.

6. Analysis of non-digestible carbohydrates

The analysis and structural characterisation of carbohydrates, involving the determination of their DP, monomeric composition and order, type of glycosidic linkage and the identification of any anomeric mixture is required to fully understand the functionality of carbohydrates and their correlation with the chemical structure. This is particularly important in the case of fermented carbohydrates as they are rarely pure and usually comprise multiple molecular structures, stressing the necessity of a comprehensive but challenging structural characterisation. Their analysis, therefore, normally requires the combination of sensitive and high-resolution separation techniques combined with powerful identification tools. Additionally, prior to structural and functional characterisation, fractionation or purification may be mandatory in most cases. These processes can be also necessary to provide enrichment for these oligosaccharides for chromatographic analysis of complex carbohydrate mixtures and for commercial production. Selection of an appropriate fractionation technique and optimisation of the methodology will depend on the analytes of interest, the type of sample and the required level of detail for each case (Moreno *et al.*, 2014a) and goes beyond this review. Instead, this section will focus on the fundamentals of the main analytical techniques currently used for the analysis of prebiotics, as well as on some relevant applications developed for the study of milk oligosaccharides and manufactured prebiotics.

Fundamentals of analytical techniques used for the analysis of dietary carbohydrates

Instrumental methods based on separation and/or spectroscopic techniques are currently the most widespread for oligosaccharide identification, quantification and characterisation. Separation techniques (chromatographic and electrophoretic) coupled to suitable detectors give

quantitative information on concentration of individual components. Among these, capillary electrophoresis (CE), gas chromatography (GC) and high-performance liquid chromatography (HPLC) are the most-widely chosen procedures for routine analysis of oligosaccharides. However, the structural knowledge afforded by separation techniques is usually limited, and therefore, spectrometric and spectroscopic techniques such as mass spectrometry (MS and tandem MS) or nuclear magnetic resonance (NMR) are frequently necessary to provide a detailed structure for an isolated compound or a simple mixture. This is particularly relevant in the case of novel synthesised or non-commercially available carbohydrates. Thus, coupling several techniques through 'hyphenated' methods (Sarker and Nahar, 2006) is often necessary to gather all the required information for a comprehensive oligosaccharide characterisation, and consequently, to gain insight into structure-function relationships. MS can be coupled to a wide range of chromatographic and electrophoretic techniques providing structural information by analysis of MS^n fragmentation patterns. Nevertheless, it is not currently easy to accurately determine the structure of an oligosaccharide by MS alone although MS/MS techniques can provide much useful information. Therefore, the technique of choice for providing detailed information about the structure of unknown compounds is NMR spectroscopy. This is particularly true in the case of oligosaccharides due to the lack of available authentic standards. NMR is able to establish structure by analysis of connectivity networks on the atomic level (Seger *et al.*, 2013; Van Leeuwen *et al.*, 2014a).

Capillary electrophoresis

CE is an attractive and powerful technique for the separation of polar compounds such as oligosaccharides, and requires limited amounts of sample, minimal solvent and reagents, as well as giving rapid throughput and high resolution (Mantovani *et al.*, 2016). Separation is achieved due to differences in migration of the analytes in a narrow fused-silica capillary filled with a conducting buffer solution under the influence of an applied high voltage electric field. There are different operation modes of CE, including capillary zone electrophoresis (CZE), micellar electrokinetic chromatography (MEKC), capillary gel electrophoresis (CGE), capillary electrochromatography (CEC) and capillary affinity electrophoresis (CAE). Recent advances in miniaturised CE have led to the development of microchip electrophoresis, decreasing oligosaccharide analysis times (Mantovani *et al.*, 2018; Matsuno *et al.*, 2014). The absence of molecular charge, ionizable molecules, chromophore or fluorophore groups in oligosaccharides makes their direct detection only viable by UV at wavelengths lower than 200 nm. However, the indirect detection of oligosaccharides by CE can be routinely accomplished using background electrolytes containing chromophoric compounds or following derivatisation of reducing carbohydrates. This is normally performed by reductive amination using amines with chromophores or fluorophores (Campa *et al.*, 2006). In consequence, the more common detectors combined with CE analysis are UV absorbance (including diode array detectors (DAD)), laser-induced fluorescence (LIF) (Kottler *et al.*, 2013) and coupled mass spectrometers.

Gas chromatography

The high resolving power, sensitivity and selectivity of GC are extremely advantageous for oligosaccharide analysis despite their low volatility. Derivatisation is required to convert polar carbohydrates into volatile derivatives making them suitable for GC analysis. A wide variety of derivatisation methods has been developed (Ruiz-Matute *et al.*, 2011). The two detectors that are mainly used in GC analyses of oligosaccharides are flame ionization (FID) and mass spectrometry (MS) detectors.

Oligosaccharide derivatives are most commonly analysed on polysiloxane based stationary phases (the so-called 'silicones') since they present good thermal stability and high permeability to solutes and are available in a wide polarity range (Sanz *et al.*, 2009). As carbohydrates are usually present in mixtures, programmed temperature elution (commonly from 60 to 330 °C) is used to separate each compound. The determination of high DP (5-7) oligosaccharides can be achieved by high temperature GC (temperatures close to or above 360-370 °C) making use of thermostable bonded stationary phases.

High-performance liquid chromatography

HPLC is one of the most-widely used techniques for oligosaccharide analysis, with separation based on degree of polymerisation, linkage pattern, monomeric composition, and isomerism. Many methods have been developed using different operation modes and detectors. As indicated above, the absence of chromophore and fluorophore groups, along with the fact that carbohydrates only absorb in ultraviolet (UV) at very short wavelengths, prevents direct detection of oligosaccharides by fluorescence or UV detectors unless a derivatisation step is performed. This usually results in higher sensitivity and improvement of oligosaccharide resolution. In most cases, therefore, it is necessary to rely on universal detectors such as refractive index (RID) or evaporative light-scattering (ELSD) detectors, commonly coupled to size-exclusion chromatographic (SEC) or hydrophilic-interaction liquid chromatography (HILIC) columns. In addition, electrochemical detectors, such as the pulsed amperometric detector (PAD), are generally the detection methods of choice for high performance anion exchange chromatography (HPAEC). These methodologies are very convenient in routine monitoring of oligosaccharide synthesis for qualitative identification and quantitative

measurements because, in most cases, sample preparation is limited to dilution and filtration, to achieve the proper carbohydrate concentration and to remove any insoluble material or impurities of the sample. Moreover, the use of MS detectors coupled to HPLC systems has considerably enriched the field of carbohydrate identification (Sanz *et al.*, 2009).

High performance anion exchange chromatography (HPAEC), hydrophilic interaction chromatography (HILIC) and graphitised carbon chromatography (GCC) are the most commonly used for prebiotic analysis. The main advantage of the latter two techniques is that the mobile phases are highly compatible with MS allowing direct identification and partial structural characterisation of oligosaccharides.

High-performance anion exchange chromatography

The advent of HPAEC in 1983 at the hand of Rocklin and Pohl (1983) revolutionised carbohydrate analysis, significantly increasing knowledge about the oligosaccharide composition of a wide variety of products. HPAEC works on the principle that carbohydrates are weak acids with pK_a values in the range 12-14. This means that the hydroxyl groups are ionisable under alkaline conditions. The most common anion-exchange columns employed in the analysis of carbohydrates by HPAEC-PAD are Dionex™ CarboPac™ columns of polymeric, non-porous, pellicular anion exchange resins which exhibit resistance to pH values higher than 13. Carbohydrate retention increases with decreasing pK_a value; so separation is largely by molecular weight but monomeric composition, glycosidic linkage and charge also have an effect. As an example, the elution order can change when families of oligosaccharides with different linkage types are mixed, e.g. isomaltohexaose elutes before maltotriose (Sanz *et al.*, 2009). During HPAEC, the mobile phase usually consists of aqueous sodium hydroxide with a gradient addition of sodium acetate or sodium nitrate.

Oligosaccharides are detected and quantified by PAD by measuring the electrical current generated on oxidation at the surface of a working electrode. Gold is often the metal of choice for this electrode for oligosaccharide detection. Oxidation products gradually damage the electrode surface, causing signal degradation. Cleaning between measurements is accomplished by applying a potential sequence that reactivates the electrode surface between measurements; this is the basis of pulsed amperometry (Thermo Scientific. DIONEX Technical Note 20).

The extremely high resolution of this approach enables detection of carbohydrates at picomole levels with excellent signal-to-noise ratios. In addition, this technique is extremely selective to isomers and oligosaccharides of high DP. Nevertheless, PAD detectors have a limited range of linear response and carbohydrates have a wide range of response factors requiring the need for specific standards for calibration. On the other hand, the salts in the mobile phase are inconvenient when coupling to MS, although their removal prior to MS detection is technically possible as it will be explained later on.

Hydrophilic-interaction liquid chromatography

HILIC has been extensively used for the analysis of oligosaccharides, even before Andrew Alpert firstly mentioned this term in 1990 (Alpert, 1990). Nowadays, similar conditions to those described by Linden and Lawhead (1975) or Palmer (1975) more than four decades ago (i.e. amino-silica stationary phase and a mixture of acetonitrile and water as mobile phase (75:25, v/v)) are still used to separate and detect oligosaccharides by LC-RID under isocratic mode or by LC-ELSD using gradient elution. Furthermore, with the growing popularity of HILIC, the commercial availability of these columns has dramatically increased, and several stationary phases bonded with other polar substituents, such as cyano, hydroxyl, amide, zwitterionic or diols, among others, have been used for carbohydrate analysis (Buszewski and Noga, 2011). HILIC is now considered to be a consolidated and established analytical tool for the separation of oligosaccharides and other polar compounds, which are poorly retained under reversed-phase liquid chromatography (RP-LC) conditions.

HILIC uses polar stationary phases and analyte retention increases with polarity. The aqueous organic mixtures used as mobile phases are totally compatible with MS. Acetonitrile is the most often used water-miscible organic solvent for HILIC separations (Jandera, 2011). Retention can be adjusted by varying the fraction of organic solvent and water in the mobile phase; the higher the aqueous content, the more soluble the sugars, resulting in decreased retention (Greco and Letzel, 2013).

Oligosaccharides generally elute in order of increasing DP. However, monomeric composition and glycosidic linkage impact on polarity of oligosaccharides with the same DP. Thus, ketose sugars elute earlier than aldoses and non-reduced oligosaccharides before reduced ones. If solubility problems do not occur, separation of oligosaccharides with high DP can be achieved, albeit with a decrease in sensitivity at long retention times.

Graphitised carbon chromatography

Although the use of columns packed with charcoal (activated carbon) has been a widely used method for preparative separation of oligosaccharides (Whistler and Durso, 1950), it was only two decades ago that graphitised carbon was introduced as stationary phase in LC (Koizumi, 1996). Although the separation mechanism is still under

debate, both hydrophobic and ionic interactions are involved (Lane and Hickey, 2014; Pabst and Altmann, 2008). The surface of this graphitic carbon is stereo-selective with the capacity to resolve geometric isomers and other structurally related compounds (Bao *et al.*, 2013). Mobile phases are like HILIC with high percentages of organic modifiers such as acetonitrile, making MS the preferable detection method.

Mass spectrometry

A thorough description of procedure for MS analysis of carbohydrates is out of scope of this review and some recent reviews on this topic have been recently published (Ruhaak *et al.*, 2018; Wang *et al.*, 2021). Briefly, there are currently a wide range of commercially available mass analysers that differ in sensitivity, resolution, dynamic range, mass accuracy and fragmentation behaviour (Sprenger and Roepstorff, 2012). The most commonly used in oligosaccharide studies are quadrupole (Q), linear ion trap (LIT/LTQ), quadrupole ion trap (QIT), time of flight (TOF) and Orbitrap, as well as tandem systems composed of two or more coupled analysers of the same or different types, such as QqQ, Q-TOF, QqQ-LIT (Hernández-Hernández and Roepstorff, 2014). In addition, recent advances have led to the use of novel type of mass spectrometers to oligosaccharides analysis, as it is the case of ion mobility mass spectrometry (IMMS) which is based on ions separation from their mass/charge, but also from their size and shape.

Nuclear magnetic resonance spectroscopy

NMR spectroscopy is a valuable tool for *de novo* structural elucidation of oligosaccharides. The technique relies on the spin properties of atomic nuclei under a strong magnetic field when stimulated with additional electromagnetic radiation and provides useful information for the structural elucidation of unknown chemical compounds. NMR works best if the investigated analyte is present in high purity; whenever mixtures are present, the number of overlapping signals of its spectra rises dramatically. Therefore, a potential disadvantage of this technique is that relatively large amounts of the purified compound are required as compared to MS. Nevertheless, this can be achieved by the preparative isolation of the oligosaccharide under investigation. Furthermore, NMR is a non-destructive technique, so the compound may be recovered after the analysis. Preferably, the sample should be dissolved in a solvent (usually in deuterium oxide, D_2O). NMR is, however, a rather specialist technique that requires careful assignment and interpretation of the acquired spectra (Seger *et al.*, 2013).

Modern high-resolution NMR instruments used for carbohydrate samples, typically 500 MHz or higher, are able to run a suite of one dimensional (1D) proton or carbon-13 (^{1}H and ^{13}C), 2D and 3D homo- and heteronuclear experiments to determine structures of oligosaccharides. The set of experiments and the strategy used for assignment and full structure elucidation largely depends on the compound to be identified. Smaller oligosaccharides, for which resonance overlap is not too serious and in which proton density is high, can be fully assigned based on homonuclear ^{1}H-^{1}H shift-correlation experiments such as COSY (correlation spectroscopy), TOCSY (total COSY), and NOESY (nuclear Overhauser effect spectroscopy), or ROESY (rotating-frame analog of NOESY). Heteronuclear single- or multiple-quantum correlation (HSQC or HMQC) and heteronuclear multiple-bond correlation (HMBC) are frequently used to assign signals from complex oligosaccharides. The introduction of these heteronuclear correlation experiments has dramatically reduced the required sample amount, so that these spectra can be recorded with milligram quantities on 1 mm microprobes (Bross-Walch *et al.*, 2005).

Applications of prebiotic analysis

Milk oligosaccharides

Human milk oligosaccharides (HMO) have been analysed either by direct UV detection at low wavelengths or after fluorescence derivatisation by CZE, MEKC (Bao *et al.*, 2007; Galeotti *et al.*, 2014; Koketsu and Linhardt, 2000; Oefner and Chiesa, 1994; Song *et al.*, 2002), CGE (Kottler *et al.*, 2013) or CAE using lectins (Kinoshita and Kakehi, 2014). CAE was also applied to the analysis of bovine colostrum oligosaccharides, and a data library was constructed for analysing unknown milk oligosaccharides (Nakajima *et al.*, 2006). Moreover, significant efforts have been made for the detection of milk oligosaccharides in biological samples, such as HMO present in faeces of breast- and formula-fed babies by CE-LIF-MS^n analysis of the fluorescent 9-aminopyrene-l,4,6-trisulfonate (APTS)-labelled carbohydrates (Albrecht *et al.*, 2011).

Several studies have developed efficient HPAEC-PAD methods for analysis of HMO as recently reviewed by Lane and Hickey (2014) and Mantovani *et al.* (2016). As relevant examples, Kunz *et al.* (1996) separated neutral and acidic oligosaccharides on a CarboPac™ PA-1 column, whereas other studies dealt with the determination of HMO in infant urine and serum and the demonstration of their minimal digestibility (Coppa *et al.*, 1990, 1999; Gnoth *et al.*, 2000). More recently, Coppa *et al.* (2011) used a CarboPac™ PA-1 column and a PAD II detector for the qualitative and quantitative characterisation of up to 18 HMO in milk samples collected at the end of the first month after delivery. For longitudinal studies of HMOS a Carbopac PA 100 was used analysing the neutral and acidic fractions separately (Thurl *et al.*, 2010).

There are multiple HILIC methods for analysis and characterisation of milk oligosaccharides. Marino *et al.* (2011) developed a method for free milk oligosaccharide profiling based on 2-aminobenzamide labelling and HILIC using bovine colostrum as a case study. In that study, up to 33 different structures were identified using a combination of HILIC, exoglycosidase digestion, desalting and offline MS/MS analyses. Fong *et al.* (2011) developed a HILIC-MS method that allowed the quantification of six different oligosaccharides (3'- and 6'-sialyllactose, 3'- and 6'-sialyllactosamine, disialyllactose, and *N*-acetylgalactoaminyl lactose) in bovine milk, bovine colostrum, and infant formulae. A HILIC-MS^n method, capable of quantifying 13 major oligosaccharides within a single run, was successfully applied to 32 bovine milk samples collected after three different feeding treatments (Liu *et al.*, 2014). Researchers have also begun to explore the oligosaccharide profile of less common animal milk samples, such as horse (Difilippo *et al.*, 2015b) or porcine (Difilippo *et al.*, 2016) by HILIC-MS^n. More recently, a HILIC-MS^n method under multi-reaction monitoring (MRM) mode was applied for the characterisation of 12 HMO whose DP ranged from 3 to 6 (Zhang *et al.*, 2019). Interestingly, other HILIC-based methods with great sensitivity have been developed after fluorescent labelling with 2-aminobenzamide (2AB) of HMO for their analysis in maternal serum in pregnant women over the course of gestation (Jantscher-Krenn *et al.*, 2019) and in cord-blood to investigate maternal-to-foetal transport (Hirschmugl *et al.*, 2019). Likewise, using the same fluorescent labelling, (Austin and Bénet, 2018) analysed up to 20 HMO by LC-MS applying minimal sample-prep and an on-line cleanup. This method has been successfully applied to several large cohort studies (Austin *et al.*, 2019; Binia *et al.*, 2021; Lefebvre *et al.*, 2020; Samuel *et al.*, 2019).

On the other hand, in the in the early 90s FAB-MS and MALDI-MS were applied to the analyses of complex oligosaccharides (Egge *et al.*, 1991; Stahl *et al.*, 1997).

A series of pioneer works published by Lebrilla and co-authors described the application of GCC for the analysis of HMO (Niñonuevo *et al.*, 2005, 2006; Totten *et al.*, 2012; Wu *et al.*, 2010, 2011). These studies were subsequently expanded to determination of HMO in urine (De Leoz *et al.*, 2013), faeces (Davis *et al.*, 2016; De Leoz *et al.*, 2013) and plasma from infants (Ruhaak *et al.*, 2014). More recently, LC-MS based methods using a porous graphitic column has been developed for the identification and quantification of 3'- and 6'-sialyllactose in human milk (Csernák *et al.*, 2020), as well as 16 neutral and acidic HMO (Tonon *et al.*, 2019). Many of these studies used nano-liquid chip-based technologies, mainly coupled to MS or tandem MS (MS/MS), allowing sensitive detection and compositional verification. Moreover, nano-LC-chip technology coupled to time of flight (TOF) mass spectrometry has been applied to the comprehensive characterisation of milk oligosaccharides present in several animal species, such as bovine (Barile *et al.*, 2010; Tao *et al.*, 2008), caprine (Martín-Ortiz *et al.*, 2016, 2017; Meyrand *et al.*, 2013) and porcine (Tao *et al.*, 2010). The micro-chips packed with graphitised carbon solid phase provide reproducible retention times and separate structural isomers. In consequence, a library containing mass, retention time, and fragmentation of neutral HMO was initially developed (Wu *et al.*, 2010), then, complemented with a library for sialylated HMO (Wu *et al.*, 2011), and, based on an UHPLC-HILIC-MS^n method, for neutral and acidic HMO (Remoroza *et al.*, 2018). This approach was also expanded to bovine (Aldredge *et al.*, 2013) and caprine (Meyrand *et al.*, 2013) milk oligosaccharides. In another study, Bao *et al.* (2013) quantified 11 neutral oligosaccharides in human milk based on the excellent separating power of GCC using a Hypercarb™ column and a single quadrupole mass spectrometer. Recently MS/MS generated MRM fragments of were applied to identify and quantify the unlabelled HMO (Mank *et al.*, 2018). This analytical approach has been further applied to the analysis of mature human milk samples collected at 6 and 16 weeks post-partum, allowing the successful characterisation of the most abundant HMO up to hexasaccharides and, for the first time, also the assignment of blood group A and B tetrasaccharides (Mank *et al.*, 2020).

A GC-MS method has been developed for the identification and quantification of 2'-FL and 3-FL in human milk as TMS-oxime derivatives (Balogh *et al.*, 2015), whilst the application of NMR spectroscopy to structural analysis of HMO and other carbohydrates have been covered in depth by Maliniak and Widmalm (2014). Finally, an approach, based on the combined use of IMMS and well-defined oligosaccharide standards, has been successful to determine the exact positions of fucosyl residues in structurally complex glycans, such as sialosyl-fucosyl oligo-*N*-acetyllactosamine derivatives, which is relevant for HMO analysis (Sastre Toraño *et al.*, 2019).

Lastly, some enlightening and comprehensive information has become available from the structural analysis of HMO by NMR and molecular simulations (Gu *et al.*, 2021; Maliniak and Widmalm, 2014; Van Leeuwen *et al.*, 2014, 2018). For further information, critical reviews on HMO analysis evaluating the advantages and limitations of these techniques have been published during the last years as those of Auer *et al.* (2021) and van Leeuwen (2019).

Manufactured prebiotics

GOS have been analysed by CE-UV-DAD with a precolumn derivatisation (Petzelbauer *et al.*, 2000; Splechtna *et al.*, 2006) and by CE-LIF after derivatisation with the fluorescent dye APTS (Albrecht *et al.*, 2010). Using a similar approach, GOS present in biological samples of piglets, such

as serum, urine, faeces and caecal digesta (Difilippo *et al.*, 2015a), as well as glucomannan oligosaccharides (Albrecht *et al.*, 2009) and carbohydrates up to DP 7 derived from the colonic microbiota fermentation of β-glucan (Beeren *et al.*, 2015) have been successfully analysed by CE-LIF.

GC has been applied for the determination of GOS synthesis catalysed by β-galactosidases from different microbial sources either as trimethylsilyl (TMS)-oxime (Cardelle-Cobas *et al.*, 2009; Corzo-Martínez *et al.*, 2013; Hernández *et al.*, 2009; Montilla *et al.*, 2012) or alditol acetate derivatives (Coulier *et al.*, 2009). GC-MS has also been used for characterisation of novel GOS derived from lactulose (Hernández-Hernández *et al.*, 2011), as well as for the determination of *in vivo* digestibility of commercial GOS and novel GOS products derived from lactulose in ileal samples of individual rats (Hernández-Hernández *et al.*, 2012a).

The composition of GOS has also been analysed by HPAEC-PAD and glucose and galactose isomers can be efficiently separated (Cardelle-Cobas *et al.*, 2008; Corradini *et al.*, 2002; Martínez-Villaluenga *et al.*, 2008; Van Leeuwen *et al.*, 2016). Recently, HPAEC-PAD has been also used to monitor GOS fermentation in piglet faecal culture (Difilippo *et al.*, 2016), whilst its unusual coupling with MS, which requires the removal of salts present in the mobile phase prior to MS detection, using an LTQ with an ESI source operated in both positive and negative mode has been also used for GOS determination (DP 2-4) (Coulier *et al.*, 2009).

A combination of hydrophilic interaction liquid chromatography (HILIC) and electrospray ionization mass spectrometry (ESI-MS) under positive polarity using a microTOF as a mass analyser was useful to determine the DP (from 3 to 7) of GOS (Sinclair *et al.*, 2009). Hernandez-Hernandez *et al.* (2012b) assessed the ability of three different stationary phases: (1) sulfoalkylbetaine zwitterionic, (2) polyhydroxyethyl aspartamide and (3) ethylene bridge hybrid (BEH) with trifunctionally bonded amide, using different solvents and modifiers for the analysis of complex mixtures of GOS. The best separation was achieved on the BEH amide stationary phase, using acetonitrile:water with 0.1% ammonium hydroxide as mobile phase, where the most of oligosaccharides were successfully resolved. An UHPLC-MS^2 approach using a porous graphitic column allowed the recognition of more than 100 different GOS structures in one single run after reduction, including reducing and non-reducing GOS isomers (Logtenberg *et al.*, 2020). Interestingly, these authors reported MS fragmentation rules to distinguish reducing GOS isomers with a mono-and disubstituted terminal glucose. Likewise, in a pioneering work, the BEH amide stationary phase in the first dimension was coupled to a reversed-phased column in the second dimension for the first application of on-line comprehensive two-dimensional LC (LC × LC) to the successful separation of previously derivatised commercial prebiotic mixtures of GOS and gentio-oligosaccharides (GEOS) (Martín-Ortiz *et al.*, 2019).

Carevic *et al.* (2016) have recently reported a complete elucidation of GOS structures from DP 2 to DP 4 based uniquely on IMMS-MS/MS. Findings coming from this paper gave insight into the mechanism of transgalactosylation and highlighted the variation of particular β-linkages during GOS synthesis. Previously, Neri *et al.* (2011) and Hernandez-Hernandez *et al.* (2012b) and Maina *et al.* (2013) provided useful MS^n data on GOS and IMOS fragmentation, respectively, with an ESI source and an ion trap mass spectrometer. A series of recent papers have provided NMR data on GOS up to DP5 from different sources (Van Leeuwen *et al.*, 2014a,b, 2016). In fact, a library of NMR data was built up from DP2 and DP3 compounds to enable fast screening of complex GOS mixtures in terms of major and minor linkage types.

Concerning FOS, a conventional GC-FID procedure for the determination of oligosaccharides up to DP 7 in different foodstuff was developed by Montilla *et al.* (2006) using a temperature program with 360 °C as the maximum temperature and an HT5 (non-polar) aluminium-clad capillary column. This method has also been applied for the quantification of GOS and FOS in several commercial infant formulas (Sabater *et al.*, 2016).

A detailed review article of HPAEC-PAD as a powerful tool to evaluate carbohydrates of food interest was published by Corradini *et al.* (2012). The suitability of HPAEC-PAD to separate FOS of different DP up to 80 and more (Finke *et al.*, 2002) has been extensively reviewed (Borromei *et al.*, 2009; Corradini *et al.*, 2004; Sangeetha *et al.*, 2005). Nevertheless, separation of FOS using new optimised HPAEC methods are still being developed (Rodríguez-Gómez *et al.*, 2015). Brokl *et al.* (2011) evaluated reversed-phased LC (Luna C18 column), HPAEC (CarboPac® PA 100), HILIC (ethylene bridge hybrid column) and GCC (Hypercarb column) for the analysis of complex mixtures of neutral oligosaccharides, including FOS, GEOS and dextransucrase cellobiose acceptor oligosaccharides. The authors concluded that HILIC and GCC were the most suitable LC modes for the analysis of neutral oligosaccharides based on their separation potentials. HILIC efficiently analysed oligosaccharides of different DP whereas GCC provided the best separation of isomeric oligosaccharides with the same molecular weight. FOS have been also efficiently separated using classical HILIC conditions, i.e. on amine modified silica columns under isocratic conditions with acetonitrile and water as mobile phase and a refractive index detector (Díez-Municio *et al.*, 2016; Rodríguez-Gómez *et al.*, 2015). Similar column and chromatographic conditions have been successfully applied

for the efficient separation of hetero-fructooligosaccharides, such as maltosyl- (Díez-Municio *et al.*, 2013), lactosyl- (Díez-Municio *et al.*, 2015) and raffinosyl-oligofructosides (Díez-Municio *et al.*, 2016). Different types of FOS together with raffinose have been also determined and characterised by different HILIC-MS methods with gradient elution in complex food matrices, such as wheat flour (Liu and Rochfort, 2015) and artichoke industrial waste (Machado *et al.*, 2015). Likewise, Harrison *et al.* (2012) described fragmentation patterns which allowed the differentiation between inulins and fructans by LIT-MS^n. MALDI MS has been applied to fructan analyses including *in situ* analyses of plant tissues (Stahl *et al.*, 1997).

Finally, Schols *et al.* (2000) demonstrated the ability of HILIC using a TSK-gel Amide-80 column to separate acetylated XOS. Westphal *et al.* (2010) developed a GCC-ELSD-MS^n method for the characterisation of a wide array of oligosaccharides derived from plant cell wall polysaccharides, including XOS, manno-oligosaccharides and galacturonic acid oligosaccharides. Similarly, AXOS have been also structurally characterised by HILIC-MS^n using a BEH amide column (Juvonen *et al.*, 2019). In an elegant study, Daas *et al.* (2000) developed an HPAEC-PAD method based on the CarboPac PA-1 column for the analysis of methyl-esterified galacturonic acid from pectin. More recently, a HILIC-ELSD/MS^n method using a LTQ as mass analyser was developed to determine pectic-oligosaccharides derived from different sources, including sugar beet pectin (Remoroza *et al.*, 2014) or rose hip fruits (Ognyanov *et al.*, 2016), whereas a method based on the coupling of HILIC to traveling-wave IMMS allowed the precise identification of isomeric sugar beet pectic oligosaccharides after separation by ion mobility and subsequent MS fragmentation (Leijdekkers *et al.*, 2015).

7. Model systems to study carbohydrate fermentation in the gut

Despite their physiological relevance, *in vivo* animal or human trials are limited for screening the effects and possible mechanism of action of such dietary ingredients and/or drugs, as they are confronted with the high complexity of the GIT environment and the lack of easy access to the gut (Sousa *et al.*, 2008). *In vivo* studies are thus often restricted to faecal samples which do not provide information on dynamic microbial processes at the site of fermentation in the gut. Therefore, even if they suffer from the absence of a physiological host environment, *in vitro* simulations, which can closely mimic the microbial composition and activity in different regions of the human gut, offer unique advantages (Macfarlane and Macfarlane, 2007; Payne *et al.*, 2012a; Venema and Van den Abbeele, 2013). Firstly, *in vitro* models provide insights in different steps of the fermentation process by allowing a dynamic sampling over time in different consecutive regions of the human colon. Moreover, as *in vitro* models are standardised, they provide results with a high reproducibility. Importantly, there are no ethical constraints for using *in vitro* models, so that pathogens, toxic or radioactive compounds can be used without ethical approval. *In vitro* approaches thus offer the possibility of mechanistic studies and developing hypotheses that need to be proven (or disproven) in human clinical trials. It is however important to create conditions that closely resemble the *in vivo* situation. The better a system can simulate the real situation, the higher is the physiological significance of the information obtained (Guerra *et al.*, 2012).

To study the effect of food components and drugs on microbial composition and activity, numerous *in vitro* experiments have been applied in the past, both for screening different products and mechanistic studies. The debate has been however, how representative the outcome of such experiments is for human (or animal) application. A first validation of conventional *in vitro* models was provided by molecular tools that allowed characterising the *in vitro* microbiota in high phylogenetic resolution. This revealed that *in vitro* models can maintain a human-like gut microbiota (Rajilić-Stojanović *et al.*, 2010; Van den Abbeele *et al.*, 2010). The microbial bias that was observed during these studies, i.e. depletion in butyrate-producing *Clostridium* cluster IV and XIVa sp. *In vitro*, was alleviated by incorporating a simulation of the intestinal mucosal surface (Van den Abbeele *et al.*, 2012a). The latter study also demonstrated that the microbial spectra unique to specific human subjects (Rajilić-Stojanović *et al.*, 2010; Van den Abbeele *et al.*, 2010) can be maintained *in vitro*. Besides overall microbial composition, specific activities can also be maintained *in vitro,* as shown for the bio-activation of phytoestrogens. Upon inoculation of *in vitro* models with a faecal sample of a person with high or low bioconversion rates *in vivo*, this specific (high or low) activity was maintained in the corresponding *in vitro* microbiota (Possemiers *et al.*, 2008). Also, when comparing microbial fermentation spectra of different prebiotic compounds in different *in vitro* models for the human GIT, similar observations were done across different *in vitro* models (Van den Abbeele *et al.*, 2013).

Finally, also results from human clinical trials, such as the bifidogenic effect of fructo-oligosaccharides (FOS) or inulin (Van den Abbeele *et al.*, 2011; Van Nuenen *et al.*, 2003), as well as the butyrogenic effect of resistant starches (Fässler *et al.*, 2006), can be reproduced *in vitro*. This broad spectrum of validation studies has encouraged researchers to use *in vitro* systems to study the role of the gut microbiota in health and disease. Faecal donations can be collected from different age groups (babies, adults, elderly), different geographical locations, or people with different disorders or diseases, such as IBD (Van Nuenen

et al., 2004), and obese versus lean individuals (Aguirre *et al.*, 2016; Venema, 2010).

As inoculum two strategies are employed: (1) faecal inocula from individual donors are used, as well as (2) pooled samples from several donors. Both have their pros and cons. When testing individual samples, the starting inoculum is always different between different experiments, unless a multitude of experiments can be run in parallel. However, with the current existing models this is not possible A pooled (and stored) microbiota that allows for approximately 100 different runs, always starting with the same starting microbiota, allows for direct comparison between experiments. Also, when collecting large quantities of microbiota is a problem, e.g. for newborns, pooling may be an outcome. For the major metabolic activities of the gut microbiota, carbohydrate and protein fermentation, it has been shown that pooling gives the same outcome as using the individual samples, likely due to the functional redundancy of microbes (Aguirre *et al.*, 2014). However, for other microbiota activities, such as polyphenol degradation, methane production and others, pooling may lead to a skewed picture. E.g. for methane production, it has been shown that this occurs only in about one-third of the human individuals (De Lacy Costello *et al.*, 2013), and is unclear what happens if a methanogenic and a non-methanogenic microbiota are combined in a pool.

Luminal models of the gut

Short-term batch incubations

The simplest and most frequently used *in vitro* models to study the gut microbiome are static batch fermentations. These are usually performed in small reactor vessels or test-tubes, in which a variety of cultures, such as specific strains, intestinal or faecal microbial communities from animal or human origin, are tested for their ability to metabolise different substrates. These systems are far from physiological and suffer e.g. from accumulation of microbial metabolites inhibiting further microbial activity so that incubations usually need to be extended to periods of 24 h or more, to get full fermentation of the compound of interest. From more complex dynamic systems, it is clear that these compounds are fermented much quicker, usually within 4 h, indicating the limitations of these batch incubations. In fact, due to changes in pH, redox potential and community structure in these incubations, these systems will not provide accurate results (Gibson and Fuller, 2000). However, inter-individual variations in microbiota composition (De Weirdt *et al.*, 2010; Possemiers *et al.*, 2007) and the high throughput of these batch incubations make them valuable systems for initial screening assays.

Single stage reactors or semi-continuous systems

The majority of semi-continuous fermenter systems used to model the human colonic microbiota have been adapted from a system described by Miller and Wolin (1981), which in turn, was adapted from a system devised for use in rumen micro-ecology studies (Slyter *et al.*, 1964). Numerous variations on this system have been used and since they have been reviewed before (Rumney and Rowland, 1992), they will not be discussed here.

Multi-compartmental continuous systems

Batch experiments and single stage reactors offer a very easy and flexible screening tool. However, they tend to over-simplify the actual complexity of the processes occurring in the GIT. The application of well-designed, continuous, dynamic models allows the in-depth study of the gut microbiome, and the effect of the activity of selected food molecules or diets on the composition and activity of the gut microbiota, under representative environmental conditions.

MacFarlane and Gibson 3-stage continuous culture system

The research in the field of multi-stage systems started with the three-stage continuous culture reactor by the group of Gibson and Macfarlane in the 1980s (Gibson *et al.*, 1988; Macfarlane *et al.*, 1989a,b). The model consists of 3 connected vessels simulating the ascending, transverse and distal colon. The pH of these vessels is 6.0, 6.5, and 7.0, respectively, while the operating volumes are 0.3, 0.5, and 0.8 l with dilution rates of 0.08, 0.048, and 0.034/h. The total retention time in the system is approximately 63 h (Gibson *et al.*, 1988). These researchers also developed a growth medium, which was subsequently used by others in the field with minor modifications. The medium consists of a source of protein, and complex carbohydrates (i.e. pectin, xylan, arabinogalactan and starch and amylopectin, the latter two representing 'resistant starch') that are not digested by the human enzymes and thus reach the colon, where they are fermented by the gut microbiota.

SHIME

The SHIME® (Simulator of the Human Intestinal Microbial Ecosystem) system (Ghent University-Prodigest, Ghent, Belgium) consists of a succession of five reactors simulating both the upper and the lower digestive tract. Technically, it is an evolution of the simulator introduced by Gibson *et al.* (1988). The first two reactors, mimicking the stomach and small intestine, follow the fill-and-draw principle adding a defined amount of nutritional medium, pancreatic enzymes and bile to the simulated stomach and small intestinal compartments. The three colon compartments are continuously stirred reactors with constant volume (0.5, 0.8 and 0.6 l, respectively) and pH control (pH between

5.6 and 5.9, 6.15-6.4, and 6.6-6.9, respectively) (Van den Abbeele *et al.*, 2010). The overall residence time of the last three vessels, simulating the large intestine, is 72 h. Upon inoculation with a faecal microbiota, an initial two-week stabilisation period allows these human faecal microbes to adapt to the imposed *in vitro* conditions and to evolve from a faecal to a colon region-specific microbiota (Molly *et al.*, 1993, 1994; Van den Abbeele *et al.*, 2010). For different SHIME-units run in parallel, this stabilisation process is reproducible and usually followed by a two-week control period, and a two/three-week treatment period. Finally, a two-week wash-out period allows evaluating the reversibility of a treatment (Van den Abbeele *et al.*, 2010).

EnteroMix

The EnteroMix semi-continuous model is comprised of four glass vessels, representing the ascending, transverse, descending and distal colon, respectively. It is possible to run eight units simultaneously in parallel using the same faecal inoculum. The vessels have small working volumes (6-12 ml). The pH levels in the vessels are similar to conditions *in vivo* (pH 5.5, 6.0, 6.5 and 7.0, respectively) (Lamichhane *et al.*, 2018; Makivuokko *et al.*, 2005). Fresh faecal inoculum is added to each unit and vessel, and with three-hourly fluid transfers the feeding fluid containing the test substance is fed to the system, while each vessel is continuously stirred under anaerobic conditions (Forssten *et al.*, 2015). After 48 h, the simulation stops, and samples are collected from each vessel. The EnteroMix system has been used both for adult and infant simulations (Forssten and Ouwehand, 2017; Salli *et al.*, 2019).

Lacroix model

The three-stage model developed by the group of Lacroix at ETH in Zurich uses an immobilised microbiota with the idea to represent the complex bacterial community in the colon which is present both at planktonic and sessile states (Cinquin *et al.*, 2006b). Faecal inocula are immobilised in 1-2 mm diameter gel beads, composed of gellan gum, xanthan gum and sodium citrate (Cinquin *et al.*, 2004). The total mean retention time in the system with a total volume of 325 ml is set at 13 h by adjusting the feed flow rate to 25 ml/h, with mean retention times of 4, 5 and 4 h in the 3 consecutive vessels, respectively. The pH in the first two vessels is set at 5.9 and 6.2, respectively. The pH in the third vessel is not controlled but stabilised at the physiological range of 6.6-6.7 (Cinquin *et al.*, 2006b). Several studies have been done with a microbiota originating from infants (Le Blay *et al.*, 2009a; Payne *et al.*, 2012a).

TIM-2

The TNO dynamic computer-controlled *in vitro* model of the proximal colon (TIM-2), developed by TNO in the Netherlands, consists of 4 connected glass-jackets with flexible silicon membranes inside. By applying pressure on the flexible walls, peristaltic movements are achieved, causing the chyme to be mixed and moved through the system. This mixing is better than that achieved by stirring in a reactor so that even viscous solutions are properly mixed and transported through the model. The pH is maintained at 5.8 and the volume is 135 ml. Multiple units can be run in parallel using the same human faecal inoculum. This is the only *in vitro* model that contains a dialysis membrane that simulates uptake of microbial metabolites by the body, which also prevents accumulation of these metabolites in the lumen, which would otherwise cause inhibition of microbial activity. This allows the system to be inoculated with a high-density microbiota. After overnight stabilisation of the microbiota, experiments are performed over a short period (1 day to generally 3 days), although the system has been shown to be stable over a 3-week period (Minekus *et al.*, 1999; Venema *et al.*, 2003). Usually, only the proximal colon is mimicked in TIM-2, although the technology allows for incubations throughout the colon.

Models incorporating the mucosal compartment

Macfarlane and colleagues have studied the effect of adding mucin to their *in vitro* model (Macfarlane *et al.*, 2005), to determine how intestinal bacteria colonise mucus and to study physiological and enzymatic factors involved in the destruction of this glycoprotein. Colonisation of mucin gels was studied simulating conditions of nutrient availability and limitation characteristic of the proximal and distal colon, respectively. Gel samples were also taken for analysis of mucin-degrading enzymes and measurements of residual mucin sugars. Mucin gels were rapidly colonised, especially by members of the *Bacteroides fragilis* group, enterobacteria, and clostridia. These populations growing on mucin surfaces were shown to be phylogenetically and metabolically distinct from their luminal counterparts. Destruction of mucin in the gels was most extensive by bacteria growing under nutrient-limited conditions (Macfarlane *et al.*, 2005).

Recently, Van den Abbeele *et al.* (2012a,b) improved the SHIME model for the luminal microbiota by incorporating a simulated mucosal environment containing mucin-covered microcosms (mucosal SHIME or M-SHIME). Firstly, the mucosal environment allowed colonisation of specific lactobacilli such as *Limosilactobacillus mucosae* and *L. rhamnosus* GG via mechanisms corresponding to the *in vivo* situation (Van den Abbeele *et al.*, 2012b). Moreover, profound characterisation of the overall microbiota revealed

that, in correspondence with *in vivo* studies (Nava *et al.*, 2010; Shen *et al.*, 2010), the mucosal microbiota of the M-SHIME was enriched with species belonging to the *Firmicutes* (especially butyrate-producing *Clostridium* cluster IV and XIV species) as opposed to *Proteobacteria* and *Bacteroidetes* (Van den Abbeele *et al.*, 2012a). This is particularly interesting as *in vitro* models without mucosal environment have been shown the opposite (Rajilić-Stojanović *et al.*, 2010; Van den Abbeele *et al.*, 2010). Incorporating a mucosal environment in a dynamic *in vitro* simulation may thus lead to more *in vivo*-like microbial communities and allow to study the unique mucosal microbiota in health and disease as demonstrated for the unravelling of microbial factors in IBD (Van den Abbeele *et al.*, 2013; Vermeiren *et al.*, 2012; Vigsnaes *et al.*, 2013).

Applications of *in vitro* models to study the gut microbiota in health and disease

In vitro models have been used for numerous applications. It goes beyond the scope of this review to discuss all of these. We will highlight some applications which are important in determining the role of the microbiota in health and disease, providing examples of each of the multi-compartmental continuous systems as described above.

Due to the limited sampling possibilities of the human colon, it is hard to study microbial activity *in vivo*. Initially, *in vitro* models were used to study the effect of food components on the genotoxic activity of the microbiota. For instance, it was shown in the three-stage model by McBain and colleagues that an increase in retention time (which ranges from 20-120 h) augments the expression of enzymes thought to be involved in the production of genotoxic compounds, such as azoreductase, beta-glucosidase and nitroreductase (McBain and Macfarlane, 1997, 1998). Downregulation of these enzymes was accomplished by adding inulin or GOS (McBain and Macfarlane, 2001). Another early aspect that was studied using *in vitro* models was the effect of dietary components on microbial composition, where the aim was to increase the levels of presumably beneficial microbes such as bifidobacteria. Practically all multi-compartmental *in vitro* models have studied this. This started with research on inulin (Grootaert *et al.*, 2009; McBain and Macfarlane, 2001; Van den Abbeele *et al.*, 2011; Van Nuenen *et al.*, 2003) and fructo-oligosaccharides (Cinquin *et al.*, 2006), the most studied 'bifidogenic' substrates. In addition, also lactulose (Venema *et al.*, 2003), starch (Rose *et al.*, 2010), polydextrose and xylitol (Mäkeläinen *et al.*, 2007; Makivuokko *et al.*, 2005), polydextrose (Forssten *et al.*, 2015; Lamichhane *et al.*, 2016, 2018), GOS (Martinez *et al.*, 2013; McBain and Macfarlane, 2001), and arabinoxylan (Grootaert *et al.*, 2009; Van den Abbeele *et al.*, 2011) have been investigated. Also, synbiotic effects of probiotics and prebiotics have been studied (Mäkivuokko *et al.*, 2010; Van Zanten *et al.*, 2012). Frequently, the models are used for screening a large number of potential functional foods for their beneficial activities (Maathuis *et al.*, 2009). In addition, the production of SCFA (primarily acetate, propionate and butyrate) has been studied heavily. Especially butyrate has attracted considerable attention, as it is an indispensable fuel for our colonocytes (Roediger, 1980). For instance, it has been shown that enemas of butyrate diminish symptoms in people that suffer from IBD (for review see Hamer *et al.*, 2010). Moreover, recently also propionate has been shown to have beneficial effects in the host (for review see Al-Lahham *et al.*, 2010). To study which SCFA are produced from which carbohydrate substrate, experiments using the stable isotope ^{13}C have been used. This has been primarily studied in TIM-2. The isotopic label allows tracing of the label into microbial biomass and metabolites that are produced by these microbes (De Graaf *et al.*, 2010; Egert *et al.*, 2007; Kovatcheva-Datchary *et al.*, 2009b). Thus, it becomes possible to link the fermentation of a certain ^{13}C-labelled substrate by specific members of the microbiota to quantitative production of microbial metabolites. These metabolites are primarily the SCFA (Egert *et al.*, 2007; Kovatcheva-Datchary *et al.*, 2009a; Lamichhane *et al.*, 2018) although label incorporation in metabolites such as ethanol and several amino acids has also been demonstrated (Binsl *et al.*, 2010; De Graaf *et al.*, 2010). Examples of ^{13}C-labelled substrates that have been studied in TIM-2 are glucose (Egert *et al.*, 2007), starch (Kovatcheva-Datchary *et al.*, 2009a), lactose and inulin (manuscript in preparation) and GOS (Maathuis *et al.*, 2012). Because the label can be traced, the exact amount of SCFA produced by the microbiota from a given substrate can be calculated, and this allows for the exact determination of the amount of energy that is extracted by the microbiota from these substrates (Venema, 2010, 2012).

Whereas carbohydrate fermentation leads primarily to the beneficial SCFA, protein fermentation leads to production of several potentially toxic metabolites, such as the branched-chain fatty acids (BCFA), ammonia, amines, several phenolic and indolic compounds and hydrogen sulphide (Verbeke *et al.*, 2015). Since most gut microbes prefer to ferment carbohydrates over proteins, the balance of saccharolytic and proteolytic fermentation can be directed towards a healthier state by adding more carbohydrate to the 'diet' of the *in vitro* models. For instance, the addition of inulin, with different degrees of polymerisation, led to a significant reduction of production of toxic proteolytic metabolites (Van Nuenen *et al.*, 2003). Interestingly, the addition of *C. difficile*, mimicking overgrowth of this species after antibiotic treatment led to a two-fold increase in proteolytic metabolites, which could be counteracted by the addition of the different inulins (Van Nuenen *et al.*, 2003). Moreover, it has been shown that the proteolytic capacity of the microbiome of IBD patients seems to be higher than that of healthy individuals, as the production of toxic metabolites by an IBD microbiota was shown to

be significantly higher on the same 'diet' (Van Nuenen *et al.*, 2004). However, a limitation of this study was that the physiological conditions for healthy individuals were also used for IBD patients. It is likely that differences may exist between healthy individual and IBD patients with respect to pH, redox potential, gut transit and dietary habit and thus the substrates that make it to the colon. That gut transit is important for microbial metabolism has for instance been studied in the three-stage model (Child *et al.*, 2006; Macfarlane *et al.*, 1998; Smith and Macfarlane, 1996). It was shown that an increase in retention time led to higher production of putrefactive metabolites. Retention time also influences expression of enzymes involved in production of genotoxic compounds as discussed earlier (McBain and Macfarlane, 1997, 1998, 2001).

Furthermore, studies have been performed on the production of the antimicrobial reuterin from glycerol (Cleusix *et al.*, 2008; De Weirdt *et al.*, 2010), the prevention of growth of *Salmonella* (Le Blay *et al.*, 2009b; Zihler *et al.*, 2010, 2011), the effect of low iron availability on the metabolism of the gut microbiota (Dostal *et al.*, 2013), prevention of oxalate production which is involved in kidney stones formation (Lewanika *et al.*, 2007), the metabolism of polyphenols by the gut microbiota and the resulting effect on anti-oxidant capacity (Bracke *et al.*, 2008; Kong *et al.*, 2009; Possemiers *et al.*, 2008), and the effect of probiotics after or concurrent with antibiotic treatment (Rehman *et al.*, 2012).

CoMiniGut

A relatively novel *in vitro* parallel gut microbial fermentation screening tool, called Copenhagen MiniGut (CoMiniGut), with a working volume of only 5 ml consisting of five parallel reactor units that can be expanded with multiples of five to increase throughput has recently been introduced (Wiese *et al.*, 2018). The functionality of the CoMiniGut was first in experiments with inulin and the lactulose. Due to its small volume, it is particularly suited to test of scarce and expensive substrates, such a (chemically synthesised or purified) human milk oligosaccharides and potential novel prebiotics (Chapter 4), which are usually present in small quantities. Apart from Wiese *et al.* (2018), to our knowledge the model has not been studied further in carbohydrate fermentation.

Limitations and challenges in modelling colonic fermentation

While the use of continuous culture systems has allowed gaining mechanistic insight in many aspects of the human colon microbiota, it is of importance for optimal data interpretation that the drawbacks that are inherently associated with these *in vitro* approaches are fully appreciated.

Firstly, *in vitro* culture systems may be an oversimplification of the *in vivo* situation as only the cecum and proximal colon exhibit characteristics of a continuous culture. These parts of the gut receive undigested material from the ileum, and act as a mixing chamber (Macfarlane and Macfarlane, 2007). Colonic material then passes to the distal bowel for storage, and further mixing is largely restricted due to water absorption. Here the bacteria are effectively growing under batch or fed-batch conditions due to restrictions in substrate supply. It is therefore difficult to properly reproduce all fermentation dynamics of the colon in its entirety *in vitro* (Macfarlane and Macfarlane, 2007).

Similarly, all *in vitro* models lack feedback mechanisms by the host. Also, epithelial cells and immune cells are missing, which are important components in host-microbe interactions. But, as we will discuss later, samples from *in vitro* models can be combined with cell culture systems to better mimic this interaction with the host.

Physiological parameters that have been used in the current models in most cases have been obtained from healthy individuals. It is not clear whether these parameters, such as pH, redox potential, transit time, are the same for patients with several disorders or diseases. In fact, for some diseases one can argue that they will definitely not be the same, such as redox potential in IBD patients. It is known that already small changes in pH affect microbial metabolism (Duncan *et al.*, 2009; Smith and Macfarlane, 1996; Walker *et al.*, 2005).

Therefore, mimicking colonic metabolism using *in vitro* models will remain a challenge. Studies performed in such models will give indications of potential effects. However, they do allow one to test hypotheses and study mechanisms, or generate new hypotheses based on the results obtained.

Future perspectives

An exciting development is the combination of dynamic luminal models with cell culture systems. Whereas simple co-culture models of single microbes with cell-line models are beyond the scope of this review, we focus on the modelling the complex microbiota in the presence of host cells which becomes increasingly feasible.

Several studies investigated the effect of samples from *in vitro* models on enterocytes (usually Caco-2 cell cultures) and/or immune cells, such as the macrophage cell line U937 (Bahrami *et al.*, 2011; Putaala *et al.*, 2011; Van Nuenen *et al.*, 2005), to study adherence (Bahrami *et al.*, 2011), cytokine production (Bahrami *et al.*, 2011; Van Nuenen *et al.*, 2005), or gene expression (Putaala *et al.*, 2011). In the study by Van Nuenen *et al.* (2005), concentrations of microbial metabolites such as those produced in TIM-2 by a microbiota originating from IBD patients (Van Nuenen *et al.*, 2004) led to a different cytokine production by

macrophages than those from healthy individuals. This was observed both in a co-culture system of Caco-2 cells with U937, as well as U937 alone, mimicking a compromised epithelial barrier, which often is the case in IBD patients.

The host-microbiota interaction (HMI™) model is an *in vitro* model that allows studying microbial interactions with host cells during long-term incubations. It consists of a two-compartment reactor, with at one side a complex microbiota from the SHIME, and at the other side human enterocytes, potentially co-cultured with immune cells (Marzorati *et al.*, 2012, 2013). The compartments are separated by a semi-permeable membrane coated with mucins to allow colonisation of mucin-adhered microbes. Moreover, the membrane allows transport of microbial or host metabolites and of oxygen from the host to the microbial compartment in order to create microaerophilic conditions in the simulated mucus layer. Finally, the membrane prevents direct exposure of host cells to intestinal microbes, thus avoiding direct toxic effects. This approach has been validated by dosing an anti-inflammatory compound to the SHIME for one week while following the production of pro/anti-inflammatory cytokines during 48h. Comparison to a control, the HMI-module connected with the SHIME treated with the test product showed a lower production of pro-inflammatory cytokines (i.e. IL8 and IL 1β; Marzorati *et al.*, 2014).

Another spectacular development that may lead to sophisticated host-microbe models is the recent establishment of a method to cultivate single intestinal stem cells into spherical crypt-like structures, called organoids, with several distinct cell types that are normally found in the gut, such as enterocytes, goblet cells, and Paneth cells (Roeselers *et al.*, 2012; Sato *et al.*, 2009). However, currently it will be challenging to combine the organoids with a microbiota, as it seems very difficult to trap microbes within the organoids. Perhaps when these cells can be grown as a single cell layer, like Caco-2 cells are usually cultured, it will become feasible to study the microbe-organoid interaction.

Finally, another fascinating *in vitro* development is the 3D-culturing of human intestinal epithelial cells using rotating wall vessels. This simulation of the epithelium has been shown to better represent the *in vivo* situation compared to monolayer models as there is a distinct apical and basolateral polarity, an enhanced expression and organisation of tight junctions, extracellular matrix, and brush border proteins. Moreover, the 3D-culture models are characterised by highly localised mucus production, and differentiation into multiple epithelial cell types including enterocytes, goblet cells, Paneth cells and M-like cells (Barrila *et al.*, 2010). The results obtained with such 3D models have been shown more predictive of key *in vivo* responses to infection by *Salmonella* Typhimurium as compared to monolayers (Höner zu Bentrup *et al.*, 2006; Radtke *et al.*, 2010).

8. Recommendations and hypotheses for future research

At the present time it is not possible to come to firm conclusions with respect to structure-function relationships among dietary carbohydrates. This is due to the following limitations in the available literature:

- In many papers on fermentable carbohydrates, the carbohydrate is very poorly characterised. In many cases a loose description such as 'apple fibre' or 'sugar beet fibre' is used which does not give information about the mix of complex carbohydrates that have actually been used. Many authors, approaching their work from a nutritional perspective have characterised the materials by their soluble fibre content. In terms of deducing structure-function relationships, this is not informative.
- There have been a wide range of microbial methods used over the years of research in this field. It is not possible to compare data obtained by plate counts with data obtained via molecular methods. Some molecular methods such as fluorescent *in vitro* hybridisation (FISH) or qPCR target anything from particular species, or particular genera, to large functional groups of microorganisms. On the contrary, most sequencing studies give relative abundance of DNA reads rather than quantify cell populations. For this reason, detailed comparative analyses from the literature are not practical. Comparisons using similar methods can be made, however, which may then give an indication of shifts or changes in the microbiota. Different methods may then be used to give confidence on these outcomes.
- Many *in vitro* studies on the fermentation properties of prebiotics or candidate prebiotics have not worked with pure materials. Some prebiotics such as inulin are essentially a pure prebiotic but one that exists as a disperse range of molecular weights. Others such as GOS are very complex in terms of the carbohydrate structures present and this complexity varies among the available commercial products. Further, most of these products contain appreciable quantities of residual lactose and monosaccharides left over from the manufacturing process. These absorbable carbohydrates will act as confounding factors in an *in vitro* fermentation if not removed. Current methods of removal of such confounding carbohydrates are generally based on size fractionations, whereas the absorption of carbohydrates from the gut is not this simple and is based around carbohydrate structure.
- It is becoming clear that the variations in the gut microbiome among human individuals have a bearing on the response of those individuals to fermentable carbohydrates. Most *in vitro* studies in the literature have not reported on comparative analyses of different

carbohydrates with the same faecal donors. The majority of human feeding studies have fed a specific carbohydrate to groups of volunteers and have not analysed the faecal microbiome responses on an individual level.

Despite the limitations in the literature, there are some general trends that can be seen:

- *Bifidobacterium* species are almost always selectively stimulated by low molecular weight oligosaccharides. This seems to be a result of these organisms possessing a wide range of carbohydrate transporters together with exo-glycosidase enzymes.
- *Bacteroides* as a genus is very capable of degrading and metabolising high molecular weight polysaccharides and has evolved very sophisticated mechanisms to handle a variety of complex structures.
- NDC potentially have many mechanisms by which they can impact on host health. These include acting as a selective fermentation substrate, inhibiting the adhesion of potential pathogens to host cells and stimulating mucosal cells to enhance barrier function. The extent to which these various mechanisms operate *in vivo* is yet to be elucidated. We recommend that future research aims to address the degree to which NDC can bring about their effects via direct interactions with cells *in vivo*.

This manuscript has examined the effects of a wide range of fermentable natural fibres on the human gut microbiota in *in vitro* and *in vivo* systems. The effects of fibres on the gut microbiota of human volunteers has recently been published (So *et al.*, 2018). This includes a total of 64 studies involving 2,099 individuals and the key findings analysis can be summarised as:

- Fructans and galactooligosaccharides led to significantly greater abundance of both *Bifidobacterium* and *Lactobacillus* and also higher faecal butyrate concentrations in healthy adults.
- Candidate prebiotics (which comprise a broader range of fibres including polydextrose and resistant starch interventions as defined by Roberfroid *et al.*, 2010) produced different effects on the abundance of these two genera, with significant effects demonstrated for *Bifidobacterium* but not *Lactobacillus* spp.
- Fibres not classified as accepted or candidate prebiotics (also based on (Roberfroid *et al.*, 2010)) did not affect the abundance of either *Bifidobacterium* or *Lactobacillus.*
- With respect to some other important bacterial groups such as *Roseburia* spp.; *A. muciniphila*; *E. hallii*; *E. rectale*; *F. prausnitzii* and *R. bromii*, the authors stress that the number of studies evaluating species of such is small and they highlight that further studies are needed to investigate the effects of fibre and other dietary components on these groups.

Based on our analysis of the literature with a view to understanding the structure-function relationships in fermentable fibres, the authors make the following recommendations for future studies:

1. *Analysis of carbohydrate composition, structure and degree of polymerisation.* It is evident that the structural characteristics of carbohydrates (i.e. type of glycosidic linkage, monomer composition and order and DP) play a key role in their fermentative properties. MS-based approaches normally have difficulties to provide accurate linkage-specific information and positional oligosaccharide isomers, whilst NMR is the gold standard for absolute *de novo* structural elucidation of carbohydrates; however, fermentable carbohydrate samples tend to be complex, resulting in uninterpretable NMR spectra. Despite the remarkable advances in the development of analytical techniques, there are still important limitations for the efficient separation of complex carbohydrate mixtures. In this context, the application of multidimensional chromatographic techniques could be a very promising alternative to overcome the drawbacks on the capability of separating complex mixtures by the monodimensional methods currently available for carbohydrate analysis. Likewise, a multi-strategy approach involving an integration of high throughput glycomic techniques based on systematic and/or sequential enzyme digestion steps and on powerful analytical and spectroscopic methods could also be very helpful to unravel fermentable carbohydrate structures

2. *Determination of the impact on the gut microbiota.* One of the most challenging aspects of attempting to identify structure function relationships in fermented carbohydrates is issue of microbiological methods used. Much of the literature on carbohydrate fermentation in the gut describes studies using targeted quantitative techniques, such as FISH or qPCR. More recent literature has focussed on untargeted approaches such as 16S sequencing.

Future studies would benefit from the rational use of multidimensional microbiology techniques. An untargeted sequencing-based overview could be followed up with a targeted approach based on FISH or qPCR to gain population sizes of the microorganisms that change in response to a fibre intervention.

3. *Determination of the metabolic fingerprint of different fibre fermentations.* It is relatively common to find *in vitro* studies determining SCFA as fermentation end products, generally focussing on acetate, propionate and butyrate and sometimes measuring branched chain fatty acids. Rarely they will include ammonia and phenolic compounds from protein fermentation too. Some studies have not even analysed the microbiota but have relied upon measurement of SCFAs as an indicator of microbial fermentation.

Once again, these are targeted approaches, and they demand *a priori* decisions over which metabolites will be measured.

We now have the capacity to determine metabolites in an untargeted manner using ^{1}H-NMR and GC-MS techniques to give an overview of metabolic changes occurring. These are, however, specialised techniques that need expert input. They are not frequently used in *in vitro* testing but have come into their own in human studies. Future studies would have maximum impact by sampling blood plasma, urine and faeces and using metabonomic techniques to obtain a balanced view of the flux of metabolites from gut to human metabolism. Such approaches can also identify bacteria-human co-metabolites that may be significant mediators of the health effects of fermentation in the gut.

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