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# Galacto-Oligosaccharides: Production, Properties, Applications, and Significance as Prebiotics

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**Abstract:** Galacto-oligosaccharides (GOS) have now been definitely established as prebiotic ingredients after *in vitro* and animal and human *in vivo* studies. Currently, GOS are produced by glycoside hydrolases (GH) using lactose as substrate. Converting lactose into GOS by GH results in mixtures containing GOS of different degrees of polymerization (DP), unreacted lactose, and monomeric sugars (glucose and galactose). Recent and future developments in the production of GOS aim at delivering purer and more efficient mixtures. To produce high-GOS-content mixtures, GH should not only have good ability to catalyze the transgalactosylation reaction relative to hydrolysis, but also have low affinity for the GOS formed relative to the affinity for lactose. In this article, several microbial GH, proposed for the synthesis of GOS, are hierarchized according to the referred performance indicators. In addition, strategies for process improvement are discussed. Besides the differences in purity of GOS mixtures, differences in the position of the glycosidic linkages occur, because different enzymes have different regiochemical selectivity. Depending on oligosaccharide composition, GOS products will vary in terms of prebiotic activity, as well as other physiological effects. This review focuses on GOS production from synthesis to purification processes. Physicochemical characteristics, physiological effects, and applications of these prebiotic ingredients are summarized. Regulatory aspects of GOS-containing food products are also highlighted with emphasis on the current process of health claims evaluation in Europe.

## Introduction

Lactose ($\beta$-D-galactopyranosyl-(1→4)-$\alpha$-D-glucopyranose) is a disaccharide present in the milk of all mammals, with only few minor exceptions, in the range of concentrations of 2.0% to 10% (w/v). The average lactose content of bovine milk is about 4.8%, ranging between 4.4% and 5.2% (Ganzle and others 2008). During cheese production, almost all the lactose in milk is transferred into whey. Unlike in the past, whey is not considered a waste anymore. Nowadays, economic and environmental considerations dictate that whey should be used efficiently. Whey can be dried to produce various whey powders or fractionated by membrane technology to produce whey protein products and a permeate stream rich in lactose (Fox 2009; Lifran and others 2009).

The most common method for large-scale production of lactose directly from whey or from whey permeate is crystallization of a supersaturated solution (Yang and Silva 1995; Paterson 2009). In 2006, about 870000 metric tons crystalline lactose were produced worldwide with an expected compound annual growth rate (CAGR) between 3% and 5% until 2010 (Affertsholt-Allen 2007). Lactose has many uses in foods (infant formulas, chocolate and confectionery products, baked items, and other processed foods including meat products) and pharmaceuticals (as excipient of tablets and, in finely granulated form, as a carrier of medicines in dry powder inhalation preparations) (Schaafsma 2008).

In humans, lactose maldigestion increases with age, reaching reported adult levels of approximately 70% of the world's adult population (Paige 2005). These individuals tend to avoid milk consumption because of the risks of serious abdominal discomfort. The observation of this physiological event was the main driving force for the development of commercial enzymes with $\beta$-galactosidase activity that permitted the production of low lactose products (Gekas and Lopez-Leiva 1985; Pivarnik and others 1995; Nakayama and Amachi 1999; Rehman 2009). Since lactose has low solubility and low sweetness, its hydrolysis can also be performed to decrease unwanted lactose crystallization events in lactose-rich products during food preservation, and to increase the range of lactose applications when sweetness is desired (Gekas and Lopez-Leiva 1985; Pivarnik and others 1995; Nakayama and Amachi 1999; Rehman 2009).

$\beta$-Galactosidase is a hydrolase that attacks the o-glucosyl group of lactose. The general mechanism of enzymatic lactose hydrolysis has a transgalactosylic nature, involving a multitude of sequential reactions with disaccharides (other than lactose) and higher saccharides, collectively named galacto-oligosaccharides (GOS), as intermediate products (Wallenfels and Malhotra 1960). In

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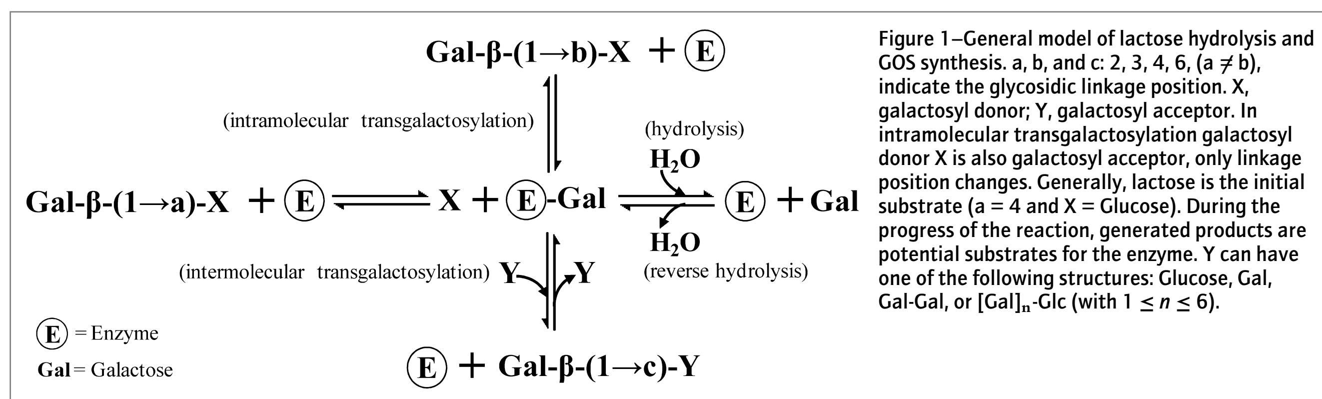


Figure 1–General model of lactose hydrolysis and GOS synthesis. a, b, and c: 2, 3, 4, 6, (a ≠ b), indicate the glycosidic linkage position. X, galactosyl donor; Y, galactosyl acceptor. In intramolecular transgalactosylation galactosyl donor X is also galactosyl acceptor, only linkage position changes. Generally, lactose is the initial substrate (a = 4 and X = Glucose). During the progress of the reaction, generated products are potential substrates for the enzyme. Y can have one of the following structures: Glucose, Gal, Gal-Gal, or $[Gal]_n$-Glc (with $1 \leq n \leq 6$).

processes for which lactose hydrolysis is required, such as for low lactose or lactose-free products, GOS appear as undesirable by-products as they can also potentially produce secondary effects. However, the ability of those oligosaccharides, when added to infant milk formulas, to replicate the bifidogenic effect of human milk, not only in bacterial numbers, but also with respect to the metabolic activity of the colonic microbiota (Knol and others 2005b), has significantly increased interest in their production and application in various food and pharmaceutical processes.

GOS were recently defined as "a mixture of those substances produced from lactose, comprising between 2 and 8 saccharide units, with one of these units being a terminal glucose and the remaining saccharide units being galactose and disaccharides comprising 2 units of galactose" (Tzortzis and Vulevic 2009). The global market size of GOS was recently estimated to be about 20.000 tons with a CAGR of 10% to 20% (Affertsholt-Allen 2007).

This review on GOS focuses on production processes from synthesis to purification. Physicochemical characteristics, physiological effects, and applications of these prebiotic ingredients are summarized.

## Synthesis Strategies for Production of GOS

It is well known that oligosaccharides can be formed from monosaccharides by the action of mineral acids (chemical synthesis). This process, known as "reversion," explains the production of oligosaccharides during acidic hydrolysis of lactose, first observed in the 1950s (Aronson 1952). The conditions suitable for oligosaccharide production during acidic hydrolysis of lactose and the resulting oligosaccharide structures formed have been well studied (Huh and others 1990, 1991). It was reported that there is formation of a complex mixture of disaccharides and trisaccharides, with a variety of linkages with $\alpha$- and $\beta$-anomeric configurations, and anhydro-sugars, as a result of this chemical process (Huh and others 1991). Probably due to the lack of product specificity and extreme conditions applied during acidic hydrolysis of lactose, this GOS production process is not used on a large scale.

The preferred mode for GOS synthesis is by enzymatic catalysis from lactose using glycosyltransferases (EC 2.4) or glycoside hydrolases (EC 3.2.1) (De Roode and others 2003). Glycosyltransferases and glycoside hydrolases are enzymes that are responsible for the transfer of glycosyl moieties from a donor sugar to an acceptor (Ly and Withers 1999). Glycosyltransferases use sugar donors containing a nucleoside phosphate or a lipid phosphate remaining group (Coutinho and others 2003; Lairson and others 2008). Although highly regio-selective, stereo-selective, and efficient, these enzymes are not used for industrial GOS production due to their unavailability, prohibitive prices of commercial enzyme preparations, and the need of specific sugar nucleotides as substrates (De Roode and others 2003).

Currently, GOS are industrially produced using the catalytic activity of glycoside hydrolases. These enzymes are more readily available than glycosyltransferases but are generally less stereo-selective (Tzortzis and Vulevic 2009). Enzymatic hydrolysis of the glycosidic bond is performed by 2 catalytic residues of the enzyme acting as a general acid and a nucleophile/base, respectively. Depending on the spatial position of these catalytic residues, hydrolysis can occur with 1 of 2 possible stereochemical outcomes: inversion of anomeric configuration, if the average distance between the 2 catalytic residues is approximately 10 Å; or retention of anomeric configuration, if the average distance between the 2 catalytic residues is about 5.5 Å. This subject has been extensively reviewed by other researchers (Koshland 1953; Sinnott 1990; Davies and Henrissat 1995; Zechel and Withers 2001).

Some retaining glycoside hydrolases are able to catalyze GOS synthesis. Mechanistically, these enzymes are thought to operate by a 2-step reaction mechanism. The 1st irreversible step involves formation of a configurationally inverted galactosyl-enzyme intermediate, followed by the exit of the "*leaving group*." The covalent intermediate is subsequently hydrolyzed, again with inversion, completing the reaction with retention of configuration via a general acid-base catalytic mechanism (Koshland 1953; Sinnott 1990; Davies and Henrissat 1995; Zechel and Withers 2001). In a well-known variant of the 2nd step, the galactosylated enzyme may be intercepted by nucleophiles other than water, potentially any sugar in solution, to form transgalactosylation products (GOS) (Figure 1) (Wallenfels 1951; Pazur 1953).

Therefore, converting lactose into GOS by $\beta$-galactosidases is a kinetically controlled reaction, by means of the competition between hydrolysis and transgalactosylation. Specifically, during this conversion, the thermodynamically favored hydrolysis of lactose, which generates D-galactose and D-glucose, competes with the transferase activity that generates a complex mixture of various galactose-based di- and oligosaccharides of different structures (Tzortzis and Vulevic 2009). Hence, knowledge of the reaction time course (or lactose conversion) is required to determine the point of maximum yield of the desired product.

Transgalactosylation involves both intermolecular and intramolecular reactions. Intramolecular or direct galactosyl transfer to D-glucose yields regio-isomers of lactose. Intermolecular or indirect transgalactosylation is the route by which disaccharides, trisaccharides, and tetrasaccharides, and eventually longer GOS, are produced from lactose (Huber and others 1976) (Figure 1).

Alternatively to transgalactosylation from disaccharide described previously, GOS can be obtained by reverse hydrolysis

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**Table 1–Archaeal sources of glycoside hydrolases used for the production of GOS.**

| ARCHAEA | | GH | $T/°C$ | pH | $L_0$ | Y | Process | Ref. |
|---|---|---|---|---|---|---|---|---|
| >**Crenarchaeota** | | | | | | | | |
| *Sulfolobus solfataricus* | $\beta$-Galactosidase (LacS), cloned | GH1 | 70 to 80 | 5.5 to 6.5 | 7 to 80 | 53 | | a |
| | $\beta$-Galactosidase (LacS), cloned | GH1 | 75 | 6.5 | 50 | 34 | TPS | b |
| | $\beta$-Galactosidase (LacS), cloned | GH1 | 70 | 5.5 | 4.5 to 17 | 23 | CMAR | c |
| >Euryarchaeota | | | | | | | | |
| *Pyrococcus furiosus* | $\beta$-Glucosidase (CelB), cloned | GH1 | 70 to 95 | 5.0 to 5.5 | 7 to 90 | 40 | | d |
| | $\beta$-Glucosidase (CelB), mutated | GH1 | 90 to 95 | 5.0 to 6.0 | 10 to 90 | 45 | | e |
| | $\beta$-Glucosidase (CelB), cloned | GH1 | 70 | 5.5 | 4.5 to 17 | 25 | CMAR | f |
| | $\beta$-Mannosidase (BmnA), cloned | GH1 | 90 to 95 | 5.0 to 5.5 | 10 to 70 | 44 | | g |

GH, glicoside hydrolase family, if available (CAZy 2009). $L_0$, initial lactose concentration (%, w/v). Y, maximum GOS yield reported (%) = GOS concentration/$L_0$ × 100 (±1%). TPS, 2-phase systems; CMAR, membrane-assisted reactor (continuous). a, Petzelbauer and others (2000b), Hansson and Adlercreutz (2001), Splechtna and others (2001), Park and others (2008); b, Hansson and Adlercreutz (2001); c, Petzelbauer and others (2002); d, Petzelbauer and others (2000b), Hansson and others (2001); e, Hansson and others (2001); f, Petzelbauer and others (2002); g, Hansson and others (2001).
DNA cloning and expression of recombinant glycoside hydrolases was performed in *E. coli*.

(equilibrium synthesis) from monosaccharides (Figure 1) (Drueckhammer and others 1991; Monsan and Paul 1995; Pivarnik and others 1995; Crout and Vic 1998). Equilibrium yields are much lower, as mainly disaccharides (around 10% to 25%, w/w) and few percentages of trisaccharides (or higher) are formed. However, from this reaction no hydrolytic side-products are obtained, and when combined with an effective separation process, a hypothetical 100% yield is possible (Bruins and others 2003).

## GOS Production

### The catalysts: glycoside hydrolases

The rapid increase since the late 1980s in the number of available amino acid sequences of glycoside hydrolases (GHs) and the availability of new sequence comparison methods permit the classification of these enzymes based on amino acid sequence similarities (Henrissat 1991; Henrissat and Bairoch 1996; Cantarel and others 2009). Currently, these enzymes constitute 115 protein families (CAZy 2009). Enzymes with $\beta$-galactosidase activity (classified as EC 3.2.1.23/108, according to substrate specificity) are grouped within the GH1, GH2, GH35, and GH42 families. Lactose is the natural substrate only to some enzymes belonging to GH1 and GH2, while families GH35 and GH42 include enzymes that act on different galactose-containing glycosides (Adam and others 2004; EFSA 2010b). Some $\beta$-glucosidases from the GH1 family, which are involved in the hydrolysis of terminal, nonreducing $\beta$-D-glucosyl residues (EC 3.2.1.21), and $\beta$-mannosidases from the GH1 and GH2 families, which are involved in the hydrolysis of terminal, nonreducing $\beta$-D-mannosyl residues (EC 3.2.1.25), also attack the o-glucosyl group of lactose, what make them suitable for GOS synthesis (see Table 1 to 3).

Glycoside hydrolases with $\beta$-galactosidase activity occur in a variety of microorganisms from the superkingdoms Archaea, Bacteria, and Eukaryota. Some of these enzymes have been expressed in host organisms, and/or purified by a combination of several conventional techniques, such as salting-out fractionation, ion exchange, gel filtration, hydroxyapatite, and hydrophobic interaction chromatographies (Nakayama and Amachi 1999). A variety of commercial glycoside hydrolases with $\beta$-galactosidase activity are commercially available for use in food processing (Richmond and others 1981; Gekas and Lopez-Leiva 1985; Pivarnik and others 1995; Panesar and others 2006), and some of them are already used in the industrial production of GOS as discussed in a section previously. Nevertheless, there is continuous interest in finding microorganisms with adequate properties for industrial uses and able to produce specific GOS mixtures with better yields.

The product spectrum obtained during lactose conversion, the linkage between the galactose units, and the efficiency of transgalactosylation all depend on the enzyme source and the physicochemical conditions in the catalytic environment. Interactions between the galactosyl acceptor and the active site of the enzyme are thought to play a major role in the formation of intermolecular galactosyl transfer products (Petzelbauer and others 2000a). In other words, the enzyme ability to accommodate the galactosyl acceptor in the neighborhood of the active site during the catalytic moment, and the spatial orientation of the galactosyl acceptor, are probable key factors in the transgalactosylation efficiency and in the position of new glycosidic linkages (see Figure 2 and Table 4).

Glucose/galactose ratio at the maximum GOS yield was determined to quantify the ability of different enzymes to catalyze the transgalactosylation reaction (to lactose or galactose acceptors) relative to complete hydrolysis (Figure 2). This ratio should be higher than 1 because some of the galactose produced by lactose cleavage was transferred to oligosaccharides, whereas all the glucose was released. It should be noted that this ratio does not take into account the transfer of the galactosyl moiety to free glucose via intermolecular reaction or via intramolecular reaction (see Figure 1 for details).

### Process improvement

Generally, the yield of GOS synthesis from lactose using glycoside hydrolases can be increased by: using highly concentrated starting lactose solution; decreasing water thermodynamic activity (for example, using a micro-aqueous environment); removing the final product and/or inhibitors from the reaction medium; and modifying the enzyme (Monsan and Paul 1995; Czermak and others 2004).

**Using highly concentrated starting lactose solution.** Data gleaned from the literature show that maximum GOS yield is largely influenced by initial lactose concentration ($L_0$) mainly, until the concentration range is 30% to 40% (w/v). For $L_0 > 30\%$ the influence of $L_0$ in GOS yield markedly decreases (Figure 3). Data from *A. oryzae*, *A. aculeatus*, and *B. longum* (not represented) are more scattered but show a similar behavior for $L_0 > 40\%$.

Since lactose solubility is relatively low at room temperature but manifestly increases with increasing temperature (Roos 2009), high temperatures are generally desired. Some studies have been focused on sourcing thermostable glycoside hydrolases. Glycoside hydrolases from *S. solfataricus* (Petzelbauer and others 2000b; Hansson and Adlercreutz 2001; Splechtna and others 2001; Park and others 2008), *P. furiosus* (Petzelbauer and others 2000b; Hansson and others 2001), *Thermus* sp. (Akiyama and others 2001), *T. caldophilus* (Choi and others 2003), *C. saccharolyticus* (Stevenson and others 1996), *T. maritima* (Kim and others 2004; Ji and others 2005) are examples of enzymes from hyperthermophil microorganisms used at temperatures around 80 °C and higher.

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**Table 2–Bacterial sources of glycoside hydrolases used for the production of GOS.**

| BACTERIA | | GH | $T/°C$ | pH | $L_0$ | Y | I | Process | Ref. |
|---|---|---|---|---|---|---|---|---|---|
| >**Actinobacteria** | | | | | | | | | |
| *Saccharopolyspora rectivirgula* | $\beta$-Galactosidase, purified | GH2 | 70 | 7.0 | 60 | 44 | | | a |
| *Bifidobacterium adolescentis* | Crude enzyme fraction | | 55 | 7.5 | 30 | 43 | | | b |
| *Bifidobacterium angulatum* | Crude enzyme fraction | | 55 | 7.5 | 5 to 30 | 44 | | | c |
| *Bifidobacterium bifidum* | $\beta$-Galactosidase (BIF3), mutated | GH2 | 37 | 6.0 | 10 to 40 | 44 | | | d |
| | Crude enzyme fraction | | 55 | 7.5 | 30 | 38 | | | e |
| | Cells, resting cells | | 39 | 6.8 | 50 | 20 | | | f |
| | Cells, toluene-treated, resting cells | | 40 | 6.2 to 6.8 | 45 to 50 | 44 | | | g |
| | Cells, toluene-treated, resting cells | | 40 | 5.0 to 5.5 | Whey, 45 to 50 | 38 | | | h |
| *Bifidobacterium longum* subsp. *infantis** | $\beta$-Galactosidase ($\beta$-GalI), cloned# | GH2 | 30 | 6.5 | 36 | 49 | | | I |
| | $\beta$-Galactosidase ($\beta$-GalI), cloned | GH2 | 30 to 60 | 7.5 | 20 to 30 | 68 | | | j |
| | $\beta$-Galactosidase, ($\beta$-GalIII), cloned | GH42 | 37 | 7.5 | 20 | 10 | | | k |
| | Crude enzyme fraction | | 55 | 7.5 | 30 | 48 | | | l |
| | Cells, in culture | | 60 | 7.5 | Milk, 35 | 43 | | | m |
| *Bifidobacterium longum* | Crude enzyme fraction | | 45 | 6.8 | 5 to 50 | 35 | | | n |
| *Bifidobacterium pseudolongum* | Crude enzyme fraction | | 55 | 7.5 | 30 | 27 | | | o |
| >Deinococcus-Thermus | | | | | | | | | |
| *Thermus* sp. | $\beta$-Glucosidase, cloned | GH1 | 70 | 7.0 | 7.5 to 30 | 40 | | | p |
| *Thermus caldophilus* | $\beta$-Glucosidase (BglA) cloned | GH1 | 70 to 80 | 6.0 | 30 to 50 | 77 | | | q |
| >Thermotogae | | | | | | | | | |
| *Thermotoga maritima* | $\beta$-Galactosidase (LacZ), cloned | GH2 | 80 | 6.0 | 20 to 50 | 19 | | | r |
| >**Firmicutes** | | | | | | | | | |
| *Bacillus circulans* | $\beta$-Galactosidase (I), purified | | 40 | 6.0 | 4.6 | 6.0 | | | s |
| | | | 40 | 6.0 | 4.3 | 40 | SG | | t |
| | $\beta$-Galactosidase (II), purified | | 40 | 6.0 | 4.6 | 41 | | | u |
| | | | 40 | 6.0 | 4.6 to 20 | 48 | SG | PBR | v |
| | | | 40 | 6.0 | 4.6 | 35 | PR | PBR | w |
| | Crude enzyme fraction | | 40 to 55 | 6.0 | 6.5 to 36 | 43 | | | x |
| *Bacillus* sp. | Crude enzyme fraction | | 50 to 55 | 5.0 | 33 to 36 | 43 | | | y |
| | | | 55 | 5.0 | 36 | 41 | Chitosan | | z |
| *Geobacillus stearothermophilus* | $\beta$-Galactosidase (BgaB) | GH42 | 37 | 6.5 | 17 | 2.4 | | | aa |
| | $\beta$-Galactosidase (BgaB), mutated | GH42 | 37 | 6.5 | 17 | 30 | | | ab |
| *Caldicellulosiruptor saccharolyticus*** | $\beta$-Glucosidase (Bgla), cloned | GH1 | 65 to 80 | 6.3 | 10 to 72 | 63 | | | ac |
| *Lactobacillus acidophilus* | $\beta$-Galactosidase (LacL+LacM), cloned | GH2 | 30 | 6.5 | 21 | 39 | | | ad |
| *Lactobacillus reuteri* | $\beta$-Galactosidase (LacL+LacM), cloned | GH2 | 23 | 6.0 | 21 | 26 | | | ae |
| | $\beta$-Galactosidase (LacL+LacM), purified | GH2 | 30 to 37 | 6.0 to 6.5 | 4.6 to 21 | 38 | | | af |
| | | GH2 | 37 | 6.0 | 21 | 29 | | CMAR | ag |
| *Streptococcus thermophilus* | $\beta$-Galactosidase (LacZ), purified | GH2 | 37 | – | Milk, 5.3 | 25 | | | ah |
| >Proteobacteria | | | | | | | | | |
| *Enterobacter agglomerans**** | $\beta$-Galactosidase (LacZ), cloned | GH2 | 50 | 7.5 | 5 to 13 | 38 | | | ai |
| *Enterobacter cloacae* | $\beta$-Galactosidase, (Bga/LacZ), cloned | GH2 | 50 | 6.5 | 28 | 49 | | | aj |
| | Cells, resting | | 50 | 6.5 | 28 | 55 | | | ak |
| *Escherichia coli* | $\beta$-Galactosidase (LacZ), purified | GH2 | 30 to 37 | 6.5 to 7.2 | 2.2 to 24 | 56 | | | al |
| | | | 37 | 7.5 | 2.2 | 44 | | RM | am |

GH, glicoside hydrolase family, if available (CAZy 2009). $L_0$, initial lactose concentration (%, w/v). Y, maximum GOS yield reported (%) = GOS concentration/$L_0 \times 100$ ($\pm$ 1%). I, immobilization support. SG, silica gel; PR, phenolformaldehyde resin; PBR, packed bed reactor (continuous); CMAR, membrane-assisted reactor (continuous); RM, dioctyl sodium sulfosuccinate/isooctane reverse micelles. a, Nakao and others (1994); b, Rabiu and others (2001); c, Rabiu and others (2001); d, Jorgensen and others (2001); e, Rabiu and others (2001); f, Tzortzis and others (2005b); g, Goulas and others (2007a); h, Goulas and others (2007a); i, Jung and Lee (2008); j, Hung and others (2001), Hung and Lee (2002); k, Hung and others (2001); l, Rabiu and others (2001); m, Roy and others (2002); n, Hsu and others (2007); o, Rabiu and others (2001); p, Akiyama and others (2001); q, Choi and others (2003); r, Kim and others (2004), Ji and others (2005); s, Mozaffar and others (1984); t, Mozaffar and others (1987); u, Mozaffar and others (1984); v, Mozaffar and others (1986); w, Mozaffar and others (1986); x, Boon and others (1999, 2000a), Cheng and others (2006c); y, Cheng and others (2006a, 2006c); z, Cheng and others (2006c); aa, Placier and others (2009); ab, Placier and others (2009); ac, Stevenson and others (1996); ad, Nguyen and others (2007); ae, Maischberger and others (2008); af, Splechtna and others (2006, 2007); ag, Splechtna and others (2007); ah, Greenberg and Mahoney (1983); ai, Lu and others (2007); aj, Lu and others (2009); ak, Lu and others (2009); al, Huber and others (1976), Chen and others (2003); am, Chen and others (2003). **Bifidobacterium longum* subsp. *infantis* synonym: *Bifidobacterium infantis*; ** *Caldicellulosiruptor saccharolyticus* synonym: *Caldocellum saccharolyticum*; *** *Enterobacter agglomerans* synonym: *Pantoea agglomerans*; DNA cloning and expression of recombinant glycoside hydrolases was performed in *E. coli* except # cloned into *Pichia pastoris*.

High temperature also seems to favor transgalactosylation relative to hydrolysis. An increase in reaction temperature significantly increases GOS yield when glycoside hydrolases from *A. aculeatus* (Cardelle-Cobas and others 2008), *S. solfataricus*, or *P. furiosus* (Hansson and Adlercreutz 2001) were used. However, other researchers found only a slight or no correlation between temperature and GOS yield using *P. furiosus* (Boon and others 1998; Petzelbauer and others 2002), *S. solfataricus* (Petzelbauer and others 2002), *B. circulans*, *A. oryzae*, *K. lactis*, and *K. marxianus* (Boon and others 2000a).

The use of microwave irradiation as a nonconventional energy source during GOS synthesis was also successfuly attempted using an immobilized crude enzyme preparation from *K. lactis* at 40 °C (Maugard and others 2003). Maximum GOS yield increased from 24% to 28% when initial lactose concentration was 16% (w/v).

**Decreasing water thermodynamic activity.** An example of using a micro-aqueous environment to decrease water thermodynamic activity during GOS synthesis is the use of reverse micelles. A similar transgalactosylation capability was obtained at low lactose concentration when reverse micelles were used, as compared to that reported at high lactose concentration in an aqueous system (Chen and others 2001, 2003).

Aqueous 2-phase systems appear to increase the transgalactosylation yield in comparison with usual aqueous solution probably due to the partitioning of the desired product, inhibitors, and the enzyme between the 2 phases of the system. The glucose/galactose ratio at maximum GOS yield using a crude enzyme fraction from *A. aculeatus* increased from 2.2 to 12.7 (Figure 2) when an aqueous 2-phase system is used, reflecting an advantageous environment for galactosyl transfer reactions (Del-Val and Otero 2003).

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**Table 3–Fungal sources of glycoside hydrolases used for the production of GOS.**

| EUKARYOTA | | GH | $T/°C$ | pH | $L_0$ | Y | I | Process | Ref. |
|---|---|---|---|---|---|---|---|---|---|
| **>Ascomycota** | | | | | | | | | |
| *Aspergillus aculeatus* | Crude enzyme fraction | | 45 to 60 | 4.5 to 6.5 | 10 to 57 | 25 | | | a |
| | | | 45 to 50 | 6.5 | 21 to 24 | 24 | | ATPS | b |
| | | | 50 | 6.5 | 30 | 15 | HAB | | c |
| *Aspergillus niger* | Crude enzyme fraction | | 40 | 7.0 | 2 to 30 | 16 | | | d |
| *Aspergillus oryzae* | Crude enzyme fraction | | 40 to 80 | 4.5 to 7.0 | 1 to 72 | 35 | | | e |
| | | | 45 | 7.0 | 10 to 50 | 51 | | RM | f |
| | | | 40 | 4.5 | 27 | 22 | | MA | g |
| | | | 50 to 55 | 4.5 to 4.7 | 17 to 30 | 31 | | CMAR | h |
| | | | 35 to 50 | 4.5 to 5.0 | Whey, 4 to 23 | 18 | | | i |
| | | | 40 | 4.0 to 4.5 | 10 to 30 | 26 | Chitosan | | j |
| | | | 40 | 4.5 | 5 to 50 | 27 | Cotton | | k |
| | | | 40 | 4.5 | 27 | 20 | Cotton | PB | l |
| | | | 40 | 4.5 | 5 to 50 | 26 | Cotton | PBR | m |
| | | | 40 | 4.5 | 5 to 50 | 26 | mPOS-PVA | | n |
| | | | 40 | 4.5 | 5 to 50 | 26 | mPOS–PANI | | o |
| | | | 40 | 4.5 | Whey, 6 to 20 | 14 | PVC-film | PBR | p |
| *Penicillium expansum* | Crude enzyme fraction | GH35 | 50 | 5.4 | 5.0 to 48 | 29 | Alginate | | q |
| | CSDS, *Saccharomyces cerevisiae*, in culture | GH35 | 25 | — | 5 to 25 | 44 | | | r |
| *Penicillium funiculosum* | Crude enzyme fraction | | 40 | 5.0 | 5.0 | 20 | | | s |
| *Penicillium simplicissimum* | $\beta$-Galactosidase, purified | | 50 | 6.5 | 30 to 60 | 30 | | | t |
| *Talaromyces thermophilus* | $\beta$-Galactosidase, purified | | 40 | 6.5 | 20 | 50 | | | u |
| | | | 40 | 6.5 | 5.0 to 20 | 41 | HAB | | v |
| | | | 40 | 6.5 | 20 | 25 | HAB | PBR | w |
| *Trichoderma harzianum* | Cells, in culture | | 30 | 7.0 | 15 | 32 | | | x |
| *Kluyveromyces lactis** | Crude enzyme fraction | | 24 to 50 | 5.0 to 7.5 | 5.0 to 40 | 38 | | | y |
| | | | 40 | 6.9 to 7.5 | 17 to 31 | 43 | | CMAR | z |
| | | | 40 | 6.5 | 8.0 to 32 | 28 | AER | | aa |
| | | | 40 | 6.5 | 16 | 28 | AER | MW | ab |
| | | | 37 | 6.6 | 3.0 to 13 | 7.4 | Cotton | PBR | ac |
| | | | 35 to 45 | 7.0 | Whey, 14 to 23 | 22 | | | ad |
| | | | 45 | 7.0 | Whey, 20 | 31 | | CMAR | ae |
| *Kluyveromyces marxianus*** | Crude enzyme fraction | | 35 to 40 | 6.2 to 7.0 | 5.0 to 50 | 45 | | | af |
| | | | 45 | 7.0 | Whey, 14 to 23 | 24 | | | ag |
| **>Basidiomycota** | | | | | | | | | |
| *Sirobasidium magnum* | $\beta$-Galactosidase, purified | | 60 | 5.0 | 20 | 36 | | | ah |
| | Cells, in culture | | 30 | 6.0 | 36 | 62 | | | ai |
| | Cells, toluene-treated, resting cells | | 50 | 6.0 | 36 | 38 | | | aj |
| *Sterigmatomyces elviae* | $\beta$-Galactosidase, purified | | 60 | 5.0 | 20 | 38 | | | ak |
| | Cells, in culture | | 30 | 6.0 | 36 | 64 | | | al |
| | Cells, toluene-treated, resting cells | | 30 | 6.0 | 36 | 38 | | | am |
| *Rhodotorula minuta* | $\beta$-Glucosidase, purified | | 60 | 5.0 | 20 | 39 | | | an |
| | Cells, in culture | | 30 | 6.0 | 36 | 64 | | | ao |
| | Cells, toluene-treated, resting cells | | 60 | 6.0 | 20 | 39 | | | ap |
| *Sporobolomyces singularis**** | $\beta$-Glucosidase (Bgla), purified | GH1 | 37 to 50 | 3.7 to 6.0 | 18 to 30 | 54 | | | aq |
| | $\beta$-Glucosidase, purified | GH1 | 45 | 4.8 | 10 | 55 | Chitosan | PBR | ar |
| | | GH1 | 37 | 6.0 | Whey, 20 | 34 | | | as |
| | Cells, resting cells | | 55 | 5.0 | 60 | 41 | | | at |
| | Cells, resting cells | | 55 | 5.0 | 60 | 40 | Alginate | | au |

CSDS, cell-surface display system ($\beta$-Galactosidase gene from *P. expansum* expressed on the cell surface of *S. cerevisiae*); GH, glicoside hydrolase family, if available (CAZy 2009). $L_0$, initial lactose concentration (%, w/v). Y, maximum GOS yield reported (%) = GOS concentration$/L_0 \times 100$ ($\pm$ 1%). I, immobilization support. HAB, hydrophilic acrylic beads (Eupergit C®); mPOS-PVA, magnetic polysiloxane-polyvinyl alcohol; mPOS–PANI, magnetic polysiloxane–polyaniline; PVC-film, polyvinyl chloride film; AER, anion exchange resin; ATPS, aqueous 2-phase systems; RM, dioctyl sodium sulfosuccinate/isooctane reverse micelles; MA, recycled membrane-assisted system; CMAR, membrane-assisted reactor (continuous); PB, packed bed assisted system; PBR, packed bed reactor (continuous); MW, microwave power 12 watt.
a, Del-Val and others (2001), Del-Val and Otero (2003), Aslan and Tanriseven (2007), Cardelle-Cobas and others (2008); b, Del-Val and Otero (2003); c, Aslan and Tanriseven (2007); d, Toba and Adachi (1978), Prenosil and others (1987); e, Prenosil and others (1987), Iwasaki and others (1996), Stevenson and others (1996), Boon and others (2000b), Chen and others (2001), Cheng and others (2006a), Gaur and others (2006), Matella and others (2006), Neri and others (2009a); f, Chen and others (2001); g, Matella and others (2006); h, Czermak and others (2004), Ebrahimi and others (2006b); i, Prenosil and others (1987), Rustom and others (1998); j, Sheu and others (1998), Gaur and others (2006); k, Albayrak and Yang (2002a, 2002b, 2002c), Matella and others (2006); l, Matella and others (2006); m, Albayrak and Yang (2002b); n, Neri and others (2009a); o, Neri and others (2009b); p, Leiva and Guzman (1995); q, Li and others (2008); r, Li and others (2009); s, Shin and Yang (1996); t, Cruz and others (1999); u, Nakkharat and Haltrich (2007); v, Nakkharat and Haltrich (2006, 2007); w, Nakkharat and Haltrich (2007); x, Prakash and others (1987); y, Burvall and others (1979), Boon and others (2000a), Kim and others (2001), Chockchaisawasdee and others (2005), Cheng and others (2006a), Martinez-Villaluenga and others (2008); z, Czermak and others (2004), Ebrahimi and others (2006a), Engel and others (2008); aa, Maugard and others (2003); ab, Maugard and others (2003); ac, Zhou and others (2003); ad, Rustom and others (1998); Foda and Lopez-Leiva (2000); ae, Foda and Lopez-Leiva (2000); af, Roberts and Pettinati (1957), Toba and Adachi (1978), Jeon and Mantha (1985), Boon and others (2000a); ag, Rustom and others (1998); ah, Onishi and Tanaka (1997); ai, Onishi and others (1996); aj, Onishi and others (1996); ak, Onishi and Tanaka (1995); al, Onishi and others (1995); am, Onishi and others (1995); an, Onishi and Tanaka (1996); ao, Onishi and Tanaka (1996); ap, Onishi and Tanaka (1996); aq, Shin and others (1998), Cho and others (2003); ar, Shin and others (1998); as, Cho and others (2003); at, Sakai and others (2008); au, Sakai and others (2008). **Kluyveromyces lactis* synonym: *Kluyveromyces marxianus var. lactis*; ** *Kluyveromyces marxianus* synonyms: *Kluyveromyces fragilis*, *Saccharomyces fragilis*; *** *Sporobolomyces singularis* synonym: *Bullera singularis*.

**Modifying the enzyme.** Optimization of the enzyme structure can contribute to increasing the maximum GOS yield from lactose. A protein engineering approach was applied to $\beta$-glucosidase from *P. furiosus*. An increase in GOS yield was observed by changing phenylalanine 426 residue to tyrosine (Table 1). By changing an additional residue, methionine 424 to a lysine, better transgalactosylation properties at low lactose concentrations were obtained (Hansson and others 2001). More recently, a similar approach was applied to the $\beta$-galactosidase from *G. stearothermophilus*. When the arginine 109 residue on the active site was changed to lysine, valine, or tryptophan, trisaccharide yield increased from 2% to 12%, 21% and 23%, respectively (Placier and others 2009).

Deletion mutagenesis of the C-terminal part turned the $\beta$-galactosidase from *B. bifidum* into a high-efficient transgalactosylating enzyme (Table 2) characteristically noninfluenced by lactose concentration (Jorgensen and others 2001). At maximum GOS yield the glucose/galactose ratio in the reaction media is much higher using this truncated $\beta$-galactosidase from *B. bifidum*

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than that obtained with other $\beta$-galactosidases from the genus Bifidobacterium (Figure 2).

Transgalactosylation activity of the $\beta$-galactosidase-1 from *B. circulans* was increased by modification of the enzyme with glutaraldehyde (Table 2), probably due to conformational changes near the active site of the enzyme (Mozaffar and others 1987). This is an example of a chemical-induced structural change, particularly interesting since glutaraldehyde is commonly used as a crosslinking agent for covalent enzyme immobilization.

**Removing the final product and/or inhibitors from the reaction medium.** Membrane-assisted, continuously stirred tank reactors have been used for GOS synthesis. An advantage of this type of scheme is that the product is continuously removed (along with water, some substrate, and simple sugar by-products) from the stirred tank using cross-flow membrane ultrafiltration, while the enzyme is retained. This configuration also allows variation of the residence time aiming at the optimization of the yield and composition of the oligosaccharides fraction (Czermak and others 2004). The yield of GOS produced by glucosidase from *S. solfataricus* at 30% and 70% lactose conversion was 3- and 1.3-fold higher, respectively, in the continuous reactor as compared to the results obtained in the batch reactor (Petzelbauer and others 2002). When compared with the batch process, higher yields of GOS using membrane-assisted continuously stirred tank reactors were also observed during lactose conversion (in solution or in whey) with enzymes from *K. lactis* (Table 3). Increases in GOS yield can be explained by a more efficient transfer of galactosyl residues to glucose and lactose, and a smaller extent of secondary degradation of GOS in the steady state, as compared to batch reactor (Petzelbauer and others 2002; Czermak and others 2004). However, this advantage was not observed using glycoside hydrolases from *P. furiosus* (Petzelbauer and others 2002), *L. reuteri* (Table 2), and *A. oryzae* (Table 3). These differences result from the distinct kinetic properties of the enzymes involved.

Contrary to the batch operation mode, where the composition of the different saccharides is changing constantly over the reaction time/lactose conversion, in the steady state of a continuous system the composition of the mixture stays constant over time. The composition of GOS mixtures can be finely tuned by controlling the residence time of a continuous system (Petzelbauer and others 2002; Czermak and others 2004; Chockchaisawasdee and others 2005; Splechtna and others 2007).

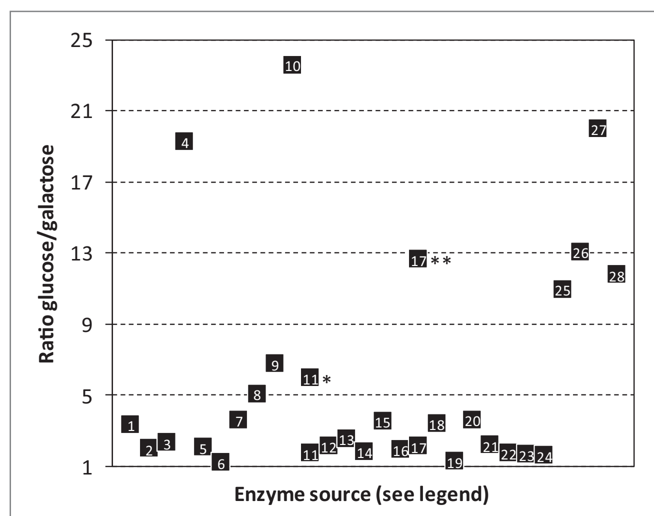


**Figure 2–Glucose/galactose ratios at maximum GOS yield of glycoside hydrolases from different microbial sources. ARCHAEA: 1, *S. solfataricus* (Petzelbauer and others 2000b); 2, *P. furiosus* (Petzelbauer and others 2000b); BACTERIA: 3, *S. rectivirgula* (Nakao and others 1994); 4, *B. bifidum* (BIF3, mutated) (Jorgensen and others 2001); 5, *B. infantis* ($\beta$-GalI) (Hung and Lee 2002); 6, *B. infantis* ($\beta$-GalIII) (Hung and Lee 2002); 7, *B. longum* (Hsu and others 2007); 8, *Thermus* sp. (Akiyama and others 2001); 9, *B. circulans* (Boon and others 1999); 10, *Bacillus* sp. (Cheng and others 2006c); 11, *G. stearothermophilus* (wild-type) (Placier and others 2009); 11*, *G. stearothermophilus* (mutated) (Placier and others 2009); 12, *L. acidophilus* (Nguyen and others 2007); 13, *L. reuteri* (Splechtna and others 2006); 14, *S. thermophilus* (Greenberg and Mahoney 1983); 15, *C. saccharolyticus* (Stevenson and others 1996); 16, *T. maritima* (Ji and others 2005); FUNGI: 17, *A. aculeatus* (Cardelle-Cobas and others 2008); 17**, *A. aculeatus* (aqueous 2-phase systems) (Del-Val and Otero 2003); 18, *A. oryzae* (Matella and others 2006); 19, *T. thermophilus* (Nakkharat and Haltrich 2007); 20, *P. expansum* (Li and others 2008); 21, *P. funiculosum* (Shin and Yang 1996); 22, *P. simplicissimum* (Cruz and others 1999); 23, *K. marxianus* (Roberts and Pettinati 1957); 24, *K. lactis* (Chockchaisawasdee and others 2005); 25, *S. magnum* (Onishi and Tanaka 1997); 26, *S. singularis* (Ishikawa and others 2005); 27, *R. minuta* (Onishi and Tanaka 1996); 28, *S. elviae* (Onishi and Tanaka 1995). (Data extracted from the literature.)**

## GOS yield and lactose conversion

As already discussed, the efficiency of the transgalactosylation reaction is kinetically controlled, as GOS are potential substrates of the glycoside hydrolase enzyme. The formation of oligosaccharides reaches a maximum time-course during a batch reaction. After the maximum point, GOS concentration decreases until approximately zero when lactose conversion is around 100%. The increase in the maximum amount of oligosaccharides can be due either to the enzyme ability to catalyze the transgalactosylation reaction relative to hydrolysis (as discussed previously) and/or to a lower rate of breakdown of the oligosaccharide products relative to lactose, probably because of the much higher km (affinity) values for GOS than for lactose. The enzyme ability could explain the differences observed in Figure 3 regarding the GOS yield (*y* axis), while the lower rate of GOS breakdown may explain the different lactose conversion rates obtained (*x* axis).

As reported in the literature, maximum yields of GOS between 15% and 77% are achieved when lactose conversion is between 45% and 95% depending on the enzyme source and the production process (Figure 3). If, with a given enzyme, a maximum yield of GOS is obtained for a high lactose conversion (examples: glycoside hydrolases from *T. caldophilus*, *S. thermophilus*, or *K. marxianus*), that is probably because it has higher km values for GOS than for lactose.

## Enzymes, cells, or resting cells

Processes for GOS production from lactose can be roughly classified into those employing enzymes extracted from microbial cells, with variable purity; those where a specific microorganism is cultured in a lactose-containing medium; and finally those that use resting cells of a lactose-utilizing microorganism acting as a so-called enzyme bag (Dombou and others 1992).

The former process requires the extraction of enzymes, which means additional costs and time consumption. Moreover, as hydrolysis of lactose and GOS is, to some extent, inevitable, accumulation of glucose and galactose as by-products occurs. The presence of these monosaccharides is generally reported as undesirable since the objective is to produce tri- and larger oligosaccharides. Galactose and glucose were reported as inhibitors of glycoside hydrolases from *K. lactis* (Chockchaisawasdee and others 2005) and from *S. elviae* (Onishi and others 1995), respectively, regarding GOS formation. However, an unexpected positive

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Table 4–Structures of oligosaccharides produced from lactose by the transgalactosylation catalyzed by microbial GH and cells.

| Structure | Enzyme | | | | | | | | | | | | | | | Cells | | | | | | | |
|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|
| | 1 | 2 | 3 | 4 | 5 | 6 | 7 | 8 | 9 | 10 | 11 | 12 | 13 | 14 | 15 | 16 | 17 | 18 | 19 | 20 | 21 | 22 | 23 |
| β-D-Gal*p*-(17→6)-D-Glc (allolactose) | ■ | ■ | | ● | | ■ | ● | ● | ■ | ● | | ■ | ● | ● | | ● | ■ | ■ | ■ | ● | | | |
| β-D-Gal*p*-(1→3)-D-Glc | ■ | ■ | | ● | | ■ | | | ■ | ● | | | | ● | | ● | ■ | | ■ | ● | | | |
| β-D-Gal*p*-(1→2)-D-Glc | | | | ● | | | | | | ● | | | | ● | | | | | ■ | | | | |
| β-D-Gal*p*-(1→6)-D-Gal | ■ | ■ | | | | ■ | ● | | ■ | ● | | ■ | ● | ● | | ● | | ■ | ■ | ● | | | |
| β-D-Gal*p*-(1→3)-D-Gal | ■ | ■ | | | | ■ | | | | ● | | ■ | | ● | | | | | | | | | |
| β-D-Gal*p*-(1→6)-β-D-Gal-(1→4)-D-Glc (6′-galactosyllactose) | ■ | ■ | | | | ■ | | | ■ | | ● | | ■ | | | | | | | ● | | ■ | |
| β-D-Gal*p*-(1→3)-β-D-Gal-(1→4)-D-Glc (3′-galactosyllactose) | ■ | ■ | ■ | | ■ | ■ | | | ■ | | ● | | | | | ● | | | | | ● | ■ | ■ |
| β-D-Gal*p*-(1→4)-β-D-Gal-(1→4)-D-Glc (4′-galactosyllactose) | | | | ● | ■ | | | | | | | | | | ● | | | | | | ● | ■ | |
| β-D-Gal*p*-(1→6) [β-D-Gal-(1→4)]-D-Glc | | | | ● | | | | | | | ● | | ● | | | ● | | | | | | ■ | |
| β-D-Gal*p*-(1→2) [β-D-Gal-(1→4)]-D-Glc | | | | ● | | | | | | | | | | | | | | | | | | | |
| β-D-Gal*p*-(1→2) [β-D-Gal-(1→6)]-D-Glc | | | | ● | | | | | | | | | | | | ● | | | | | | | |
| β-D-Gal*p*-(1→3) [β-D-Gal-(1→6)]-D-Glc | | | | ● | | | | | | | | | | | | | | | | | | | |
| β-D-Gal*p*-(1→4)-β-D-Gal-(1→6)-D-Glc | | | | ● | | | | | | | ■ | | | | | | | | | | | | |
| β-D-Gal*p*-(1→6)-β-D-Gal-(1→6)-D-Glc | | | | | | | | | | | | | ● | | | | | | | | | | |
| β-D-Gal*p*-(1→4)-β-D-Gal-(1→3)-D-Glc | | | | ● | | | | | | | | | | | | | | | | | | | |
| β-D-Gal*p*-(1→4)-β-D-Gal-(1→2)-D-Glc | | | | ● | | | | | | | | | | | | | | | | | | | |
| β-D-Gal*p*-(1→6)-β-D-Gal-(1→6)-D-Gal | | | | | | | | | | | | | ● | | | | | | | | | | |
| β-D-Gal*p*-(1→4)-β-D-Gal*p*-(1→4)-β-D-Gal-(1→4)-D-Glc | | | | | | | | | | | | | | | | | | | | | | | ■ |
| β-D-Gal*p*-(1→6)-β-D-Gal*p*-(1→6)-β-D-Gal-(1→4)-D-Glc | | | | | | | | | | | ● | | ● | | | | | | | | | ■ | |
| β-D-Gal*p*-(1→3)-β-D-Gal*p*-(1→6)-β-D-Gal-(1→4)-D-Glc | | | | | | | | | | | ● | | | | | | | | | | | | |
| β-D-Gal*p*-(1→6)-β-D-Gal*p*-(1→3)-β-D-Gal-(1→4)-D-Glc | | | | | | | | | | | ● | | | | | | | | | | | | |
| β-D-Gal*p*-(1→3)-β-D-Gal*p*-(1→3)-β-D-Gal-(1→4)-D-Glc | | ■ | | | | | | | | | | | | | | ● | | | | | | | |
| β-D-Gal*p*-(1→6)-β-D-Gal*p*-(1→6)-β-D-Gal*p*-(1→6)-β-D-Gal-(1→4)-D-Glc | | | | | | | | | | | ● | | | | | | | | | | | | |
| β-D-Gal*p*-(1→3)-β-D-Gal*p*-(1→3)-β-D-Gal*p*-(1→3)-β-D-Gal-(1→4)-D-Glc | | | | | | | | | | | | | | | | ● | | | | | | | |
| β-D-Gal*p*-(1→3)-β-D-Gal*p*-(1→3)-β-D-Gal*p*-(1→3)-β-D-Gal*p*-(1→3)-β-D-Gal-(1→4)-D-Glc | | | | | | | | | | | | | | | | ● | | | | | | | |
| β-D-Gal*p*-(1→3)-β-D-Gal*p*-(1→3)-β-D-Gal*p*-(1→3)-β-D-Gal*p*-(1→3)-β-D-Gal*p*-(1→3)-β-D-Gal-(1→4)-D-Glc | | | | | | | | | | | | | | | | ● | | | | | | | |

● or ■ indicate that the corresponding sugar has been identified in the transfer products; ● data compiled by Nakayama (Nakayama and Amachi 1999). Archaeal enzymes are: 1, *S. solfataricus* (Petzelbauer and others 2000b); 2, *P. furiosus* (Petzelbauer and others 2000a; Petzelbauer and others 2000b). Bacterial enzymes are: 3, *B. longum* subsp. *infantis* (Hung and others 2001) or *Thermus* sp. (Akiyama and others 2001); 4, *B. circulans* (Yanahira and others 1995); 5, *G. stearothermophilus* (mutated) (Placier and others 2009); 6, *L. acidophilus* (Nguyen and others 2007) or *L. reuteri* (Maischberger and others 2008); 7, *S. thermophilus* (Greenberg and Mahoney 1983); 8, *E. coli* (Huber and others 1976). Fungal enzymes are: 9, *A. aculeatus* (Cardelle-Cobas and others 2008); 10, *A. niger* (Toba and Adachi 1978); 11, *A. oryzae* (Toba and others 1985; Chen and others 2001); 12, *T. thermophilus* (Nakkharat and Haltrich 2007); 13, *K. lactis* (Asp and others 1980); 14, *K. marxianus* (Pazur and others 1958; Toba and Adachi 1978); 15, *S. elviae* (Onishi and Tanaka 1995) or *C. laurentii* (Ohtsuka and others 1990a). Bacterial microorganisms are: 16, *B. bifidum* (Dumortier and others 1994); 17, *L. helveticus* or *L. lactis* (Toba and others 1981); 18, *L. acidophilus* or *L. casei* or *L. plantarum* (Toba and others 1981); 19, *L. bulgaricus* or *S. thermophilus* (Toba and others 1981). Fungal microorganisms are: 20, *Penicillium chrysogenum* (Ballio and Russi 1960); 21, *Chaetomium globosum* (Gorin and others 1966); 22, *T. harzianum* (Prakash and others 1987; Prakash and others 1989); 23, *S. singularis* (Gorin and others 1964).


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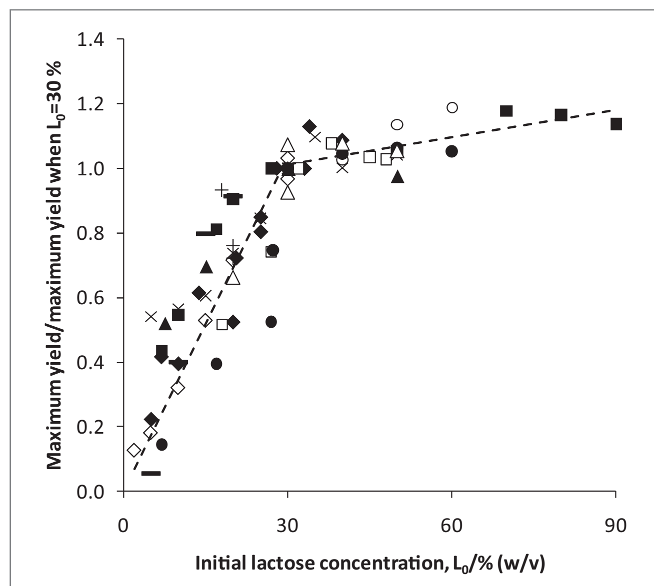


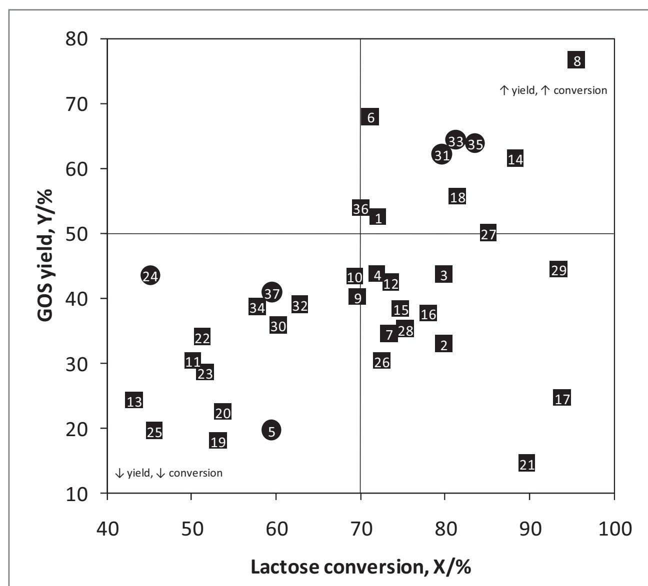


**Figure 3–The effect of initial lactose concentration on maximum GOS yield.•, *S. solfataricus* (Petzelbauer and others 2000b; Splechtna and others 2001; Park and others 2008); ■, *P. furiosus* (Petzelbauer and others 2000b; Hansson and others 2001); –, *B. angulatum* (Rabiu and others 2001); ▲, *T. caldophilus* and *Thermus* sp. (Akiyama and others 2001; Choi and others 2003); △, *T. maritime* (Kim and others 2004; Ji and others 2005); ◇, *A. niger* (Toba and Adachi 1978; Prenosil and others 1987); □, *P. expansum* (Li and others 2008); ∘, *P. simplississum* (Cruz and others 1999); ◆, *K. lactis* (Burvall and others 1979; Boon and others 2000a; Chockchaisawasdee and others 2005; Cheng and others 2006a; Martinez-Villaluenga and others 2008); ×, *K. marxianus* (Roberts and Pettinati 1957); +, *S. singularis* (Shin and others 1998; Cho and others 2003; Ishikawa and others 2005). Maximum GOS yield is normalized to reduce the enzyme source effect.**

**Figure 4–Maximum reported yield of GOS as a function of reported conversion. ■, enzyme; •, cells. Data extracted from the literature. ARCHAEA: 1, *S. solfataricus* (Park and others 2008); 2, *P. furiosus* (Petzelbauer and others 2000b); BACTERIA: 3, *S. rectivirgula* (Nakao and others 1994); 4, *B. bifidum* (BIF3, mutated) (Jorgensen and others 2001); 5, *B. bifidum* (Tzortzis and others 2005b); 6, *B. infantis* (β-GalI) (Hung and Lee 2002); 7, *B. longum* (Hsu and others 2007); 8 *T. caldophilus* (Choi and others 2003); 9, *Thermus* sp. (Cheng and others 2006c); 10, *B. circulans* (β-GalII) (Mozaffar and others 1984); 11, *B. circulans* (Boon and others 1999); 12, *Bacillus* sp. (Cheng and others 2006a); 13, *G. stearothermophilus* (Placier and others 2009); 14, *C. saccharolyticum* (Stevenson and others 1996); 15, *L. acidophilus* (Nguyen and others 2007); 16, *L. reuteri* (Splechtna and others 2006); 17, *S. thermophilus* (Greenberg and Mahoney 1983); 18, *E. coli* (Huber and others 1976); 19, *T. maritima* (Ji and others 2005); FUNGI: 20, *A. aculeatus* (Cardelle-Cobas and others 2008); 21, *A. niger* (Toba and Adachi 1978); 22, *A. oryzae* (Iwasaki and others 1996); 23, *P. expansum* (Li and others 2008); 24, *P. expansum* (β-galactosidase gene from *P. expansum* expressed on the cell surface of *S. cerevisiae*) (Li and others 2009); 25, *P. funiculosum* (Shin and Yang 1996); 26, *P. simplicissimum* (Cruz and others 1999); 27, *T. thermophilus* (Nakkharat and Haltrich 2007); 28, *K. lactis* (Cheng and others 2006b); 29, *K. marxianus* (Roberts and Pettinati 1957); 30, *S. magnum* (Onishi and Tanaka 1997); 31, *S. magnum* (Onishi and others 1996); 32, *S. elviae* (Onishi and Tanaka 1995); 33, *S. elviae* (Onishi and others 1995); 34, *R. minuta* (Onishi and Tanaka 1996); 35, *R. minuta* (Onishi and Yokozeki 1996); 36, *S. singularis* (Shin and others 1998); 37, *S. singularis* (Sakai and others 2008).**

effect of these monosaccharides on GOS yield was observed when using *B. bifidum* resting cells (Goulas and others 2007a).

Culture-based processes can be conducted if glycoside hydrolases expressed by the microorganism are cell-bound or secreted into the culture medium. One of the advantages of cultured-based processes is that glucose and galactose can be consumed during cell growth reducing their content in the final saccharide mixture, thus improving GOS yield. Higher GOS yields were reported using fermentation systems with microorganisms from the species *S. elviae*, *S. magnum*, or *R. minuta* growing on lactose comparing to processes using resting cells or purified enzymes from the same microorganisms (Table 3 and Figure 4) (Onishi and others 1995, 1996; Onishi and Tanaka 1996, 1997). These researchers concluded that these improved GOS yields result from the fact that the glucose is consumed for cell growth, since glucose has been found to inhibit GOS synthesis. More recently, an innovative strategy for GOS synthesis by anchoring β-galactosidase from *P. expansum* on the cell surface of *S. cerevisiae* as an immobilized enzyme was also successfully applied (Li and others 2009).

On the other hand, GOS must be separated from accumulated secreted microbial products and from other ingredients essential or useful for cell growth incorporated in the culture medium (nitrogen sources, vitamins, trace elements, among others).

Industrial GOS producers use either cells or enzyme extracts. For example, *C. laurentii* resting cells entrapped in calcium alginate gels are used for industrial-scale production of GOS. Furthermore, GOS are also produced by means of a sequential reaction of a lactose solution with *A. oryzae* β-galactosidase and *S. thermophilus* β-galactosidase, respectively (Nakayama and Amachi 1999).

Nevertheless, it is important to notice that the production of GOS mixtures using microbial cultures can occur as a consequence of the multiple glycoside hydrolases expressed by some microorganisms. Thus, this combined activity of several enzymes limits the fermentation process modeling and control. As examples, 2 glycoside hydrolases from *B. circulans* (Mozaffar and others 1984), 3 from *A. niger* (Widmer and Leuba 1979), 3 from *B. bifidum* DSM20215 (Moller and others 2001), and 4 from *B. bifidum* NCIMB41171 (Goulas and others 2007b) have been reported.

## High-content GOS production

GOS mixtures produced by transgalactosylation always contain considerable amounts of nonreacted lactose and monosaccharides. The efficient removal of these non-GOS impurities allows the commercialization of added-value GOS products (Crittenden and Playne 2002).

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**Table 5–Chemical composition of commercial GOS (w/w dry matter).**

| | | | | GOS | | | | | | |
|---|---|---|---|---|---|---|---|---|---|---|
| | Glucose | Galactose | Lactose | DP2 | DP3 | DP4 | DP > 4 | Total | Enzyme source | Ref. |
| CUP-oligo | | 25 to 30[i] | | – | – | – | – | 70 | *Cryptococcus laurentii* | a |
| Oligomate 55 | 18 to 39[ii] | | 10 to 22 | 15 to 17 | 18 to 24 | 10 to 16 | 2 to 5.4 | 50 to 60 | *A. oryzae* and *S. thermophilus* | b |
| TOS-100 | 0[ii] | | 0 to 1 | 0 | 55 | 33 to 35 | 12 to 14 | 99 to 100 | *A. oryzae* and *S. thermophilus* | c |
| Vivinal GOS | 19 to 22 | 0.8 to 1.3 | 10 to 23 | 19 to 27 | 22 to 23 | 11 | 6.0 to 7.6 | 57 to 59 | *Bacillus circulans* | d |
| Bimuno | 18 | 12 | 22 | 25 to 29 | 12 to 14 | 6.7 to 7.7 | 3.8 to 4.4 | 48 to 55 | *Bifidobacterium bifidum* | e |
| Purimune | 0 to 1.0 | 0 to 0.5 | 7.0 to 10 | 16 to 21 | 38 to 51 | 25 to 29[iii] | | 90 to 92 | *Bacillus circulans* | f |
| Promovita GOS | – | – | – | – | – | – | – | – | – | |

Nissin Sugar Manufacturing Co. (Tokyo, Japan): Cup-Oligo in syrup (Cup-Oligo H70, 75% solids) and powder format (Cup-Oligo P); Yakult Honsha (Tokyo, Japan): Oligomate 55 syrup (75% dry matter); Oligomate 55P (powder); TOS-100. Yakult now also uses *B. circulans* to produce $\beta 1 \rightarrow 4$ linked products. A preparation containing trans-galactosylated disaccharides has been reported and manufactured using $\beta$-galactosidase from *S. thermophilus* (Ito and others 1993); Friesland Foods Domo (Zwolle, The Netherlands) is offering Vivinal GOS in a syrup (73% dry matter) and 2 powder formats, Vivinal 10 and Vivinal 15, obtained co-spray-drying Vivinal GOS syrup and whey protein concentrate or maltodextrin, respectively; Clasado Ltd. (Milton Keynes, U.K.) offers a powder GOS product, Bimuno, as well as a syrup version; Corn Products Intl., Inc. (Ill., U.S.A.): Purimune (powder); Fayrefield Food (Crewe, U.K.) and First Milk (Paisley, U.K.): Promovita GOS (syrup);
[i] glucose + galactose + lactose; [ii] glucose + galactose; [iii] DP $\geq$ 4; (-) no data available.
a, Hartemink and others (1997), Playne and Crittenden (2009); b, Ito and others (1990), Katta and others (2000), Sar and others (2004), Splechtna and others (2006); c, Ito and others (1990), Rowland and Tanaka (1993); d, Goulas and others (2007a), Coulier and others (2009), Sinclair and others (2009); e, Vulevic and others (2008), Silk and others (2009), Tzortzis (2009); f, GTC Nutrition (2009).

Large-scale separation of monosaccharides is usually conducted by a chromatographic process with ion-exchange resins or activated charcoal (Hernandez and others 2009; Nobre and others 2009). Regarding ion-exchange chromatography, the cation-exchange resins are the most used as they have the highest affinity for monosaccharides, and therefore oligosaccharides are the first to elute from the column. Activated charcoal has been reported to possess a higher affinity for oligosaccharides compared to mono- and disaccharides, which makes their operation at the industrial level more advantageous, since regeneration can take place off-line without large substrate losses.

Recently, a comparison of fractionation techniques to obtain high-content GOS mixtures, at preparative scale, concluded that size-exclusion chromatography was the most appropriate method to obtain fractions with high purity, enabling the purification of GOS with different degrees of polymerization (DP) (Hernandez and others 2009). Although not in a mature stage, membrane techniques, particularly nanofiltration, also show potential for large-scale fractionation of oligosaccharides from complex mixtures (Goulas and others 2002, 2003; Feng and others 2009). Supercritical fluid extraction technology also has shown satisfactory performances in the isolation of monosaccharides, disaccharides, and higher saccharides from complex mixtures (Montanes and others 2009).

Furthermore, other approaches based on the selective fermentation characteristics for a given microorganism have been proposed. During these fermentation processes, depending on the microorganism, ethanol can be produced and, if in toxic concentrations, its activity can be compromised. *S. cerevisiae* was used to improve the purity of a commercial mixture of GOS obtained with $\beta$-galactosidase from *B. circulans* by completely removing the monosaccharides (Hernandez and others 2009). The same approach was applied to a GOS mixture produced by *B. bifidum* biomass (Goulas and others 2007a). *K. marxianus* was used to improve the purity of a GOS mixture produced by $\beta$-galactosidase from *B. circulans* from 38% to 97% by selective fermentation of mono- and disaccharides (including lactose) (Cheng and others 2006a). A combination of *S. cerevisae* and *K. lactis* was used to improve the purity of a GOS mixture produced by $\beta$-galactosidase from *P. expansum* from 29% to 98% by selective fermentation of monosaccharides and lactose (Li and others 2008).

The separation of lactose from a disaccharide fraction has proven to be difficult by all the reported processes, and usually results in large losses of GOS products, mainly nonlactose disaccharides. To overcome this difficulty, a process where lactose is efficiently separated from other sugars by anion-exchange chromatography, after its selective oxidation into lactobionic acid using a fungal cellobiose dehydrogenase, was proposed by Splechtna and others (2001). However, with this process, total losses of GOS around 17% occur, since the fungal cellobiose dehydrogenases used are not specific for lactose (Splechtna and others 2001; Maischberger and others 2008).

### Commercially available GOS

GOS have been manufactured and commercialized by Yakult Honsha and Nissin Sugar Manufacturing from Japan, by Corn Products Intl. from the United States, and by Borculo Domo Ingredients and Clasado from Europe. Also, some companies have been reported to produce GOS for incorporating in their own products rather than for commercial purposes (Playne and Crittenden 2009). Recently, new players have come into the market, but available information on their products is scarce (Table 5). Food-grade GOS are usually transparent syrups or white powders. In both cases, they are mixtures containing oligosaccharides of different DP, the nonreacted lactose and monomer sugars (glucose and galactose) (Playne and Crittenden 2009; Tzortzis and Vulevic 2009). Purified products with more than 90% (w/w) GOS are available from some manufacturers.

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 (Table 5). The Oligomate range products offer mainly GOS with $\beta 1 \rightarrow 6$ linkages; the Bimuno product contains mainly $\beta 1 \rightarrow 3$ linkages, whilst Cup-Oligo, Vivinal GOS, and Purimune offer mainly $\beta 1 \rightarrow 4$ linkages (see Table 4 for more details about glycosidic linkage according to enzyme source).

## GOS Properties

### Physicochemical properties of GOS

Since commercially available food grade-GOS are mixtures (Table 5), it is expected that their physicochemical properties and physiological actions will, to some extent, depend on the mixture composition, which in turn will determine its proper application. Sweetness, solubility, osmolality, crystal formation ability, and reactivity (Maillard reactions) decrease as the molecular size increases, contrary to viscosity (Playne and Crittenden 2009). Table 6 presents a summary of the commonly reported GOS properties.

### Physiological properties of GOS

**Indigestibility.** Several *in vitro* and *in vivo* experiments have demonstrated the indigestibility and stability to hydrolysis by digestive enzymes of GOS. In a consensus report, it was concluded that more than 90% of GOS passes into the colon (van Loo and others 1999).

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**Table 6–General physicochemical properties of GOS. Adapted from Macfarlane and others (2008), Playne and Crittenden (2009), and Tzortzis and Vulevic (2009).**

| | |
|---|---|
| Solubility | Water-soluble, about 80% (w/w) |
| Appearance | Translucent/colorless |
| Viscosity | Similar to that of high-fructose corn syrup |
| Heat stability | Stable to 160 °C for 10 min at pH 7; stable to 100 °C for 10 min at pH 2; stable to 37 °C at pH 2 for several months |
| Freezing point | Reduces the freezing point of foods |
| Humectant properties | High moisture retaining capacity preventing excessive drying |
| Sweetness | Typically 0.3 to 0.6 times that of sucrose |

Tri- and tetra- GOS were not hydrolyzed *in vitro* by human salivary $\alpha$-amylase, artificial gastric juice, $\alpha$-amylase of hog pancreas, and rat intestinal acetone powder. Contrarily, disaccharides were partially digested by the intestinal enzymes (Chonan and others 2004). In another study, only a very small amount of 4′-galactosyllactose was digested by the homogenate of the rat small intestinal mucosa (Ohtsuka and others 1990b).

Most of the *in vivo* human data regarding the nondigestibility of GOS were obtained by hydrogen breath tests. Breath hydrogen excretion is likely to be a dose-dependent effect. Using this noninvasive technique, several studies reported an increased breath hydrogen excretion when the ingested GOS amount was between 15 to 35 g/d, indicating that GOS escaped digestion and were fermented by the colonic microbiota (Tanaka and others 1983; Alles and others 1999; Chonan and others 2004). However, Bouhnik and others (1997) showed reduced breath hydrogen after administration of a 10-g daily dose of GOS but, at the same time, bifidobacterial numbers were increased, indicating that the GOS were fermented by colonic microbiota (Bouhnik and others 1997). It has also been shown that dietary GOS can be detected in feces of infants fed with formula containing GOS (Moro and others 2005).

European regulation on food labeling obliges the manufacturers of GOS-containing food products to clearly identify these ingredients as dietary fiber. According to the recent legal definition, fiber means "carbohydrate polymers with 3 or more monomeric units, which are neither digested nor absorbed in the human small intestine obtained from food raw material by physical, enzymatic, or chemical means and which have a beneficial physiological effect demonstrated by generally accepted scientific evidence" (European Directive 2008). Usually, the amount of dietary fiber that appears on food labels is determined according to the methodology outlined in the Official Methods of Analysis from the Association of Official Analytical Chemists (AOAC 1995; DeVries 2004). However, this methodology is not suitable for measuring nondigestible oligosaccharides requiring the development of new methods. Specifically, for GOS analysis, a method based on the enzymatic treatment with $\beta$-galactosidase followed by high-performance anion-exchange chromatography, using pulsed amperometric detection (HPAEC-PAD), has been adopted (De Slegte 2002; AOAC 2005).

Because GOS are indigestible, but fermentable (see below), the caloric value of GOS, as well as that of other nondigestible oligosaccharides, has been estimated to be 1 to 2 kcal/g (Roberfroid and others 1993; Cummings and others 1997). For food labeling purposes, in Europe, GOS should be given a caloric value of 2 kcal/g (European Directive 2008).

**Prebiotic properties.** The main physiological effects of GOS are related with their impact on the composition and activities of the intestinal microbiota. The human intestinal tract harbors a complex community of bacteria, eukaryotic microorganisms, archaea, viruses, and bacteriophages, collectively referred to as the intestinal microbiota. Bacteria account for the majority of these microorganisms: their total number in the human gut is estimated at $10^{14}$ cells mainly present in the colon (Backhed and others 2005; Lupp and Finlay 2005). Colonization of the gastrointestinal tract with intestinal microbiota occurs immediately after birth and lasts a lifetime. In adults, the composition of the colonic microbiota is generally estimated to comprise more than 500 bacterial persistent and transient species (based on worldwide observations, not 1 individual person), although it is thought that only 30 to 40 of them predominate (McCartney and Gibson 2006). The majority of the members of the colonic microbiota are obligate anaerobic genera, including *Bacteroides*, *Bifidobacterium*, *Clostridium*, *Enterococcus*, *Eubacterium*, *Fusobacterium*, *Peptococcus*, *Peptostreptococcus*, and *Ruminococcus* (McCartney and Gibson 2006).

Recent advances in defining the quality, quantity, and physiologic activity of the intestinal microbiota have been possible as a result of the advent of metagenomics (the analysis of collective genomes of microbes), an emerging field in which the power of genomic analysis is applied to entire microbial consortia (Gill and others 2006). Collectively, human microbiota contains at least 100 times as many genes as the human genome corresponding to a degree of metabolic activity that is increasingly being recognized (Gill and others 2006; Wikoff and others 2009). This metabolic activity includes: the ability to metabolize, otherwise indigestible, saccharides of our diet by fermentation to various short-chain fatty acids (SCFAs); influencing host genes that regulate energy expenditure and storage; contributing to several biosynthetic pathways (including vitamins and isoprenoid precursors); production of conjugated linoleic acid (CLA) isomers, and bioactive peptides, with recognized health effects; promoting host homeostasis; and decontaminating the intestine, thereby minimizing exposure to toxic substances that could result in malignancies (Sekirov and Finlay 2006; Ross and others 2010).

Production of SCFAs is one of the most important physiological actions mediated by the microbiota. These are absorbed resulting in energy salvage from indigested food. Moreover, SCFAs affect colonic epithelial cell transport, energy transduction in colonocytes, growth, and cellular differentiation. These trophic properties have important physiological implications, in addition to maintaining the mucosal defense barrier against invading organisms. When absorbed into the bloodstream, they affect the hepatic control of lipid and carbohydrate metabolism and provide energy to muscle, kidney, and brain (Cummings 1995).

The colonic microbial ecosystem is quite stable but can be influenced by genotype, age, diet, and health status (Lupp and Finlay 2005). Three approaches are used to beneficially modulate the gastrointestinal microbiota: (1) by directly supplementing the intestinal microbiota by consuming live bacteria, "probiotics"; (2) by consuming dedicated dietary components selectively used by resident microorganisms, "prebiotics"; or (3) by combining both strategies, "synbiotics." A prebiotic is defined as "a selectively fermented ingredient that allows specific changes, both in the composition and/or activity in the gastrointestinal microbiota

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that confers benefits upon host wellbeing and health" (Gibson and Roberfroid 1995; Gibson and others 2004a). The selective properties of prebiotics are supposed to relate to the growth of bifidobacteria and lactobacilli at the expense of other groups of bacteria in the gut (Macfarlane and others 2006). Along with inulin, fructo-oligosaccharides (FOS), and lactulose, GOS are also food ingredients that have been consistently established as prebiotic ingredients from several studies conducted *in vitro* and *in vivo* (animal and human) (Gibson and others 2004a; Rastall 2006; Roberfroid 2007; Tzortzis and Vulevic 2009).

It was shown in Table 4 and 5 that the differences in DP and structures of the oligosaccharides present in GOS mixtures occur mainly due to the different enzymes used for their production. These differences are expected to be important when it comes to GOS assimilation by bifidobacteria in the colonic microbiota (Tzortzis and Vulevic 2009). In a recent study, the administration of a GOS mixture (3.6 g/d) containing mainly $\beta 1 \rightarrow 3$, as well as $\beta 1 \rightarrow 4$ and $\beta 1 \rightarrow 6$ linkages, proved to have a better bifidogenic effect than a GOS mixture (4.9 g/d) containing mainly $\beta 1 \rightarrow 4$, as well as $\beta 1 \rightarrow 6$, after 1 wk of intake by healthy humans (Depeint and others 2008). Both mixtures had mainly di- and trisaccharides. Both these GOS mixtures had low polymerization degree with DP $\geq 4$ accounting for less than 12% and 19% of total saccharides, respectively.

Most of the prophylactic health effects proposed for GOS arise from their selective consumption by bifidobacteria and lactobacilli, as a fermentative substrate. These reported health effects have been recently reviewed by other researchers (Gibson and others 2004b; Macfarlane and others 2008; Playne and Crittenden 2009; Tzortzis and Vulevic 2009) and include protection against enteric infections; increased mineral absorption; immunomodulation for the prevention of allergies and gut inflammatory conditions; trophic effects of SCFAs on the colonic epithelium; fecal bulking; and reduced toxigenic microbial metabolism that may reduce risk factors for colon cancer.

**GOS in infant milk formulas.** The significance of a bifidobacteria-predominant microbiota in healthy breast-feed infants is well accepted (Fanaro and others 2003). At that time, the microbiota organisms are believed to be particularly important for the correct functioning of the gut and maturation of the immune system. Bifidobacteria and, to a lesser degree lactobacilli, may account for as much as 90% of the total microbiota in breast-fed infants, while formula-fed infants have a more diverse microbiota composition with greater numbers of more potentially harmful organisms such as clostridia and enterococci (Harmsen and others 2000). Oligosaccharides in human milk can reach concentrations as high as 8 to 12 g/L, which is 100 times greater than in cow's milk (Kunz and others 2000), mainly composed of sialic acid, N-acetylglucosamine, L-fucose, D-glucose, and D-galactose (Boehm and others 2005). One characteristic of human milk oligosaccharides is the large amount of galactose. The backbone structure is based on lactose (galactose-glucose) plus a further galactose residue forming the 3 different GOS, namely, 3′-galactosyl-lactose, 4′-galactosyl-lactose, and 6′-galactosyl-lactose (Boehm and others 2005). The preponderance of bifidobacteria in breast-fed babies is thought to result from their abilities to use oligosaccharides in breast milk, including GOS (Newburg 2000; Fanaro and others 2003).

The recognized bifidogenic effect of human milk can be replicated using GOS, alone or with FOS, to fortify infant cow's milk-based formulas (Bakker-Zierikzee and others 2005; Fanaro and others 2005, 2009; Knol and others 2005a, 2005b; Boehm and Moro 2008). Additionally, the ability of GOS to resemble glycoconjugate structures on cell surface receptors, used by pathogens for adherence in the gut, may also protect the colonization and growth of pathogens during this vulnerable period (Kunz and others 2000; Newburg 2000; Boehm and Moro 2008; Macfarlane and others 2008).

## GOS Applications

GOS are mainly used in infant milk formula, follow-on formula, and infant foods (Playne and Crittenden 2009). Supplemented infant formulas usually contain 6.0 to 7.2 g/L GOS together with 0.6 to 0.8 g/L FOS (Rastall 2006; Playne and Crittenden 2009).

Because of their stability, in addition to infant foods, GOS can also be incorporated into a wide variety of other foods. Recently, they have been used in beverages (fruit juices and other acid drinks), meal replacers, fermented milks, flavored milks, and confectionery products (Affertsholt-Allen 2007). Bread, as most other baked goods, is a suitable candidate for GOS incorporation because during the fermentation and baking processes, GOS molecules are not cleaved or consumed. Furthermore, due to the high moisture retaining capacity of GOS, excessive product drying is prevented conferring this bread a better taste and texture. Specialized foods for the elderly and hospitalized people are also promising fields of application of GOS (Sako and others 1999).

As other nondigestible oligosaccharides, GOS have a pleasant taste and can increase the texture and mouthfeel of foods providing bulk properties similar to sucrose. GOS are resistant to salivary degradation and are not used by the oral microbiota and can therefore be used as low-cariogenic sugar substitutes. Being indigestible they have negligent impact on blood glucose (Prapulla and others 2000).

Besides the food sector, other areas, such as the cosmetic and pharmaceutical industries, can also exploit the physicochemical and physiological properties of GOS. Indeed, prebiotic oligosaccharides can selectively stimulate "beneficial" bacteria on human skin and some formulations for that purpose have already been developed (Bockmühl and others 2007; Krutmann 2009).

The use of nondigestible oligosaccharides in the livestock feed and pet food industries is also increasing. GOS are finding increased use in the poultry (Biggs and others 2007; Jung and others 2008; Yang and others 2009), pig (Houdijk and others 1997, 1998; Tzortzis and others 2005a; Modesto and others 2009), and aquaculture (Burr and others 2008; Grisdale-Helland and others 2008) industries for improving health and growth; improving gut microbial ecology, minimizing the use of antibiotics; prevent early mortality; and reduce fecal odor. GOS have also been applied to suppress methane production by ruminants (Mwenya and others 2004a, 2004b; Santoso and others 2004; Sar and others 2004; Iqbal and others 2008).

## Regulatory and Safety Aspects of GOS

GOS have a generally recognized as safe (GRAS) status in the United States, a non-Novel Food status in the EU, and are regarded as foods for specific health use (FOSHU) in Japan, (Tzortzis and Vulevic 2009) due to the fact that they are components of human milk and traditional yogurt and can be produced from ingested lactose by the resident intestinal bacteria. The only adverse effect of GOS known so far is transient osmotic diarrhea that occurs when an excess of GOS is consumed, similar to unabsorbed sugar alcohols or lactose (in symptomatic lactose-intolerant individuals). The amount of GOS that does not induce osmotic diarrhea has

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been estimated to be approximately 0.3 to 0.4 g/kg body weight, or about 20 g per human body (Sako and others 1999).

Actually, in Europe, health claims on foods, specifically "general function" claims are being regulated under Article 13.1 in Regulation (EC) 1924/2006 of the European Parliament and of the Council of 20 December 2006 on nutrition and health claims made on foods (European Regulation 2006). The European Food Safety Authority (EFSA) is evaluating the fundamental science beyond the claims provided by all member states. Although not yet authorized, in the meantime, 3 claims on GOS have already passed EFSA's pre-screening, namely: "maintains a healthy normal digestive system," "prebiotic/bifidogenic," and "calcium absorption" (EFSA 2010a, 2010b). However, the following claims on GOS: "helps support a healthy immune system in an ageing population"; "helps to manage the symptoms associated with irritable bowel syndrome"; and "energizes your immunity boosting bacteria" or "helps boost your body's self-defense" did not pass through the EFSA pre-screening.

## Conclusions

Several microbial glycoside hydrolases have been proposed for the synthesis of GOS from lactose. In this context, these enzymes fundamentally differ in their ability to catalyze the transgalactosylation reaction relative to hydrolysis, and in their affinity for the GOS formed as compared to the affinity for lactose. The final product spectrum obtained during lactose conversion and the glycosidic linkage between the monomers depends on the enzyme source and the physicochemical conditions in the catalytic environment.

Current commercially available GOS products are mixtures of galactose-based oligosaccharides with varying degree of polymerization and linkage configuration with glucose, galactose, and lactose. GOS mixtures are well-established prebiotic ingredients. However, depending on their oligosaccharide composition, GOS products vary in terms of their bifidogenic and other protective actions.

Recent and future developments in the production of GOS aim at delivering purer and more efficient mixtures, desirably with narrow and specific target ranges. A better understanding of what constitutes a "healthy" intestinal microbiota composition certainly would contribute to that goal.

GOS are stable in wide pH and temperatures ranges and are suitable for several applications in the food, feed, and pharmaceutical industries. Infant and geriatric nutrition offer the most promising opportunities for GOS applications.

GOS are safe and well-tolerated ingredients up to intake levels of 20 g/d; they have GRAS status in the United States, FOSHU status in Japan, and can be included in the dietary fiber content of foods.

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