7 Galacto-Oligosaccharide Prebiotics

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7.1 Introduction

The wide recognition of bifidobacteria as health promoting bacteria (Boesten and de Vos, 2008) has attracted a lot of interest in identifying substances that can selectively promote their growth. Many studies using conventional culture and molecular techniques for bacterial identification have shown that breast-fed infants are characterized by an intestinal microbiota that is dominated by bifidobacteria (Benno et al., 1984), which is different from that of infants fed on cow's milk in that their microbiotas are characterized by lower counts of bifidobacteria, with greater numbers of more potentially harmful organisms such as clostridia and enterococci (Lunderquist et al., 1985). As a result of this difference in the microbiota composition, higher levels of ammonia, amines and phenols and other potentially harmful substances have also been found in infants fed cow's milk products (Lunderquist et al., 1985).

This has led, at the beginning of the last century, to the belief that there are molecules in the human milk that can promote the growth of this specific type of intestinal bacteria, leading to attempts to isolate and characterize those bifidogenic factors (Hamosh, 2001). Although bifidogenic nucleotides have been detected in human milk the predominance of bifidobacteria in breast-fed babies is thought to result from their abilities to utilize the oligosaccharides fraction of breast milk (Sela et al., 2008). Oligosaccharides are the third largest component of human milk and high levels are found in the colostrum where these substances constitute up to 24% of total colostrum carbohydrates (Bode, 2006). Total oligosaccharides in breast milk can reach concentrations as high as 8–12 g/l, which is 100 times greater than in cow's milk, and their concentrations steadily decrease to between 19% and 15% in the first 2 months after birth (Kunz et al., 1999). Milk contains a greater proportion of neutral compared to acidic oligosaccharides and the principal sugar components of oligosaccharides are sialic

acid, N-acetylglucosamine, L-fucose, D-glucose and D-galactose, resulting in a complex mix of over 130 different oligosaccharides, due to the great variety of different sugar combinations that are possible (Bode, 2006). Amongst those oligosaccharides, human milk contains a large amount of galactose with the backbone structure based on lactose (galactose–glucose) plus a further external galactose residue that leads to the formation of three galactosyl-lactoses, 1→3, 1→4 and 1→6-galactosyl-lactose, with 1→6 galactosyl-lactose being found in amounts ranging between 2.0 and 3.9 mg/l. The total concentration of lactose-derived oligosaccharides in human milk has been estimated to approximately 1 g/l (Boehm et al., 2005).

The ability of those oligosaccharides to replicate the bifidogenic properties of breast milk (Knol et al., 2005) and to resemble glycoconjugate structures on cell surface receptors used by pathogens for adherence in the gut (Rudloff et al., 2002, Morrow et al., 2005) has attracted a lot of interest in further studying their physiological properties and production. As a result, galacto-oligosaccharides have attracted significant interest for their inclusion in various foods as health promoting ingredient, particularly in Japan and Europe.

7.2 Production Process

7.2.1 Transgalactosylation Reaction

Galacto-oligosaccharides are defined as a mixture of those substances produced from lactose, comprising of between two and eight saccharide units, with one of these units being a terminal glucose and the remaining saccharide units being galactose, and disaccharides comprising of two units of galactose.

They can be synthesized by classical chemical synthesis methods from simple sugars, but the preferred mode for their synthesis is by enzymatic catalysis from lactose using an appropriate β-galactosidase enzyme. The two types of enzyme that can be used in the preparation of GOS are the glycosyltransferases (EC 2.4) and the glycohydrolases (EC 3.2.1).

Galactosyltransferases catalyze the transfer of sugar moieties from activated donor molecules to specific acceptor molecules, forming glycosidic bonds. These enzymes are highly regio-and stereo-selective and can produce high yield of GOS, but the fact that they are not readily available and their requirement for sugar nucleotides, make their use for industrial GOS production prohibiting due to the cost involved.

Galactohydrolases are much more readily available than glycosyltransferases but are generally less stereo-selective. They transfer the galactosyl moiety of a substrate to hydroxyl acceptors and are able to catalyze hydrolysis or transgalactosidation depending on the relative concentration of hydroxyl acceptors from water or other carbohydrates respectively. Synthesis of GOS with β-galactosidases is a general characteristic of retaining galactohydrolases that during lactose hydrolysis use a double-displacement mechanism to form a covalent glycosyl-enzyme intermediate, which is subsequently hydrolyzed via oxocarbenium ion-like states. The active site of the enzyme contains a pair of carboxylic acids, serving as a proton donor and a nucleophilic base, with an average distance of ≈5.5 Å apart. Acid base catalysis is important for this enzyme class and is provided by a single carboxyl group at the active site, which is functioning as the acid catalyst for the first glycosylation step and as the base catalyst for the second deglycosylation step. During the glycosylation step one carboxyl acid, the acid catalyst, protonates the glycosidic oxygen, whereas the other carboxyl acid mediates the aglycon departure by acting as the nucleophile. During the deglycosylation step, the produced glycosyl-enzyme intermediate is hydrolyzed by a water molecule activated by the first carboxyl acid which now behaves as the base catalyst. However, in the presence of other carbohydrates in the reaction mixture, especially at elevated concentrations, they can act as acceptors for the glycosyl moiety resulting in the elongation of the carbohydrate acceptor to a higher degree of polymerization.

The enzymatic conversion of lactose into GOS by β-galactosidases is thus a kinetically controlled reaction during which the thermodynamically favored hydrolysis of the substrate generates D-galactose and D-glucose in competition to the transferase activity that generates a complex mixture of various galactose based di- and oligosaccharides of different structures.

In addition to this transferase activity, another mechanism of GOS synthesis that leads directly to formation of the disaccharide allolactose is by the direct internal transfer of galactose from the position 4, found in lactose, to the position 6 of the glucose moiety without the release of the glucose moiety from the active site. Quantitatively, allolactose is one of the major oligosaccharides formed by neutral pH β-galactosidases and although this mechanism has been demonstrated only for β-galactosidase enzymes from *E. coli*, it has been proposed also for other β-galactosidases with similarities to the LacZ enzyme.

During this enzymatic reaction, the amount and nature of the formed oligosaccharide mixture is affected by the ratio of hydrolytic and transferase activities of the enzyme. This ratio depends on the enzyme source, the concentration and

nature of the substrate and the reaction conditions (pH, temperature and time) following the general principle that the transferase activity is favored at high lactose concentration, elevated reaction temperature and lower water activity. The source of the enzyme is directly influencing the type of glycoside bond formed between the galactose moieties of the produced GOS, and is also setting the range of pH and temperature conditions available for the synthesis reaction.

7.2.2 Microbial β-Galactosidase

The most extensively studied β-galactosidases for GOS synthesis are of microbial origin (β-galactohydrolase, EC 3.2.1.23). Enzymes from species belonging to *Kluyveromyces*, *Aspergillus*, *Bacillus*, *Streptococcus* and *Cryptococcus* have been used for the synthesis of GOS from lactose showing differing requirements for reaction conditions in terms of pH and temperature and differing product formation in terms of the glycoside bonds formed between the galactose moieties and the degree of polymerization (DP) of the synthesized oligosaccharides. Usually 55% of the initial lactose is converted into a mixture of products containing glucose and galactose due to the hydrolytic activity of the enzyme, un-reacted lactose, disaccharides of galactose and glucose with different β-glycoside bonds from lactose due to direct internal transfer, and trans-galactosylation products such as galactobiose, galactotriose, galactosyl lactose, tetra- to octasaccharides of similar rearrangement and/or side chain formations (Playne, 2002).

6′-galactosyl lactose is the main product when the yeast β-galactosidase from *Kluyveromyces* (*K. marxianus* subsp. *lactis*, *K. fragilis*) is used, whilst 3′- and 6′-galactosyl lactose are formed when the fungal lactase of *Aspergillus oryzae* is used. Enzymes from *Bacillus circulans* or *Cryptococcus laurentii* form mainly 4′-galactosyl lactose and enzymes from *Streprococcus thermophilus* 3′-galactosyl lactose. Another interesting approach for sourcing microbial β-galactosidase, has been explored in species of probiotic bacteria. The rationale behind the use of β-galactosidases from probiotic bacteria is that since the origin of the enzyme used in this type of manufacturing is important in the final GOS mixture composition and therefore functionality, the use of enzymes originating from probiotic bacteria as synthetic catalyst will produce oligosaccharide mixtures that will be more readily metabolized by the producing organism, resulting so in higher selectivity towards that organism. Following this approach, enzymes from various *Bifidobacterium bifidum* strains have been used to produce mixtures of linear 3′-galactosyl lactose as well as branched oligosaccharides.

The pH conditions of the reaction vary from very acidic to neutral with the fungal β-galactosidase from *A. oryzae* showing optimum GOS formation at pH as low as 3.5, whilst the enzymes from yeast or bacterial origin have more neutral pH optima between 6 and 7.5.

The origin of β-galactosidase is also influencing the temperature tolerability of the enzyme. Generally high temperatures are preferred in order to speed the reaction, to increase the solubility of the lactose substrate and prevent its crystallization and also to reduce the viscosity of the reaction mixture so that the transferase activity is favored over the hydrolytic activity. At the same time, the higher lactose concentration is reducing the water activity of the reaction solution which in turn is influencing the degree of polymerization of the formed GOS products, since lower water activity conditions are favoring the production of trisaccharides and higher water activity is required to synthesize GOS of greater length. Therefore, considerable efforts have been focused on sourcing thermostable β-galactosidases as a mean for the improvement of the reaction yields.

Another approach to improve the reaction yields through improving the transferase/hydrolytic ratio of β-galactosidases has been attempted through genetic engineering. Jørgensen et al. (2001) investigated the functional importance of the C-terminal part of BIF3 β-galactosidase by deletion mutagenesis and expression of truncated variants using *E. coli* cells as the host. Deletion of approximately 580 amino acid residues from the C-terminal part converted the enzyme from a normal hydrolytic β-galactosidase into a highly efficient transgalactosylating enzyme. Quantitative analysis showed that the truncated β-galactosidase utilized approximately 90% of the reacted lactose for production of GOS while hydrolysis constituted a 10% side reaction. This 9:1 ratio of transgalactosylation to hydrolysis was maintained at lactose concentrations ranging from 10% to 40% (w/w), suggesting that the truncated β-galactosidase is behaving as a true transgalactosylase even at low lactose concentrations.

7.2.3 Production Process

Various reactor designs and configurations have been reported for GOS synthesis, including the batch reactor, continuous stirred-tank reactor (CSTR), CSTR coupled with crossflow filtration, hollow fiber membrane reactor, fixed-bed and fluidized-bed reactor (Boon et al., 2000). The batch modes of operation prevail so far in the scientific literature on trans-galactosylation processes, mainly because of their ease of operation, but also the reduction in the risk of possible microbial

contaminations that long-term continuous processes, especially under realistic reaction conditions of moderate temperatures, have or fouling that can occur in systems where membranes are used for the retention of the enzyme.

Continuous systems, however, have been proposed as a way for reducing the process cost by offering a more efficient usage of the enzymes that can be very expensive. In the batch process, the initially added to the reaction mixture enzyme is usually lost at the end of the reaction. In the continuous process re-usage of the enzyme can be achieved by immobilizing it on a carrier and thus limiting the loss of enzyme activity or by retaining the soluble enzyme in the reactor with the use of an ultra-filtration membrane.

Another very interesting aspect, regarding the mode of operation, is the effect on the composition of the produced mixture. In batch systems, the composition and concentration of possible galactosyl acceptors are changing constantly over the reaction time whilst in the steady state of a continuous system the concentrations of possible galactosyl acceptors stay constant over the entire reaction time. This leads to more defined GOS mixtures being expected in a continuous process system and has been suggested as the reason why a larger fraction of trisaccharides is formed in batch production compared to continuous ones. Once the conversion of lactose to GOS mixture has been completed, the efficient removal/inactivation of the β-galactosidase is an important factor in order to prevent product hydrolysis that takes place if the reaction is continued beyond the peak of oligosaccharide formation.

For the commercialization of GOS-based products the purification of the produced oligosaccharides from the reaction mixture is a significant and challenging step. On a larger scale, monosaccharides can be separated using chromatographic applications such as ion-exchange resins or activated carbon. In the case of ion exchange chromatography, cation-exchange resins are mainly used since they have the highest affinity for monosaccharides and therefore oligosaccharides are the first to elute from the column. Activated carbon has a higher affinity for oligosaccharides, compared to mono- and disaccharides, which makes their operation at industrial level more preferable, since regeneration can take place off-line without large substrate losses. The separation of lactose from the disaccharide fraction of the GOS products has been proven to be extremely difficult and usually results to large losses of GOS products. Lactose-free GOS mixtures are of great interest considering that 70% of the world population lack β-galactosidase in the small intestine and are therefore sensitive to lactose. An approach based on the selective enzymatic oxidation of lactose into lactobionic acid using a fungal cellobiose dehydrogenase has been described

(Splechtna et al., 2001). The produced lactobionic acid can then be easily separated from the non-ionic sugars of the mixture with the use of anion exchange chromatography, whilst the monosaccharides are subsequently removed in a single chromatographic step, yielding to purified GOS mixture with only minor losses of the main components of interest.

7.2.4 Commercially Available GOS

GOS have been used as food ingredients in Japan and Europe for at least 30 years and their application is currently expanding rapidly. At present, Japanese companies still dominate worldwide galacto-oligosaccharide production and development activity, although, European interest in GOS based products is increasing with several companies currently producing or planning to produce GOS mixtures. In contrast, GOS production in the USA at present remains negligible. The major companies manufacturing GOS are still located in Japan. Yakult Honsha (Tokyo, Japan), Nissin Sugar Manufacturing Company (Tokyo, Japan) and Snow Brand Milk Products (Tokyo, Japan) together with Friesland Foods Domo (ex Borculo Domo ingredients) in the Netherlands and Clasado Ltd in the UK are the main manufacturers. Most of the manufacturers produce several classes of products in terms of GOS purity in either syrup and/or powder format.

Yakult is producing three GOS product: Oligomate 55 in syrup form, Oligomate 55P in powder form and TOS-100 a purified version of 99% oligosaccharide content. Nissin is producing Cup-Oligo in syrup (Cup-Oligo H70) and powder format (Cup-Oligo P) and Snow Brand produces GOS that is incorporated into its infant milk formula P7L, without offering sales outside its organization.

In Europe, Friesland Food Domo is offering Vivinal GOS in a syrup format containing 57% oligosaccharides on dry matter and in a powder format containing 29% oligosaccharides on dry matter. Clasado Ltd is offering mainly a powder GOS product, Bimuno, with 52% galacto-oligosaccharide content on dry matter, as well as a syrup version of that product.

Besides the differences in the purity amongst the commercially offered products, there are differences also in the linkages of the oligosaccharide chain due to the different enzymes used in their production. The Oligomate range is produced with enzymes originating from *Aspergillus oryzae* offering mainly β 1–6 linkages, the Bimuno product is produced using enzymes from *Bifidobacterium bifidum* and contains mainly β 1–3 linkages whilst Cup-Oligo and Vivinal offer

mainly β 1–4 linkages as a result of the activity of enzymes from *Bacillus circulans* for the latter and *Cryptococcus laurentii* for the former GOS product. Yakult is also considering dual enzymes systems combining the activity of enzymes from *A. oryzae* and *B. circulans* to produce GOS mixtures of β 1–4 and β 1–6 linkages.

Although different enzymes are used in the production of the various commercially available GOS products the overall process flow chart of them is very similar (*Figure 7.1*). 20–40% (w/v) lactose solution is incubated with the β-galactosidase enzyme in either batch or continuous reactor until the optimum of lactose conversion into oligosaccharides has been reached. The solution is then decolorized and demineralized before being further processed for removal of

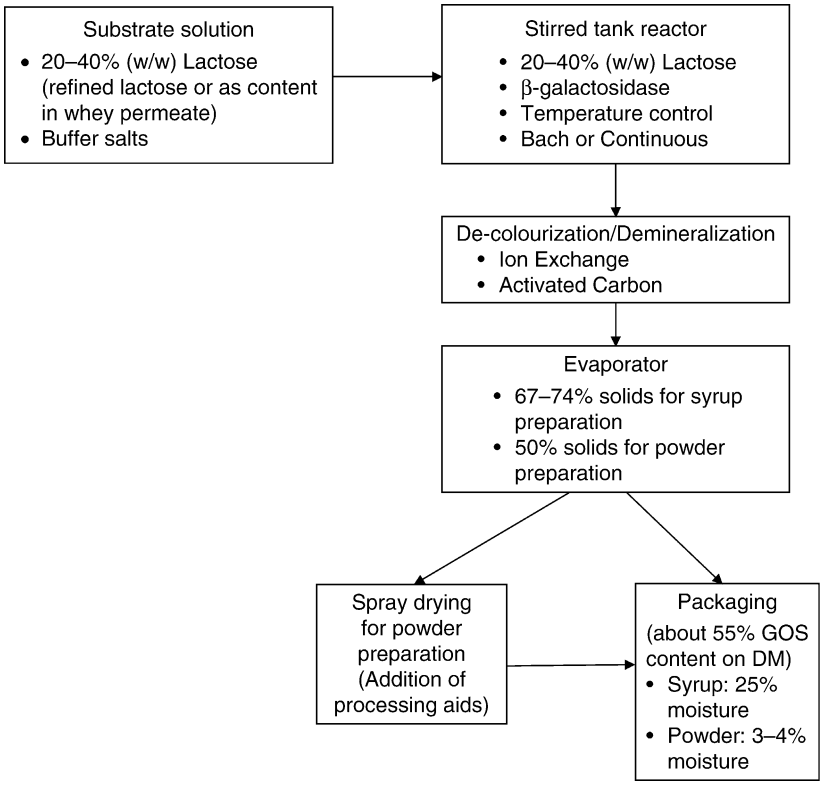

Figure 7.1
Process flow schematic for the manufacturing of GOS from lactose.

monosaccharides, either by chromatographic separation or nanofiltration, to increase the oligosaccharide purity. The resultant solution is concentrated by evaporation usually to 67–75% total solids to produce the syrup format or spray dried to produce the powder format.

A wide range of granted patents or patent applications related to the conditions as well as problem solving during the production of GOS have taken place and are available on-line (www.uspto.gov, www.epo.org).

7.3 Safety-Toxicity of GOS

In terms of safety and toxicity, GOS has been evaluated in both acute and chronic toxicity tests in rats. Oral administration of GOS did not show any toxicity as a single dose of 20 g/kg of body weight and a daily dose of 1.5 g/kg of body weight for 6 months. Using the Ames' test and the Rec assay no mutagenicity was found and the only known adverse effect of GOS is transient diarrhea due to osmotic pressure, which occurs when excess oligosaccharides are consumed. In the case of GOS, the amount of oligosaccharides which does not induce osmotic diarrhea is estimated to be approximately 0.3–0.4 g/kg body weight, or about 20 g/human body. When GOS syrup was administered to rats by gavage at 2,500 or 5,000 mg/kg of body weight daily for 90 days, no significant adverse toxicological effects attributable to treatment were noted. Clinical signs were unremarkable, and there were no ocular findings in any of the animals. Statistical analysis of clinical pathologies, including blood biochemistries, hematology, urinalysis and coagulation did not reveal any significant effects (Anthony et al., 2006).

Galacto-oligosaccharides have a generally regarded as safe (GRAS) status in the USA, a non Novel Food status in the EU and are regarded as foods of specific health use (FOSHU) in Japan.

7.4 Physicochemical Properties

GOS provide several physicochemical and health benefits, which make their use as food ingredients particularly attractive. Since commercially available food grade GOS are mixtures their specific physicochemical and physiological properties will to some extend vary depending on the mixture of oligosaccharides of different degree of polymerization, the un-reacted lactose and the generated

monosaccharides. Overall, galacto-oligosaccharides are colorless, water soluble with a viscosity similar to that of high fructose syrup leading to improved body and mouth-feel. They are stable to pH 2 at 37°C for several months making their application in non-refrigerated fruit juice matrices possible, whilst the presence of β type linkages makes them ingredients of increased resistance to high temperature in acidic medium. They remain unchanged after treatment at 160°C for 10 min at neutral pH and after treatment at 120°C for 10 min at pH 3 or 100°C for 10 min at pH 2, offering potential for a wide range of food applications. They are mildly sweet, typically 0.3–0.6 times the sweetness of sucrose and can be used in very sweet foods as bulking agent to enhance other food flavors. GOS are resistant to salivary degradation and are not utilized by the oral microbiota and can therefore be used as low cariogenic sugar substitutes in chewing gums and confectionary. They are not hydrolyzed by pancreatic enzymes and gastric juice passing the small intestine offering reduced glycemic index and a calorific value lower than 50% that of sucrose, which makes them suitable for low-calorie diet food and for consumption by individuals with diabetes. Galacto-oligosaccharides have a high moisture retaining capacity preventing excessive drying that can be useful in baked goods especially bread where GOS are not broken down during fermentation with yeast and the baking process, providing the bread with excellent taste and texture. Their low water activity can help in controlling microbial contamination and depending on the molecular weight of the oligosaccharide content, they can alter the freezing temperature of frozen foods and reduce the amount of coloring, due to Maillard reactions, in heat processed food as relatively fewer reducing moieties are available.

Analysis of galacto-oligosaccharides in different foods matrices is of high significance in terms of inclusion in various food products and a method, based on the principle of the quantification of FOS in food matrices, is available (AOAC 2001.02). This two stage method relies on the enzymatic treatment of a test solution with a β-galactosidase enzyme, followed by the quantitative determination of galactose by high-performance anion exchange chromatography with pulsed amperometric detection (HPAEC-PAD), in order to overcome the lack of sensitivity and selectivity that HPLC suffers. In the first stage, the free galactose and lactose are determined in the initial test solution and in the second stage, the total amount of galactose released from GOS and lactose is determined in the β-galactosidase treated solution. The GOS content is then calculated from the concentrations of lactose and galactose before and after the enzymatic treatment. The method has been tested and evaluated in dairy, fruit juice, pet candy, biscuit and infant formula matrices.

7.5 Functional Properties

Apart from the advantages provided to food manufacturers by the physicochemical properties of galacto-oligosaccharides, GOS offer a number of health promoting properties mainly due to their ability to modulate the balance of the colonic microbiota and promote the proliferation of intestinal bifidobacteria. Especially this bifidogenicity is making the inclusion of GOS on its own or as part of a mixture with FOS in infant milk formula, follow on formula and infant food the major application of GOS at the current time.

7.5.1 GOS Bifidogenicity

From the early steps of research into bifidobacteria, it has been recognized that those bacteria express higher activity of β-galactosidase than many other members of the colonic microbiota (Desjardins and Roy, 1990). This has been further supported by the isolation and characterization of various β-galactosidases from different *Bifidobacterium* species and subsequently confirmed at the genome level through the sequencing and annotation of numerous β-galactosidase encoding genes present in many *Bifidobacterium* strains of human origin. Within the available literature it is apparent that most bifidobacterial strains make use of more than one β-galactosidase isoenzyme for their growth. Tochikura et al. (1986) isolated and characterized two different β-galactosidases from *B. longum* biovar *longum* 401, whereas the genome sequence of *B. longum* biovar *longum* NCC2705 (Schell et al., 2002) revealed the existence of four different isoenzymes belonging to either GH 2 or GH 42 family. Furthermore, the cloning and sequencing of three genes from *B. bifidum* DSM20215 (Møller et al., 2001) and two genes from *B. longum* biovar *infantis* HL96 (Hung et al., 2001) confirm the ability of bifidobacteria to make extensive use of these enzymes for their growth. Amongst those bifidobacterial β-galactosidases many have been proven to be lactases, hydrolyzing mainly lactose into its simpler glucose and galactose moieties, but some of them, the ones belonging to the GH 42 family seem to preferentially hydrolyze galacto-oligosaccharides other than lactose, as it has been demonstrated with a β-galactosidase extracted from *B. adolescentis* DSM20083. This enzyme was shown to preferentially hydrolyze mainly β-D-(1→4) linked di- and galacto-oligosaccharides with reasonable Km values (2.2–6.4 mM) and be able to liberate galactose from β-D-(1→3), β-D-(1→6) and β-D-(1→1) galactobiose, but not from lactose. The presence of β-galactosidases with different

activity within the same cell of *Bifidobacterium* strains enables them to better utilize GOS mixtures by working in a synergistic fashion. Enzymes belonging to the GH 42 family liberate galactose moieties from the galacto-oligosaccharide molecule whereas enzymes belonging to the GHF 2 family act on lactose backbone of the molecule. The ability of bifidobacteria to utilize GOS molecules if further supported by the presence of appropriate membrane transport mechanisms which facilitate the internalization of the released carbohydrates into the cell. Although, not much research has been carried out, the presence of glucose and/or galactose transport systems has been identified in many species of bifidobacteria. In *B. bifidum* DSM20082, galactose crosses through the membrane by diffusion, whereas glucose is incorporated by a cation symport which is regulated by K^+ ions. Two glucose transport systems have been identified in *B. longum* biovar *longum* NCC2705, whereby one of them additionally participates in the incorporation of galactose even though it is repressed by the presence of lactose. Those transport systems are rapidly fed with simple carbohydrate moieties that exist in the environment of the cells through the action of extracellular enzymes that are able to degrade non-digestible galacto-oligosaccharides. *B. bifidum* DSM20215 has a putative extracellular β-galactosidase (GHF 2) which contains at their N-terminal part signal peptides that enable it to be extracellularly translocated by the cells, whereas the C-terminal part consists of domains that most probably mediate its attachment to the cell wall. Another simpler isoenzyme, in terms of protein domain structure (GHF 42), has been identified as being extracellular in *B. adolescentis* (van Laere et al., 2000). Extracellular location of the different β-galactosidase isoenzymes allows the cells to have better access and ability to degrade galacto-oligosaccharides into their simpler moieties (galactose and glucose) and subsequently internalize them via the described transport mechanisms.

However, many studies have demonstrated the preference of bifidobacteria towards di- or even oligosaccharides over their simpler moieties, suggesting that they have developed a mechanism for internalizing complex oligosaccharides into the cell and thus complement the extracellular degradation. Kim et al. (2005) and Parche et al. (2006) demonstrated the preference of *B. longum* biovar *longum* for lactose over glucose in growth culture experiments containing both carbohydrates. They showed that lactose was consumed first whereas assimilation of glucose was repressed until all lactose disappeared from the growth medium. This mechanism involves a transcriptional down-regulation of the glucose transport system in the presence of lactose that is most probably internalized by a lactose transferase. Gopal et al. (2001) and Amaretti et al. (2007) also

demonstrated the preference of *B. animalis* biovar *lactis* and *B. adolescentis* for oligosaccharides over glucose, suggesting the induction and expression of permeases specific for tri- and tetrasaccharides. Lactose transport systems have been investigated in *B. bifidum* DSM20082 and proved to be most probably based on a proton symporter. Moreover, using proteome analysis of *B. longum* biovar *longum* NCC2705 it was revealed the involvement of 19 permeases for diverse carbohydrates uptake (Parche et al., 2007). Amongst them, three putative lactose transport systems were identified. Through the function of these transporter mechanisms bifidobacteria can internalize a variety of galactooligosaccharide molecules and subsequently degrade them in the cell, avoiding thus the competition of nutrients and cross feeding of other bacteria that can occur when glucose and galactose moieties are liberated in the extracellular cell environment.

Although little data are available, the expression of β-galactosidases, as well as, galactoside transporters seems to occur in a controlled fashion in the *Bifidobacterium* cell. Growth of *B. adolescentis* DSM20083 on galacto-oligosaccharides results in the induction of β-galactosidase activity. When glucose, galactose or lactose were present, the expression of a lactose hydrolyzing enzyme was induced, whereas in the presence of galacto-oligosaccharides *B. adolescentis* DSM20083 expressed a galacto-oligosaccharide hydrolyzing isoenzyme (van Laere et al., 2000). In *B. bifidum* DSM20082, the presence of lactose slightly stimulated the β-galactosidase activity (1.5 times) that was accompanied by higher lactose incorporation into the cell, suggesting higher expression of a lactose permease. Induction of β-galactosidase activity by the substrate has also been reported for *B. longum* biovar *longum* CCRC15708 which contains four putative lactose operons in its genome (Schell et al., 2002). Two of those operons consist of a β-galactosidase gene, an LacI-type transcriptional regulator and ABC-type oligosaccharide transporters (BL0258-BL0260 and BL1167-BL1169). The third operon consists of a putative lactose permease (BL0976) in the opposite direction to a β-galactosidase gene (BL0978; *lacZ*), whereas the fourth, which is probably not functional, consists of a *lacI*-type repressor gene (BL1774) and a cryptic β-galactosidase-like gene (BL1775) (Parche et al., 2006; Schell et al., 2002). Microarray data analysis has indicated that those genes are either constitutively expressed or induced by the presence of lactose but none of them is significantly repressed by glucose (Parche et al., 2006). However, contrary to the above observations which indicate constitutive or inducible expression of β-galactosidases, the gene annotation of *B. longum* biovar *longum* NCC2705 has shown that this strain predominantly uses repressors for the negative

transcriptional regulation of β-galactosidase gene expression, something that is in contrast to most prokaryotes, but has been justified a way to allow a quicker and higher stringent response to environmental changes.

7.5.2 Non-digestibility of GOS

To exert an effect and to come into contact with bacteria growing in the colon, any prebiotic, by its definition, must escape digestive processes in the stomach and small intestine. The non-digestibility of prebiotics can be demonstrated *in vitro* by subjecting them to treatment with pancreatic and other gastrointestinal digestive enzymes. In the case of GOS, several *in vitro* experiments have shown its non-digestibility and stability to hydrolysis by such enzymes. A European consortium studied the effects of various non-digestible oligosaccharides and concluded that more than 90% of GOS arrives into the colon (van Loo et al., 1999).

The non-digestibility of prebiotics *in vivo* for human subjects can be demonstrated with ileostomized volunteers, because the digestion in these individuals is limited to the small intestine and the remainder of the food bolus can be collected from the pouch. However, very few prebiotics have been tested in this way and most of *in vivo* data is available by means of the hydrogen (H_2) breath tests. In general, studies report increased breath H_2 excretion following ingestion of GOS (Tanaka et al., 1983). These studies, therefore, give an indication that GOS is fermented by the colonic bacteria. However, the measurements of breath H_2 excretion do not provide information on the amount of GOS that actually escapes digestion and arrives into the colon intact. In the study by Bouhnik et al. (1997), reduced breath H_2 excretion was observed after daily dose of 10 g of GOS was administered for 21 days to eight volunteers. Nevertheless, the bifidobacterial numbers were increased, demonstrating that GOS was fermented by bacteria in the colon.

The calorific value of prebiotics, such as GOS, has been estimated to be 1–2 kcal/g (Cummings et al., 1997). According to the Japanese standard methods, calorific value of GOS has been calculated to be 1.73 kcal/g.

7.5.3 Prebiotic Properties of GOS

Along with inulin, FOS and lactulose, GOS is one of the prebiotics that has been the most thoroughly investigated and its prebiotic effect has been proven.

At present, this prebiotic effect is defined as the selective stimulation of the growth of bifidobacteria in complex microbial communities that exist in the large intestine. Bifidobacteria and lactobacilli are recognized as health-promoting organisms and are widely used as probiotics. Although, their specific health promoting effects are yet to be fully explained, no adverse effects related to their consumption have been reported.

7.5.3.1 *In Vitro* Effects

Methods for the assessment of prebiotic fermentation *in vitro*, range from simple static batch cultures to the multiple-stage continuous cultures inoculated with either single/mixed bacterial strains or fecal homogenates. Such fermentation studies describe the prebiotic capabilities through switches in the composition of purportedly beneficial and detrimental microbiota of fecal homogenates and the fermentation of prebiotics in pure cultures. Summary of *in vitro* data for the prebiotic effect of GOS is shown in *Table 7.1*.

Early studies used pure cultures and showed that GOS was selectively metabolized by all the *Bifidobacterium* strains tested compared to lactulose and FOS whose specificity was less remarkable (Ohtsuka et al., 1989). However, Hopkins et al. (1998) tested abilities of various bifidobacterial isolates to utilize various prebiotics and showed that substrate utilization was highly variable with considerable interspecies and interstrain differences. This finding was confirmed in a study by Gopal et al. (2001), where various strains of lactic acid bacteria were tested for their abilities to utilize GOS, and in addition to bifidobacterial species, some lactobacilli and pediococci strains were also able to utilize these substrates. Authors showed a perfect correlation between the ability of strains to utilize GOS and the presence of β-galactosidase, as well as the ability of *B. lactis* to preferentially utilize tri- and tetrasaccharides whereas *L. rhamnosus* preferred di- and monosaccharides when grown with GOS as growth substrate. Similar finding was observed in another study where *B. adolescentis* was able to degrade tri- and tetrasaccharides, while *B. longum* bv. *infantis* and *L. acidophilus* could only utilize those with a DP < 3 (van Laere et al., 2000). The ability of *B. adolescentis* to utilize GOS more efficiently was attributed to the presence of a novel β-galactosidase with activity towards GOS but not lactose. Indeed, strains belonging to bacteroides and clostridia have also been shown to utilize GOS in pure cultures (Tanaka et al., 1983).

While pure culture studies can offer an insight into various mechanisms involved in the utilization or growth processes, they can not be used to show a

Table 7.1

Effect of GOS on the colonic microbiota *in vitro* (*Cont'd* p. 223)

Method	Substrate	Effect	Reference
Test tubes, pure cultures (various strains)	GOS, glucose, inulin, lactose, lactulose	GOS selectively utilized by all *Bifidobacterium* strains tested and to a lesser extent by *L. acidophilus* but not by other bacteria, compared with lactulose and inulin whose specificity was less remarkable	Ohtsuka et al. (1989)
Static, temperature controlled batch culture (pure *Bifidobacterium* strains)	FOS, GOS, inulin, pyrodextrin, SOS, XOS	Substrate utilization highly variable between species	Hopkins et al. (1998)
Test tubes, pure cultures (various strains of lactic acid bacteria and bifidobacteria)	GOS	Other than bifidobacteria, some lactobacilli and pediococci strains able to utilize GOS. Perfect correlation observed between the ability of strains to utilize GOS and presence of β-galactosidase. *B. lactis* utilizes tri- and tetrasaccharides, and *L. rhamnosus* prefers mono- and disaccharides	Gopal et al. (2001)
Stirred, pH/temperature controlled, anaerobic batch cultures, fecal homogenates	FOS, GOS, IMO, inulin, lactulose, SOS, XOS	All prebiotics increased bifidobacteria. XOS and lactulose produced the highest population of bifidobacteria and FOS resulted in the largest increase in lactobacilli. GOS resulted in the largest decrease in clostridia	Rycroft et al. (2001)
Stirred, pH/temperature controlled, anaerobic batch cultures, fecal homogenates	FOS, fructose, GOS	No differences in the number of bifidobacteria between substrates. *B. adolescentis*, *B. longum* and *B. angulatum* comprised the dominant bifidobacterial species	Sharp et al. (2001)

Table 7.1

Method	Substrate	Effect	Reference
MPE (measurement of changes in dominant bacteria, substrate assimilation and SCFA), using batch cultures and fecal homogenates	FOS, V-GOS, IMO, SOS, ChOS, Psyllium, Benefiber, Sunfiber, FOS:V-GOS (50:50), FOS: Benefiber (90:10), V-GOS:Benefiber (90:10)	V-GOS in combination with FOS and alone resulted in highest MPE, suggesting the best prebiotic effect of the tested substrates	Vulevic et al. (2004)
Three-stage gut model, pH/temperature controlled to represent proximal, transverse and distal colons	B-GOS	In vessels corresponding to proximal and transverse colons, strong bifidogenic effect was observed. There was no effect on bifidobacterial population in the third vessel, due to complete utilization of substrate in previous vessels	Tzortzis et al. (2005)

prebiotic effect. As mentioned above, under these conditions GOS, as indeed most oligosaccharides, can support the growth of various bacteria and thus the selectivity can not be demonstrated. Batch and continuous culture studies using fecal homogenates offer a better *in vitro* assessment of a prebiotic potential.

Rycroft et al. (2001) used various prebiotics as substrates, in pH/temperature controlled anaerobic batch cultures inoculated with fecal homogenates, and showed that all prebiotics increased the numbers of bifidobacteria but GOS resulted in the largest decrease of clostridial population. In another study, fecal homogenates were grown in batch cultures in the presence of FOS, fructose or GOS and no differences were observed in the number of bifidobacteria between the treatments (Sharp et al., 2001). However, quantitative analysis, termed a measure of the prebiotic effect (MPE), which takes into an account a number of dominant bacterial groups, fermentation end products such as short-chain fatty acids (SCFA) and substrate assimilation was developed by Vulevic et al. (2004). Here it was shown that out of 11 substrates tested, V-GOS on its own or in combination with FOS (V-GOS:FOS, 50:50) resulted in the highest MPE suggesting the best prebiotic effect of the tested substrates. Recently, B-GOS mixture (1%, w/v) was shown to have a strong bifidogenic effect in a three-stage gut model, in vessels corresponding to the proximal and transverse colons but not in the third vessel (Tzortzis et al., 2005). The lack of the effect in the last gut model vessel, which represents distal colon, was attributed to low molecular weight and complete assimilation of B-GOS by the bacteria in previous vessels.

The results obtained from *in vitro* fermentation with fecal homogenates are important in prebiotic investigations, particularly when choosing or evaluating substrates to be used *in vivo*. However, *in vitro* models do not provide a full picture of the complex ecosystem that exists within the large intestine.

7.5.3.2 *In Vivo* Human Studies

Table 7.2 summarizes *in vivo* human studies investigating the prebiotic effect of GOS. Several studies showed that administration of various GOS mixtures to healthy humans result in significant increases in the population numbers of bifidobacteria following ingestion (Bouhnik et al., 1997; Gopal et al., 2003; Ito et al., 1993; Tanaka et al., 1983). This increase in bifidobacteria was sometimes accompanied by increased lactobacilli numbers (Gopal et al., 2003; Tanaka et al., 1983) and decreased bacteroides numbers (Tanaka et al., 1983) without any significant effect on the other bacterial groups assessed. Initially higher doses (10 g/day) of GOS were used (Bouhnik et al., 1997; Tanaka et al., 1983), however Ito et al. (1993) showed that lower dose of GOS (2.5 g/day) was sufficient to observe a bifidogenic effect when the initial number of the bifidobacteria population is low. This was confirmed in a study by Gopal et al. (2003), where it was shown that 4 weeks of supplementation with 2.4 g of GOS per day resulted in increased numbers of both bifidobacteria and lactobacilli. However, Bouhnik et al. (2004) studied the effect of 1 week intake of C-GOS (Cup-oligo) at concentrations of 0, 2.5, 5.0, 7.5 and 10.0 g/day and observed no dose-dependent effect, although the low initial numbers of bifidobacteria were associated with overall better prebiotic effect compared to the other oligosaccharides tested. In addition, this study also showed that the effect of GOS on bifidobacterial population level was higher than that observed with other prebiotics (i.e., inulin, FOS, lactulose) used in the study (Bouhnik et al., 2004). In a recent study, the administration of B-GOS mixture (3.6 g/day) was recently shown to result in a better bifidogenic effect than V-GOS (4.9 g/day) after 1 week of intake by healthy humans (Depeint et al., 2008). Moreover in the same study, a different composition of bifidobacteria was suggested after the two GOS treatments which was indicated through the isolation of different fecal *Bifidobacterium* species The difference in the magnitude and quality of the effect on bifidobacteria between the two GOS mixture was attributed to the difference in structures of the oligosaccharide present in the mixtures due to the different enzymes used for their production. Similar effect on bifidobacterial population of B-GOS was

Table 7.2

Bacteriological changes after GOS administration in human volunteer studies *in vivo*

Dose per day	Number of subjects	Treatment period	Effect	Method	Reference
3 or 10 g Oligomate	5	3 weeks	Increased bifidobacteria and lactobacilli. Decreased bacteroides	Agar plate counts	Tanaka et al. (1983)
2.5 g Oligomate	12	3 weeks	Increased bifidobacteria	Agar plate counts	Ito et al. (1993)
8.1 g V-GOS	10	2 weeks	No effect on endogenous bifidobacterial population	Qualitative DGGE analysis and sequencing	Satakori et al. (2001)
2.4 g BPOligo	10	4 weeks	Increased bifidobacteria and lactobacilli. No effect on clostridia and enterobacteria	Agar plate counts	Gopal et al. (2003)
2.5, 5.0, 7.5, 10.0 g Cup-oligo, FOS, IMOS, inulin, lactulose, SOS	8	1 week	10 g of GOS resulted in a best effect on bifidobacterial numbers. No dose-dependent effect was observed, but lower initial bifidobacterial numbers associated with better prebiotic effect	Agar plate counts	Bouhnik et al. (2004)
4.9 g V-GOS	29	1 week	Increased bifidobacteria with both but B-GOS significantly higher than V-GOS. Dose-dependent effect observed in bifidobacterial numbers with B-GOS	FISH	Depeint et al. (2008)
1.9, 3.6 g B-GOS	30				

observed even at a lower dose of 1.9 g/day in this study, and the effect was found to be dose dependent in the type of microbiota with the initial numbers of bifidobacteria being within normal range. It is clear that not all GOS mixtures necessarily result in the same effect on the microbiota, since differences in their degree of polymerization as well as structure composition can become significant when it comes to their assimilation by bifidobacteria in the complex colonic ecosystem.

Additionally, attempts have been made to perform the qualitative analysis (using DGGE and subsequent DNA sequencing) of bifidobacterial population following human intake of GOS. Satokari et al. (2001) showed that 2 weeks of supplementation with a relatively high dose (8.1 g/day) of V-GOS did not affect the qualitative composition of the endogenous bifidobacteria.

7.5.4 Metabolism in the Colon

The major end-products of carbohydrate fermentation are SCFAs, of which acetate, propionate and butyrate are quantitatively the most important in the human colon. All SCFAs are rapidly absorbed from the large intestine and stimulate salt and water absorption: principally, the gut epithelium, liver and muscle metabolize them, with virtually none appearing in the urine and only small amounts appearing in the feces. The three major SCFAs are trophic when infused into the colon, and these trophic properties have important physiological implications in addition to maintaining the mucosal defense barrier against invading organisms. The amount and type of SCFA produced in the colon will depend on the type of substrate as well as the composition of the microbiota. Because SCFA are rapidly absorbed from the gut, measurements in feces do not provide useful information about the fermentative abilities of different substrates. This has been demonstrated in human trials where the administration of GOS resulted in increased bifidobacteria but with no effect on fecal SCFA concentrations (Ito et al., 1993). However, *in vitro* studies, using fecal homogenates, can be useful models for studying the fermentation profiles. Bouhnik et al. (1997) showed that the addition of 10 g of GOS to batch cultures inoculated with human fecal homogenates resulted in increased acetic and lactic acid productions which were not observed in control fermenters. The authors suggested the change of microbiota composition (i.e., increase in the population numbers of bifidobacteria) was responsible for this. Similar findings were reported in other studies using batch cultures. For example, at concentrations of 10 g/l, FOS and GOS were shown to increase acetate and butyrate formation, with transient accumulation of lactic and succinic acids (Hopkins and Macfarlane, 2003). Studies with rats inoculated with human fecal microbiota show clear demonstration of decreased pH and increased SCFA and other organic acids in the caecal contents following consumption of GOS (Kikuchi et al., 1996). In these models, the major increases are observed in the production of lactic and succinic acids suggesting that these may be responsible for the lowering of the pH. In a study with piglets

(Tzortzis et al., 2005), inclusion of 4% (w/w) B-GOS in the diet resulted to the significant decrease of pH in the proximal colonic content, which was attributed to the significant increase of acetic acid and total SCFA concentration. This effect though could not be seen in the distal colonic content as well.

7.5.5 Physiological Effects of GOS

7.5.5.1 Stool Improvement

GOS has been shown to beneficially affect fecal nature and improve some parameters related to constipation. Deguchi et al. (1997) studied the effect of administering 2.5 and 5 g of GOS (Oligomate) daily for 1 week to 75 women who had a tendency to be constipated. Higher dose of GOS was found to improve the defecation frequency significantly. Similar findings were reported by Korpela and colleagues who performed series of studies in both healthy adults and constipated elderly (Sairanen et al., 2007; Teuri et al., 1998). This research group showed that consumption of yoghurt containing high doses (9–15 g) of V-GOS daily for 2–3 weeks, can increase the defecation frequency, however in younger adult subjects gastrointestinal symptoms, such as flatulence, also increased (Teuri et al., 1998).

The frequency of defecation does not provide information on the improvement of stool weight, however, and studies that have examined the effect of GOS on mean daily stool weight did not report any significant changes regardless of the dose (Bouhnik et al., 1997). Furthermore, no differences between of inulin, FOS and V-GOS at an intake of 15 g on mean daily stool weight were also reported (van Dokkum et al., 1999). It is worth noting here that both studies examining the effect of GOS on mean daily stool weight have used healthy adults and not constipated subjects who should provide a better group to study the laxative effect of any prebiotic. The possibility that GOS may have an improving effect in the treatment of constipation may, therefore, not be excluded and further trials are necessary to fully answer this question.

7.5.5.2 Mineral Absorption

Calcium and magnesium can be absorbed from both small and large intestines and their adequate supply and bioavailability are important factors determining the healthy bone structure. Deficiency of these minerals can lead to problems,

such as osteoporosis, later in life and especially in postmenopausal women. It has been noted that these minerals when present in the large intestine exist in forms that are poorly absorbable and that acidification caused by bacterial fermentation increases their solubility and thus absorption. Since prebiotics, including GOS, have an effect on reducing the pH and supporting the growth of lactic acid-producing bacteria, it has been shown that this types of substrate have an enhancing effect on the metabolism of calcium and magnesium as well.

Current works involving GOS are summarized in *Table 7.3*, and as shown the majority of evidence comes from animal models. Chonan and colleagues conducted series of experiments in rats and showed that calcium absorption was stimulated by ingestion of GOS when normal but not low dietary concentrations were used. This calcium absorption in turn increased bone mass and calcium content (Chonan and Watanuki, 1996; Chonan et al., 1995, 1996). Chonan and colleagues have also investigated and showed increased magnesium absorption

Table 7.3

Effect of GOS supplementation on mineral metabolism and bone mineralization

Dose per day	Study	Aim/method	Result	Reference
0.5, 5.0 g per 100 g	Rats	Ca absorption with normal and high dietary levels of GOS	Ca absorption stimulated when normal but not low dietary concentrations were used	Chonan and Watanaki (1996)
5 g per 100 g	Rats	Effect of GOS in overiectomized rats on Ca absorption and bone weight	Ca absorption stimulated and bone mass and Ca content increased	Chonan et al. (1995)
5 g per 100 g	Rats	Effect in Mg deficient rats on Mg absorption, concentration of Mg in bones and accumulation of Ca in the kidney and heart	Mg absorption and bone concentration increased and Ca accumulation in the kidney and heart reduced	Chonan et al. (1996)
15 g V-GOS, FOS, inulin	Humans (n = 12) 3 weeks	Effect on Ca and Fe absorption in healthy man using 24 h urine collections for Ca isotopes measurements	No effect on Ca and Fe absorption	van den Heuvel et al. (1998)
20 g V-GOS	Humans (n = 12) 9 days	Effect on Ca absorption in menopausal women using urine collections for administered Ca isotopes	Increased Ca absorption	van den Heuvel et al. (2000)

and bone concentration in rats fed GOS which in turn suppressed the calcification of the heart and kidney under conditions of high phosphorus and calcium dietary conditions (Chonan et al., 1996).

Although the rat models show clear beneficial effects of GOS on mineral absorption, human studies are scarce and with inconsistent results. In one study, male volunteers were fed 15 g/day of FOS, inulin and V-GOS for 3 weeks and no effect on iron and calcium absorption were observed with any of the tested prebiotics (van den Heuvel et al., 1998). In another study, postmenopausal women received 15 g/day of V-GOS for 9 days and calcium absorption was significantly increased (van den Heuvel et al., 2000). Therefore, current results offer promise, especially in relation to osteoporosis, however more human trials are needed to offer definitive answers. It is worth noting that FOS has been used more widely in human studies with good results.

7.5.5.3 Lipid Metabolism

Several animal studies have shown that administration of prebiotics, namely inulin and FOS or fermented milk products is effective in lowering blood cholesterol levels. However, *in vivo* results are variable, with some studies reporting lowering effects and others no effect on total serum cholesterol levels. Thus far, GOS has been used in two trials where serum cholesterol levels were investigated and in both no significant effect could be seen. In one trial, the effects of administration of 15 g/day of V-GOS, FOS and inulin were compared in healthy humans but no significant changes in serum lipids or glucose absorption were observed (van Dokkum et al., 1999). Recently, it was shown that 5.5 g of a GOS mixture administration to healthy elderly had no effect upon total serum cholesterol and HDL cholesterol levels (Vulevic et al., 2008). However, most studies reporting a decrease in the levels of serum cholesterol and/or increase in the levels of HDL cholesterol, following either pro- or prebiotic administration, have used subjects with initial elevated serum cholesterol levels. To common knowledge, GOS has not been used in this context and it is, therefore, not excluded that it may have an effect in these subjects.

7.5.5.4 Carcinogenesis

It has long been suggested that the human gut microbiota plays an important role in the metabolism and toxicity of both dietary and endogenous substrates.

In many cases the products of bacterial metabolism in the large intestine are associated with detrimental effects on the host and could lead to initiation and/or promotion of colon carcinogenesis. In particular, bacterial enzymes such as β-glucosidase, β-glucuronidase and nitroreductase have been associated with production of carcinogens. Since prebiotics are known to support the growth of probiotic bacteria and lower the pH in the colon it is to be expected that they would have an effect on the production of genotoxic enzymes. However, there are very few studies with GOS that have looked at these effects and most data originates from *in vitro* and animal models (› *Table 7.4*).

An *in vitro* study using a three-stage continuous gut model system showed that 5% (w/v) V-GOS was only weakly bifidogenic, but it strongly supported the growth of lactobacilli in the vessel corresponding to the proximal colon (McBain and Macfarlane, 2001), without any significant effects being observed in the other two vessels corresponding to transverse and distal colons. Fermentation of V-GOS decreased the activities of β-glucosidase and β-glucuronidase in all three vessels, however nitroreductase and azoreductase activities were increased. At the same time, this study showed that inulin exerted similar effects on the beneficial microbiota, nitroreductase and azoreductase activities, but inulin increased enterobacteria and *C. perfringens* and had no significant effect on β-glucosidase and β-glucuronidase (McBain and Macfarlane, 2001).

Studies in human-microbiota associated rats have shown that GOS (5%, w/w) administration for 4 weeks, increases caecal bifidobacteria and lactobacilli and decreases enterobacteria and the pH (Rowland and Tanaka, 1993). This change in the microbiota was followed by significant decreases in the activities of β-glucuronidase and nitroreductase, but not of β-glucosidase activity that showed to increase during GOS fermentation. Kikuchi et al. (1996) showed increased levels of β-galactosidase, lactic acid and caecal pH in human-microbiota associated rats fed GOS (10%, w/w) accompanied by a decrease in the levels of ammonia and β-glucuronidase activity.

The above results from *in vitro* and animal models suggest that GOS may have reducing effect upon the production and activities of some genotoxic enzymes. However, to date, there is only one human *in vivo* study that has looked at the effect of feeding healthy humans with 15 g/day of inulin, FOS or V-GOS on β-glucuronidase production (van Dokkum et al., 1999). Indeed, inulin and V-GOS were found to have a reducing effect upon β-glucuronidase concentration, whereas FOS had no effect in this study.

The effect of V-GOS was also assessed on the development of aberrant crypt foci in rats and it was found that at increased intake levels of V-GOS (10%, w/w),

Table 7.4

Studies on the effect of GOS on colon carcinogenesis

Dose	Study	Aim/method	Result	Reference
5% (w/v) V-GOS, inulin	*In vitro*	Effect on microbiota and activities of genotoxic enzymes in three-stage gut model	V-GOS and inulin weakly bifidogenic but increased lactobacilli. Inulin also increased enterococci and *C. perfringens*. V-GOS decreased β-glucosidase and β-glucoronidase, inulin had no effect. Both substrates increased azoreductase and nitroreductase	McBain and Macfarlane (2001)
5% (w/w) Oligomate	Rats	Effect of feeding human-microbiota colonized rats for 4 weeks on microbiota and genotoxic enzymes	Increased bifidobacteria, lactobacilli and β-glucosidase. Decreased enterobacteria, cecal pH, β-glucoronidase and nitroreductase	Rowland and Tanaka (1993)
10% (w/w) Oligomate	Rats	Effect of feeding human-microbiota colonized rats for 4 weeks on fermentation metabolites and genotoxic enzymes	Increased β-galactosidase and lactic acid. Decreased cecal pH, β-glucoronidase and ammonia	Kikuchi et al. (1996)
5, 10% (w/w) V-GOS	Rats	Fischer rats injected twice with AOM to induce colorectal tumors and fed low or high concentration V-GOS. At 4 weeks, 18 rats in each group were killed and scored, half of the remaining group swapped to the other diet. Six weeks later 9 rats in each group were killed, the rest were killed after 10 months	At first count, number of aberrant crypt foci reduced with higher compared to low V-GOS. After diet change total number of aberrant crypt foci increased, but less with animals swapped onto high V-GOS diet	Wijnands et al. (2001)
15 g per day V-GOS, FOS, inulin	Humans (n = 12) 3 weeks	Originally aimed at assessing the effect of prebiotic administration to healthy young men on serum lipids	Decreased β-glucoronidase with inulin and V-GOS but not FOS	van Dokkum et al. (1999)

it had a reducing effect, thus suggesting its potential as a protective agent against the development of colorectal tumors (Wijnands et al., 2001). However, human studies are still lacking in this respect and indeed in respect of identifying the specific anti-cancer effects of GOS and prebiotics in general, in order to draw any definite conclusions at the present time. It is worth mentioning that one of the possible and very important effects of GOS, in respect of anti-cancer as well as anti-inflammatory potential, may be its immunomodulatory properties.

7.5.5.5 Immunomodulation

The immune system protects the body against foreign agents and invasion by pathogens. It can be divided into the innate or non-specific and adaptive or acquired (specific) immune system. The innate immune system acts as first line of defence and it comprises physical barriers such as skin, and phagocytic, dendritic, inflammatory and natural killer (NK) cells as well as soluble mediators such as complement proteins and cytokines. The adaptive immune system becomes active in response to a challenge to the innate immune system. This system is more antigen-specific and it consists of two major cell types, T- and B-lymphocytes. T-lymphocytes develop into functionally different cell types with specific cytokine patterns, whereas B-lymphocytes are a part of the memory of the immune system and they produce only one type of antibody matching a specific type of antigen. T-lymphocytes can further be divided into those who mediate immunity to intracellular pathogens (Th1 cells) and those responsible for extracellular pathogens (Th2 cells).

The largest component of the immune system is situated in the gut. It is called the gut-associated lymphoid tissue (GALT), and it contains about 60% of all lymphocytes in the body. It also contains large amounts of secretory immunoglobulin A (sIgA), which plays a key role in the defence of the gut against adherence and invasion of pathogenic bacteria and viruses. GALT is constantly in contact with the microbiota and their metabolic by-products, thus dietary substrates reaching the large intestine that are able to influence the microbiota should affect the GALT.

7.5.5.6 GOS and the Immune System

The idea that prebiotics could help the intestinal defence system originated from the observations that newborn babies, who have an underdeveloped intestinal

host defence system, lack an appropriate capacity to defend themselves against intestinal infections. Furthermore, infants consuming their mother's milk were found to have a greatly reduced risk of diarrhea diseases, and a lower risk of respiratory and other infections. Human milk contains various protective components and active ingredients, including non-digestible oligosaccharides, which represent the third largest component of human milk and have been identified as the main factors involved in the development of an appropriate colonization process in infants, which in turn stimulates the maturation of intestinal host defenses.

Although it is known that human milk oligosaccharides can exert a prebiotic effect (Sela et al., 2008), research into the immunomodulatory actions of prebiotics is very recent, with most data originating from animal models and in relation to FOS and its prebiotic effect (i.e., bifidogenicity and SCFA production). However, there are few studies and lines of evidence that either suggest or demonstrate the effect of GOS on the immune system. This effect could either be direct in the form of interactions with immune, mucosal or epithelial cells and/or indirect through the species or even strain selective modulation of the microbiota and their metabolic products.

As outlined earlier, several studies have shown that GOS increases the population numbers of bifidobacteria, lactobacilli and subsequent SCFA production what are known to have immunomodulatory effect. Butyric acid is known to suppress lymphocyte proliferation, inhibit cytokine production of Th1-lymphocytes and upregulate IL-10 production, to suppress the expression of the transcription factor NF-κB and upregulate Toll-like receptors (TLR) expression, as well as to protect against colon cancer as it inhibits DNA synthesis and induces cell differentiation (Hoyles and Vulevic, 2008). Pharmacological doses of acetic acid when administered intravenously to healthy individuals and cancer patients increase NK cell activity and peripheral blood antibody production (Ishizaka et al., 1993).

Specific probiotic species belonging to either bifidobacteria or lactobacilli, when administered orally, are known to increase the secretion of sIgA in the small intestine and the feces, and to stimulate Peyer's patches (PP) B lymphocyte IgA production (Hoyles and Vulevic, 2008). In one recent study 57 infants, split into three groups, were fed either standard formula alone, standard formula containing a probiotic (*B. animalis* – Bb12) or formula containing a prebiotic mixture (V-GOS:inulin; 90:10) for 8 months (Bakker-Zierikzee et al., 2006). Measurements of fecal sIgA were made at regular intervals during the course of the study, and it was shown that babies fed the probiotic formula had variable levels of sIgA,

whereas babies receiving the prebiotic mixture demonstrated a trend towards higher levels of sIgA compared to the controls. Apart from formula fed infants, another group that could potentially benefit from the immunomodulatory effects of GOS is the elderly who are known to generally have reduced levels of beneficial bacteria and impaired immune system. A very recent study investigated the effect of feeding 5.5 g of GOS (B-GOS) to 44 healthy elderly volunteers on the microbiota composition and immune function (NK cells, phagocytosis and cytokines) (Vulevic et al., 2008). This study showed that B-GOS administration led to a significant decrease in the numbers of the less beneficial bacteria (i.e., bacteroides, *C. perfringens*, *Desulfovibrio* spp., *E. coli*) and a significant increase in the numbers of the beneficial bacteria, especially bifidobacteria. The study also found significant positive effects upon the immune response, evidenced by an improvement in NK cell activity and phagocytosis, increased secretion of the anti-inflammatory cytokine, IL-10, and decreased secretion of pro-inflammatory (IL-6, IL-1β and TNF-α) cytokines by stimulated PBMC. Additionally, a clear positive correlation between the number of bifidobacteria and both NK cell activity and phagocytosis was demonstrated. This was the first time that the immunomodulatory effect of GOS has been demonstrated in humans and it was shown that dietary intervention, such as B-GOS, can be an effective and attractive option for the enhancement of both gastrointestinal tract function and immune system (innate and adaptive) function.

7.5.5.7 Inflammatory Bowel Disease (IBD)

IBD principally includes Crohn's disease (CD) and ulcerative colitis (UC). Although, the cause of IBD is not yet known, it is generally accepted that a combination of factors such as genetic susceptibility, priming by enteric pathogens and immune-mediated tissue injury, result in its pathogenesis. Reduced numbers of beneficial bacteria accompanied by increases in the numbers of other less beneficial bacteria, such as *E. coli*, have been reported in both feces and mucosa microbiota of IBD patients. In patients with IBD Th1 and Th2 pattern of cytokine formation seem to be modified or increased in comparison to healthy individuals. For example, IBD patients seem to have increased production of tumor necrosis factor α (TNF-α) which triggers inflammation via the transcription factor NF-κB. There are very few studies with prebiotics in general, that have looked at their effect in human IBD. The use of V-GOS, however, as a therapy for immunomodulation in IBD was tested in one animal study.

The trinitrobenzene sulphonic acid (TNBS)-induced colitis rat model was used to test the effect of feeding rats 4 g/kg of body mass per day either whey or V-GOS, 10 days before the induction of colitis, or dexamethasone at colitis induction, as a control (Holma et al., 2002). Fecal bifidobacteria numbers, myeloperoxidase activity and macroscopic damage were assessed 72 h after the induction of colitis, and it was found that the bifidobacterial numbers increased with the V-GOS administration, but inflammation was not reduced. Although, this study did not show any effect from the V-GOS administration on IBD, this one animal model is not enough to draw any conclusions and more studies are needed to fully determine the potential of GOS in preventing or treating IBD.

7.5.6 Allergy

Improved hygiene and reduced exposure of infants to microorganisms are one of the suggested reasons for the observed increases in the incidence rates of allergic diseases in developed countries during recent decades. Studies indicate that there may be a link between the colonic microbiota and allergy, since reduced numbers of bifidobacteria have been found in the feces of allergic infants (Kirjavainen et al., 2002). These infants have IgE-mediated food allergies, and a Th2-biased immune response. Studies have found that FOS can reduce Th2 response in children, however GOS has not been used in this context and only indirect evidence for the effect of GOS currently exists.

Recently, 1,223 pregnant women carrying children at high risk of allergy, were divided into two groups, and fed for 2–4 weeks, before delivery, either a placebo or a mixture of 4 probiotics (*L. rhamnosus* GG, *L. rhamnosus* LC705, *B. breve*, *Propionbacterium freudenreichii* subsp. *shermanii* JS) (Kukkonen et al., 2007). Their infants received the same probiotic mix combined with 0.9 g V-GOS or a placebo for 6 months after birth, and at 2 years of age their IgE levels and the incidence of allergic diseases were evaluated. It was found that the synbiotic preparation had no effect on the incidence rates of allergy, however, incidences of eczema and atopic eczema were reduced, whilst a tendency towards the reduction of other atopic (IgE-associated) diseases was noted (Kukkonen et al., 2007).

In another study, the effect of a prebiotic mixture (V-GOS: inulin; 90:10) was assessed on the development of atopic dermatitis (AD), which is usually the first manifestation of allergy development during early infancy. Infants (259) at risk of atopy were fed a prebiotic mixture, or a placebo, from birth and for 6 months and

they were examined for clinical evidence of AD (Moro et al., 2006). Additionally, a smaller group of infants (98) was selected to assess the effect of the treatments on the fecal microbiots. The results showed a significant decrease in the incidence of AD in the V-GOS:Inulin group with 10 infants developing AD compared to the 24 infant that developed AD in the placebo group. In terms of the effect on the fecal microbiota, a significantly higher, compared to placebo, number of bifidobacteria was observed in the prebiotic group without any significant effect on lactobacilli.

7.5.7 Anti-pathogenic Activity of GOS

Studies have suggested that prebiotics could directly be involved in protecting the gut from infection and inflammation through the inhibition of the attachment and/or invasion of pathogenic bacteria or their toxins to the colonic epithelium. This attachment is necessary before pathogens can colonize and cause disease, and it is mediated by glycoconjugates on glycoproteins and lipids present on the microvillus membrane. Certain prebiotic oligosaccharides contain structures, similar to those found on the microvillus membrane, that interfere with the bacterial receptors by binding to them and thus preventing bacterial attachment to the same sugar moiety of the microvillus glycoconjugates. For example, α-linked GOS, present in human milk, are known to have anti-adhesive properties and be capable of toxin neutralization (Newburg et al., 2005, Morrow et al., 2005). B-GOS contains an oligosaccharide in alpha anomeric configuration, and it was shown to significantly decrease the attachment of enteropathogenic *E. coli* (EPEC) and *Salmonella enterica* serovar Typhimurium to HT-29 epithelial cell line (Tzortzis et al., 2005). The same GOS mixture was further studied in an oral challenge experiment, during which BalbC mice were fed either a placebo or B-GOS prior to *Salmonella enterica* serovar Typhimurium infection (Searle et al., 2009). It was shown that the animals fed the GOS mixture did not develop clinical symptoms of salmonellosis, even though the pathogen could be recovered in the feces. Furthermore the histopathology and structure of the epithelium were completely protected and translocation of the pathogen to other organs was limited compared to the placebo. In another study, GOS (Oligomate) was shown to inhibit the adhesion of EPEC to Hep-2 and Caco-2 epithelial cell lines more effectively than inulin, FOS, lactulose or raffinose (Shoaf et al., 2006). However, the anti-adhesive properties may be a result of GOS binding to pathogens and not a direct modulation of host immune system.

7.5.8 Neonates and Infants

The significance of a bifidobacteria predominant microbiota in healthy breast fed infant is well accepted. Infants become progressively colonized over a series of weeks and months by different bacteria, and by 2 years of age, the establishment of a more adult microbiota composition begins. Until then, however, bifidobacteria predominate in breast-fed infants, while formula fed infants have a more diverse microbiota composition. As mentioned previously, human milk oligosaccharides are thought to be responsible for this pattern of colonization in breast-fed infants (Hamosh, 2001; Sela et al., 2008), and therefore attempts have been made to develop infant formulas containing GOS mixture that will promote a microbiota pattern similar to that of breast feeding. Thus far, the most studied formulation is the combination of GOS and inulin at a ratio 9:1.

Preterm infants of about 31 weeks' gestational age and about 1 week old, were studied to compare standard formula with formula containing 1% (w/v) of the GOS-inulin mixture whilst a separate group fed fortified human milk was studied in parallel. Within 1 month of feeding the number of fecal bifidobacteria and lactobacilli in the GOS-inulin formula group increased to levels similar to the breast-fed group. In addition to this, the difference in the composition of the fecal flora between the standard formula and the GOS containing formula group was highly significant, with the latter being closer to the breast fed one. Moreover, stool consistency and stool frequency were also found to be similar between the GOS fed group and breast-fed infants (Boehm et al., 2002).

A number of similar studies, using the same prebiotic mixture, were performed in term infants and even toddlers showing similar results on the fecal microbiota (Veereman-Wauters, 2005). Interestingly, FOS was also tested in term infants at concentrations of 200, 400 and 600 mg per bottle for 2 weeks and it was found to exert no effect (Veereman-Wauters, 2005). It is generally accepted by the Scientific Committee on Food of the European Commission that the addition of V-GOS:inulin prebiotic mixture at a concentration of 0.8 g/dl to infant formula is considered safe, and this prebiotic mixture has been widely used in the last few years in Europe.

Although, a vast number of studies suggest the ability of a prebiotic mixture containing inulin and V-GOS to increase fecal bifidobacterial populations in infants, relatively few studies have looked at other possible effects, such as disease prevention. Atopy, as explained earlier, is an area that has been studied in some details and an area that offers a promise for use of GOS-based prebiotic mixtures. However, further studies are required in order to identify

the mechanism of action of these substrates, if GOS-based prebiotic mixtures are to be considered and used in a treatment strategy.

7.6 Under-researched and Possible Beneficial Properties of GOS

The full potential of health related and health promoting benefits of GOS is yet to be fully ascertained. As the interactions of the colonic microbiota, especially the beneficial effects of *Bifidobacterium* spp and *Lactobacillus* spp with the host are elucidated, the increased bifidogenicity of GOS is becoming a significant property for its application as a health promoting food ingredient. Consequently there are many potential areas of research where GOS has not been used and where, indeed, the prebiotics in general have not been studied.

Irritable bowel syndrome (IBS) is a common chronic functional gastrointestinal disorder that exhibits a broad spectrum of severity, ranging from mild symptoms to severe and intractable symptoms. IBS is characterized by recurrent abdominal pain and discomfort associated with alterations in bowel habit. The etiology and physiology of IBS are not fully understood, but it is most likely multifactorial. Alterations in gastrointestinal motility, visceral perception, and psychosocial factors contribute to overall symptom expression. Currently there is no single therapeutic modality of proven benefit to all IBS patients and treatment is based on the physician's understanding of the individual patient's symptom pattern and the associated psychosocial factors. Mixed probiotic combinations, mainly using specific *Bifidobacterium* species, have been used successfully in the treatment of IBS symptoms, however prebiotics have not. The possibility that GOS, through its increased bifidogenicity, could help ameliorate some of the symptoms associated with IBS as has been shown by Silk and colleagues (2009) could not be excluded and this should definitely create one area for the future research.

A common side effect of the use of antibiotics is antibiotic-associated diarrhea (AAD), which presents a particular problem in hospitalized, and especially vulnerable elderly, patients. Diarrhea associated with *Clostridium difficile* is a leading cause of hospital outbreaks of diarrhea and it considerably increases mortality and healthcare costs for inpatients. The condition occurs when patients are treated with antibiotics, for an underlying infection, which result in the disruption of the barrier of normally protective colonic microbiota. Probiotics have been successfully used to reduce the incidence of AAD. Lewis et al. (2005)

investigated the effect of 12 g of FOS on the incidence and relapse of *C. difficile* in elderly patients. Although differences between the prebiotic and placebo group were not observed on the incidence rate, there was a significant reduction in the rate of relapse in the prebiotic group accompanied by a significant increase in bifidobacteria numbers. It is clear that more prebiotic trials and involving of GOS are needed to fully explain the potential in treating or preventing AAD, as well as other GI pathogen related conditions such as travellers' diarrhea and these also offer potential areas for the future research.

Arthritis is associated with a broad spectrum of clinical and experimental intestinal inflammation, ranging from infections with intestinal pathogens, overgrowth and changes to the normal commensal colonic microbiota, injection of purified bacterial cell wall components and dietary manipulation to chronic idiopathic IBD. The common feature of all those conditions is increased exposure of the lamina propria and systemic circulation to colonic microbiota and their products, either through increased proliferation or mucosal permeability, pathogenic invasion, or immune modulation. Experimental data assessing the potential of prebiotics in arthritis is still lacking and limited to animal models. However, α-GOS has been used in adjuvant-induced arthritis Wistar rats, and type II collagen-induced arthritis in DBA/1 J mice. The dose-dependent beneficial effect was observed in erythema, swelling of limbs and on histological findings in the hind paw joints (Abe et al., 2004). This study also showed reduced levels of nitrite and nitrate in blood, although the production of IL-1 by macrophages was increased. The results indicate the potential of using GOS to immunomodulate the inflammation seen in arthritis through either direct effect or via the modulation of the colonic microbiota. However, more research and involving human subjects is needed to clarify the potential.

7.7 Summary

- Commercially available GOS products are mixtures of galactose based oligosaccharides of varying DP and linkage configuration with glucose, galactose and lactose.
- The oligosaccharide composition varies amongst GOS mixtures depending on the origin of the β-galactosidase enzyme used as well as the mode of production. Developments in the production of GOS aim to delivering purer and more efficient mixtures.
- GOS consist of oligosaccharides that are pH and heat stable offering a wide range of food application potential.

- GOS are safe well tolerated ingredients up to levels of 20 g intake per day, have a GRAS status in the USA and FOSHU status in Japan and could be included in the dietary fiber content of foods.
- GOS mixtures are well established prebiotic ingredients with increased selectivity towards *Bifidobacterium* species. Depending on their oligosaccharide composition, GOS products vary in terms of quantitative and qualitative bifidogenic properties.
- Besides through their bifidogenic effect, GOS contribute to the health of the host through their direct interaction, leading to increased immunomodulatory and anti-pathogenic properties.
- Infant and elderly nutrition offer the highest opportunity for GOS applications based on their functional properties (bifidogencicty, protection from pathogens, regulation of the immune function) and the host's requirements.

List of Abbreviations

AD	atopic dermatitis
CSTR	continuous stirred-tank reactor
DP	degree of polymerization
EPEC	enteropathogenic *Escherichia coli*
FOS	fructooligosaccharides
GALT	gut associated lymphoid tissue
GOS	galacto-oligosaccharides
NF κB	nuclear factor κB
NK cell	natural killer cells
SCFA	short chain fatty acids
TLR	toll like receptors
TNF-α	tumor necrosis factor α

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