Lactose and lactose-derived oligosaccharides: more than prebiotics?

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Abstract

Valorization of lactose is achieved by enzymatic conversion to lactose derivatives with nutraceutical properties. This review summarizes recent developments on composition and functional properties of lactose-derived oligosaccharides. Lactose conversion by β-galactosidase yields galacto-oligosaccharides (GOS); hetero-oligosaccharides with structural and / or functional similarity to human milk oligosaccharides are synthesized by transglycosylation with β-galactosidase, sialidase, glucansucrase, fructansucrase or α-fucosidase using lactose as donor or acceptor sugar. Application development for GOS was based on their prebiotic properties; intestinal fermentation of GOS to short-chain fatty acids confers health benefits. Several novel oligosaccharides exhibit additional potent biological activities that are based on direct interaction of oligosaccharides with glycan-binding domains of bacterial adhesins and toxins, and are highly specific to the oligosaccharide structure. Particularly the use of lactose-derived oligosaccharides to prevent binding of bacterial toxins or adhesins is supported by *in vivo* studies.

1. Introduction

The disaccharide lactose occurs almost exclusively in the milk of mammals (Gänzle, Haase, & Jelen, 2008; Jelen, 1979). Because lactose is the major component of cheese whey; the main routes of lactose valorization rely on the purification for pharmaceutical applications or the chemical or enzymatic conversion to lactose derivatives with nutraceutical properties (Gänzle et al., 2008). Commercial lactose derivatives include galacto-oligosaccharides (GOS), lactitol, lactulose, and lactosucrose. Application development for hetero-oligosaccharides derived from lactose is currently emerging (Díez-Municio, Herrero, Olano, & Moreno, 2014; Gänzle, 2012).

The term GOS is used for β-linked oligosaccharides with a degree of polymerization (DP) of 2 to 9 that are composed of galactose and may contain one glucose unit, typically at the reducing end (Dixon, 1982; Gänzle, 2012; van Leeuwen, Kuipers, Dijkhuizen, & Kamerling, 2016). In keeping with IUPAC nomenclature, the term GOS is used to include disaccharides, however, lactose is typically excluded because it is digestible in human infants. GOS are produced by β-galactosidase (β-Gal)-catalyzed transgalactosylation with lactose as glycosyl-acceptor and -donor (Gänzle, 2012; Gosling, Stevens, Barber, Kentish, & Gras, 2010). Hetero-oligosaccharides are obtained by transgalactosylation of carbohydrates other than lactose, or by transglycosylation of lactose with enzymes other than β-Gal (reviewed by Gänzle, 2012).

GOS are low caloric and non-cariogenic, non-digestible, and prevent attachment of some pathogens to intestinal cells (Gänzle, 2012). Application development of GOS was based on human milk oligosaccharides (HMO) as the conceptual template. HMO modulate infant microbiota based on their prebiotic activity, they also prevent the adhesion of pathogens,\ and stimulate the immune systems (Bode, 2012). The structure of GOS is less complex and less

diverse when compared to HMO, however, GOS are used in infant formula to mimic the functions of HMO (Barile & Rastall, 2013).

Production, structure, and applications of GOS and other lactose derivatives have been studied for more than 4 decades; several reviews cover enzyme selection and process engineering to increase the yield of GOS (Gänzle, 2012; Gosling et al., 2010), the development of lactose derivatives (Gänzle, 2012), and prebiotic properties of GOS (Macfarlane, Steed, & Macfarlane, 2008). The structure of most compounds in commercial or experimental GOS preparations, however, has been elucidated only recently (van Leeuwen, Kuipers, Dijkhuizen, & Kamerling, 2014; van Leeuwen et al., 2016); these recent data allow a novel perspective on structure-function relationships of GOS. Moreover, current discussions on the definition of prebiotics necessitate a revision of the prebiotic activity of GOS (Bindels, Delzenne, Cani, & Walter, 2015). The increasing number of studies related to the production of hetero-oligosaccharides from lactose (Díez-Municio, Herrero, et al., 2014) also allows to produce structural and functional analogues of HMO. This review aims to summarize recent development and concepts on composition and functional properties of lactose and lactose-derived oligosaccharides.

2. Galactooligosaccharide-intolerance or GOS as prebiotics?

Lactose digestion in humans relies on the brush border lactase, which is is specific for the β(1→4) linked lactose and cellobiose and thus differs from microbial β-galactosidases that also hydrolyze other GOS (Hooton, Lentle, Monro, Wickham, & Simpron, 2015; Mantei et al., 1988; Schwab & Gänzle, 2011). Lactase activity decreases with age and approximately 70% of human adults do not digest lactose; lactase activity and the ability to digest lactose persists in 30% of human adults (Corgneau et al., 2015). Undigested GOS and lactose are fermented by large intestinal microbiota (Venema, 2012); vigorous fermentation of GOS and lactose results in

formation of gas and microbial metabolites that cause intestinal discomfort, bloating and flatulence, and osmotic diarrhea (Venema, 2012). Adverse effects are observed after consumption of more than 10 – 15 g of lactose or GOS per day (Corgneau et al., 2015; Macfarlane et al., 2008; Venema, 2012). Tolerance of lactose and GOS corresponds well to the maximum tolerated dose of other non-digestible oligosaccharides, which was reported as 0.3 g / kg body weight (Oku & Nakamura, 2009). Tolerance of lactose and GOS can be increased by gradual adaptation of the intestinal microbiome (Corgneau et al., 2015; Davis, Martínez, Walter, & Hutkins, 2010). The effects of lactose and GOS fermentation are considered beneficial to host health if diarrhea is avoided (Corgneau et al., 2015; Macfarlane et al., 2008; Venema, 2012).

GOS and lactose were described as (conditional) prebiotics that exert health benefits through selective stimulation of intestinal bifidobacteria and lactobacilli (Macfarlane et al., 2008; Szilagyi, 2004; Venema, 2012). Previous definitions of the term “prebiotic”, however, were recently questioned or modified (Bindels et al., 2015; Louis, Flint, & Michel, 2016). Comprehensive analysis of intestinal microbiota through high-throughput sequencing of 16S rRNA gene fragments demonstrated that GOS consumption increased the intestinal abundance not only of *Bifidobacterium* but also of other *Firmicutes* and *Fusobacterium;* this effect varies strongly among individuals (Davis, Martínez, Walter, Goin, & Hutkins, 2011; Louis et al., 2016; Monteagudo-Mera et al., 2016). Moreover, prebiotic health benefits relate to the function rather than the composition of intestinal microbiota. Independent of selective stimulation of specific members of intestinal microbiota, lactose and GOS are metabolized to short chain fatty acids (Bruno-Barcena & Azcarate-Peril, 2015; Venema, 2012), which are major mediators of physiological benefits of dietary fibre and non-digestible oligosaccharides (Bruno-Barcena & Azcarate-Peril, 2015; Mudgil & Barak, 2013). Acid production in the large intestine modulates the composition of gut microbiota by decreasing the intestinal pH, and may protect against

intestinal pathogens (Fukuda et al., 2011). Moreover, short chain fatty acids and particularly butyrate are a main energy source for the colonic mucosa, and have anti-inflammatory properties (Bindels et al., 2015; Bruno-Barcena & Azcarate-Peril, 2015; Venema, 2012; Vinolo et al., 2011). Accordingly, the new definition of prebiotics no longer requires "specific stimulation" of bacterial taxs but emphasizes that health benefits are derived through microbial metabolism (Bindels et al., 2015; Louis et al., 2016). This definition not only includes GOS and β-fructans but generally includes non–digestible oligosaccharides and dietary fibre (Bindels et al., 2015).

It is noteworthy that GOS are not included in the definition of dietary fibre in the U.S., and that health claims for prebiotic carbohydrates including GOS are not approved in the U.S., Canada, or the European Union (Anonymous, 2016; EFSA Panel on Dietetic Products, Nutrition and Allergies, 2011 and 2014). The discussion related to prebiotic GOS, lactose intolerance, and intestinal health is further confounded by the suggestion that diets low in fermentable oligosaccharides, disaccharides, monosaccharides and polyols (FODMAPs) relieve symptoms of the irritable bowel syndrome (Gibson & Shepherd, 2005). GOS are included in the FODMAPs although the effect of GOS on the irritable bowel syndrome and intestinal barrier properties may be opposite to the effect ascribed to FODMAPs (Akbari et al., 2015, 2016; Alizadeh et al., 2016; Gibson & Shepherd, 2005). A reduction of the FODMAP intake over the long term also decreases gut bifidobacteria (Staudacher et al., 2012). A low FODMAP diet might be a short-term strategy to relief the symptoms but is not recommended as a long-term treatment option (Barrett, 2013; Halmos et al., 2015; Tuck, Muir, Barrett, & Gibson, 2014).

Adverse and health-promoting properties of dietary carbohydrates that are described with the terms "dietary fibre", "prebiotics", "lactose intolerance" and "FODMAP" are based on overlapping concepts and mechanisms. The recognition of adverse effects of lactose and GOS or the adjusted definition of the term prebiotic do not challenge the evidences for beneficial health

effects of GOS and other non-digestible oligosaccharides. However, further application development of GOS and other lactose derivatives requires detailed functionality description based on structure identification of GOS or novel hetero-oligosaccharides.

3. Composition of GOS synthesized by βGal

Microbial βGal are found in the glycoside hydrolase (GH) families GH1, GH2, GH35, and GH42 (Gänzle, 2012; Gosling et al., 2010). Commercial GOS production uses of βGal from *Kluyveromyces lactis*, *Bacillus circulans* (Vivinal GOS), *Bifidobacterium bifidum* (Bimuno), *Aspergillus oryzae* and *Streptococcus thermophilus* (oligomate55) (Torres, Gonçalves, Teixeira, & Rodrigues, 2010). Table 1 summarizes the composition of GOS produced by commercial βGal preparations and three experimental and purified βGal. Different βGal differ in their substrate specificity and the corresponding spectrum of GOS (Table 1). Some commercial βGal preparations contain several isoforms of βGal differing in catalytic activities and specificities, for instance, βGal from *Bc. circulans* and bifidobacteria (Arreola et al., 2014; Goulas, Goulas, Tzortzis, & Gibson, 2009; Warmerdam, Paudel, Jia, Boom, & Janssen, 2013). In commercial GOS, oligosaccharides including lactose with DP2 to DP4 constitute >90% of the total solids (van Leeuwen et al., 2016). *Bc. circulans* βGal preparation generates predominantly β-(1→4) linked GOS with a high DP while *K. lactis* and GH2 LacLM type βGal produce mainly β-(1→6) linked GOS with DP 2 – 4 (Table 1). The GH2 LacZ type βGal synthesize mainly β-(1→6) and β-(1→3) linked GOS; GOS from *A. oryzae* βGal include β-(1→6), β-(1→3) and β-(1→4) linked GOS (Table 1). The ratio of GOS with different DP and linkage types also depends on the reaction conditions. For example, Gal-β(1→4)-Gal-β(1→4)-Glc was converted to Gal-β(1→3)-Gal-β(1→4)-Glc during conversion of lactose with βGal from *A. oryzae* (Carević et al., 2016). Similarly, the main product Gal-β(1→4)-Gal-β(1→4)-Glc was decreased after 70 h while the

concentration of β(1→3 or 6)-Gal-β(1→4)-Glc increased (Rodriguez-Colinas, Poveda, Jimenez-Barbero, Ballesteros, & Plou, 2012).

4. Hetero-oligosaccharides synthesized by glycoside hydrolases

HMO represent 1-2% (w/v) of total human milk and play a crucial role in the development of infants (Bode, 2012; Smilowitz, Lebrilla, Mills, German, & Freeman, 2014). Most HMO contain lactose at the reducing end and are elongated with galactose and N-acetyglucosamine units to form linear or branched oligosaccharides (Smilowitz et al., 2014). HMO can be further fucosylated with α-(1→2)/(1→3)/(1→4) linkages and/or sialylated with α-(2→3)/(2→6) linkages (Bode, 2012). Variation of monomer composition, linkage type and degree of polymerization generates more than 1000 structures of HMO. HMO structure, particularly the structures of fucosylated HMO, varies among individuals and is related to the Lewis blood type of mothers (Venema, 2012). The most prevalent HMO structures are summarized by Bode (2012) and Kunz, Rudloff, Baier, Klein, & Strobel (2000).

HMO are specifically metabolized by *Bifidobacterium longum* ssp. *infantis*, however, their benefit to infant health is not limited to their bifidogenic effect. HMO prevent the attachment of pathogens to the intestinal mucosa, stimulate the immune system, and provide sialic acid as an essential nutrient for infants (Bode, 2012). Because structure and function of HMO differ substantially from GOS, recent efforts to synthesize HMO-like structures involve transglycosylation with lactose as donor or acceptor as outlined below.

4.1. Hetero-oligosaccharides synthesized by βGal with lactose as galactosyl-donor

Transgalactosylation by β-galactosidases from lactose to other acceptor sugars such as fructose, glucose, N-acetylglucosamine, chitosan, sucrose and sucralose yields a variety of hetero-oligosaccharides (Table 2). Lactulose (Gal-β(1→4)-Fru) is produced by chemical

isomerization or enzymatic synthesis, and is applied in treatment of hepatic encephalopathy and as prebiotic (Panesar & Kumari, 2011). Lactulose production by transgalactosylation of fructose with βGal also generates allolactulose (Gal-β(1→6)-Fru) and GOS (Shen et al., 2012) (Table 2). The yield of lactulose depends on the ratio of lactose:fructose (Guerrero, Vera, Plou, & Illanes, 2011). Lactulose and allolactulose also act as galactosyl-acceptors to yield fructosyl-GOS (Guerrero, Vera, Conejeros, & Illanes, 2015). *Bc. circulans* βGal only synthesized low amount of fructosyl-GOS (Guerrero et al., 2015), the mixture contained oligosaccharides up to DP 4, including Gal-β(1→6)-lactulose and Gal-β(1→4)-lactulose (Corzo-Martínez, Copoví, Olano, Moreno, & Montilla, 2013). βGal from *K. lactis* and *K. marxianus* predominantly synthesized trisaccharides (Cardelle-Cobas, Corzo, Martínez-Villaluenga, Olano, & Villamiel, 2011; Guerrero et al., 2015). Gal-β(1→6)-lactulose and Gal-β(1→1)-lactulose were the main trisaccharides produced by βGal from *K. lactis* and *K. marxianus* (Guerrero et al., 2015).

Lactosucrose (Gal-β(1→4)-sucrose), a non-reducing trisaccharide produced from transgalactosylation of sucrose or transfructosylation of lactose (described in section 3.2), is commercially available as a low-calorie sweetener and prebiotic. Commercial βGal from *Bc. circulans* generates lactosucrose and several by-products from lactose and sucrose (Table 2). Lactosucrose is hydrolyzed during the reaction, or elongated to produce Gal-β(1→4)-lactosucrose. The lactosucrose analogue Gal-β(1→3)-sucrose, however, increased over time. Reaction time is thus important to control the yield of lactosucrose. A yield of 146 g transgalactosylated products /L was obtained with βGal from *Bc. circulans* after 4 h of reaction and a molar lactose to sucrose ratio of 1:1 (Li et al., 2009).

The core structures of HMO including lacto-N-biose (Gal-β(1→3)-GlcNAc) or N-acetyllactosamine (Gal-β(1→4)-GlcNAc, LacNAc) (Bode, 2012) were synthesized by transgalactosylation of GlcNAc with microbial βGal (Arreola et al., 2016; Bayón, Cortés,

Berenguer, & Hernáiz, 2013; Black et al., 2012; Bridiau & Maugard, 2011; Schwab, Lee, Sørensen, & Gänzle, 2011) or by trans(N-acetyl)glucosaminylation (described in Section 4.2). LacNAc is the main product of transglycosylation of GlcNAc with βGal from *Bc. circulans* (Table 2); The LacNAc homologues from DP2 to DP4 are produced as by-products. The glycosidic bonds formed by *Bc. circulans* βGal are dependent on the enzyme concentration. βGal at 140 U mL^{-1} preferably synthesized LacNAc (15 g L^{-1}) as the main product but higher levels of βGal directed the reaction towards formation of allo-LacNAc (Bridiau & Maugard, 2011). Unlike β-(1→4) linked LacNAc, the β-(1→3) linked lacto-N-biose is not commonly synthesized as main product. Microbial βGal favour formation of β-(1→6) and/or β(1→4) linkages (Arreola et al., 2016); however, recombinant βGal-3 from *Bc. circulans* forms lacto-N-biose as the main product (Bayón et al., 2013).

Chitin-oligosaccharides [$(GlcNAc)_n$ or $(GlcN)_n$] are also suitable acceptor carbohydrates for microbial βGal to form galactosylated GlcNAc- or GlcN oligosaccharides (Black et al., 2014). Biosynthesis of lacto-N-neotetraose (Gal-β(1→4)-GlcNAc-β(1→3)-Gal-β(1→4)-Glc) was achieved with a di-enzyme system using chitinbiose and lactose as substrates. GlcNAc-β(1→3)-Gal-β(1→4)-Glc, generated by transferring a GlcNAc moiety to lactose by βN-acetylhexosaminidase, was further galactosylated by *Bc. circulans* βGal (Zeuner, Nyffenegger, Mikkelsen, & Meyer, 2016). Alternative chemo-enzymatic routes for production of lacto-N-biose do not employ lactose as substrate (Yu et al., 2010; Li & Kim, 2014).

βGal-catalysed transgalactosylation of other acceptor sugars including mannose, galactosamine, fucose and sialic acid also generates hetero-oligosaccharides (Table 2) (Arreola et al., 2016; Schwab et al., 2011). Sucralose, an artificial sweetener derived from sucrose with several hydroxyl group substituted by chlorine, is also transgalactosylated by βGal; the reaction generated 41% Gal-β(1→6)-sucralose (Lu et al., 2012). This new product may combine

properties of a sweetener and a prebiotic (Lu et al., 2012). UDP-activated sugars with a DP ranging from 2 to 4 were synthesized by *Bc. circulans* βGal and UDP-glucose, UDP-GlcNAc or UDP-GalNAc as acceptor sugars (Kamerke, Pattky, Huhn, & Elling, 2012, 2013). All transgalactosylation reactions with lactose as donor accumulate glucose in the reaction mixture. Isomerization after transgalactosylation effectively converts glucose and GOS to fructose, tagatose, lactulose and a variety of lactulose derived oligosaccharides (Cardelle-Cobas, Corzo, Villamiel, & Olano, 2008; Padilla et al., 2015).

Optimization of transgalactosylation of acceptor sugars is comparable to GOS optimization; reaction temperature, time, pH, substrate concentration and water activity influence the product yield and composition (Guerrero et al., 2015). An additional parameter for synthesizing hetero-oligosaccharides and minimizing GOS formation is the ratio of galactosyl-donor to –acceptor. A maximum yield of lactulose, 0.28 g lactulose / g lactose, was obtained with a molar lactose to fructose ratio of 1:8 (Guerrero et al., 2011) but the highest yield of galactosylated GlcNAc was obtained when the ratio of GlcNAc to lactose was 1:1 (Guerrero et al., 2015). The affinity of different microbial βGal for acceptor sugars also influences the yield of hetero-oligosaccharides. βGal from *Lactobacillus* and *Bifidobacterium* prefer glucose and GlcNAc over lactose as galactosyl-acceptors; GalNAc and fucose are weak acceptors (Arreola et al., 2016). βGal from *K. lactis*, *A. aculeatus*, *A. niger*, *A. oryzae* preferentially galactosylate lactulose over lactose while βGal from *Bc. circulans* prefers lactose as galactosyl-acceptor (Guerrero et al., 2015). In conclusion, the molar ratio of galactosyl-acceptor to -donor and the choice of acceptor sugar and enzyme influence the yield hetero-oligosaccharides.

4.2. Hetero-oligosaccharides synthesized with lactose as acceptor

Lactose is a suitable acceptor carbohydrate for various glycoside hydrolases, allowing the synthesis of hetero-oligosaccharides by transglycosylation with lactose acceptor. An overview of the diversity of oligosaccharides produced by this enzymatic route is provided in Table 3.

Acidic HMO are sialylated with N-acetylneuraminic acid (Neu5Ac) to form 3'/6' linked oligosaccharides (ten Bruggencate, Bovee-Oudenhoven, Feitsma, van Hoffen, & Schoterman, 2014). Sialic acid is a group of neuraminic acid with N- or O-substitutions. Sialic acid substrates include the bovine κ-casein-derived glycomacropeptide which contains mainly Neu5Ac (Wang et al., 2015; Wilbrink et al., 2014), glycoproteins from bovine blood plasma (45% Neu5Ac, 55% Neu5Gc) (Wilbrink et al., 2015) and fetal calf serum fetuin (Lee, Shin, & Kim, 2002). Neu5Ac is more suitable for food applications than Neu5Gc as the latter has been linked to immune problems in humans (Padler-Karavani et al., 2008). Sialidase and trans-sialidase transfer sialic acid from donor carbohydrates to lactose or GOS. Trans-sialidase is homologous to sialidase but exhibits increased transferase activity (Paris et al., 2005). Trans-sialidases are mostly found in *Trypanosoma* spp., the trans-sialidase from *Trypanosoma cruzi* belongs to GH33. Neu5Ac or Neu5Gc are transferred as α-(2→3)linked monomers to terminal galactosyl unit of lactose or GOS (Wilbrink et al., 2014, 2015), or to internal galactosyl units of Gal-β(1→6)-lactose (Wilbrink et al., 2014). Disialylation of GOS is also catalyzed (Wilbrink et al., 2015). Shorter GOS are better acceptors than longer GOS (Holck et al., 2014). Trans-sialidase produced 40 mg 3'-sialylactose / g lactose with a high transfer efficiency from the donor glycoside when lactose was present in large excess (Holck et al., 2014; Wilbrink et al., 2014).

Transfructosylation of lactose by fructansucrase yields lactosucrose. β-Fructofuranosidase of *Microbacterium saccharophilum* K-I belongs to GH68 and is homologous to levansucrases

(Tonozuka et al., 2012). This enzyme acted as hydrolase when sucrose is the sole substrate but synthesize lactosucrose when lactose is present (Tonozuka et al., 2012). Levansucrase and inulosucrase are also GH68 enzymes and produce β-(2→6) or β-(2→1) linkages, respectively. Microbial levansucrases also produce lactosucrose with lactose as acceptor; a yield of 224 g lactosucrose L^{-1} was obtained with levansucrase of *L. mesenteroides* (Li et al., 2015). Inulosucrase prefers lactosucrose over lactose as acceptor and transfructosylates lactosucrose to yield β-(2→1) linked lactosyl-oligofructosides (Díez-Municio et al., 2015).

Glucansucrases belong to GH70; enzymes were characterized in the genera *Leuconostoc*, *Lactobacillus*, *Streptococcus* and *Weissella*. Dextransucrases from *Leuconostoc* and *Weissella* synthesize α-(1→6) linked polymers from sucrose; the reaction is shifted to oligosaccharide synthesis in presence of suitable acceptor sugars. Transglucosylation of lactose by dextransucrase yields Glc-α(1→2)-lactose as main product (Díez-Municio et al., 2012; Shi et al., 2016). Glc-α(1→2)-lactose was applied for kojibiose synthesis (Díez-Municio, Montilla, Moreno, & Herrero, 2014), the α(1→2) linkage is not digested in the human intestinal tract (García-Cayuela et al., 2014). In order to decrease dextran production and increase the yield of acceptor products, high concentration of lactose and sucrose (ratio at 1) were applied (Díez-Municio et al., 2012).

The backbone of HMO is usually fucosylated with α-(1→2), α-(1→3) or α-(1→4) linkages and 2'-fucosyllactose (Fuc-α(1→2)-lactose) is the main fucosylated sugar in HMOs. Fucosylated HMO analogues are synthesized with GH29 α-fucosidases. Transfucosylation of lactose or LacNAc from *para*-nitrophenyl-fucose (pNP-Fuc) by α-fucosidase from *Thermotoga maritime* yielded α-(1→2), α-(1→3) or α-(1→6) fucosylated HMO analogues (Osanjo et al., 2007). *Alcaligenes* sp. α-fucosidase synthesized Fuc-α(1→3)-lactose or Fuc-α(1→3)-LacNAc with lactose or LacNAc as acceptor sugar in reactions with a high acceptor to donor ratio (around 33:1) (Murata, Morimoto, Zeng, Watanabe, & Usui, 1999). Similar reactions catalyzed by porcine liver

α-fucosidase yielded α-(1→2), α-(1→3) and α-(1→6) linked fucosyl-lactose and fucosyl-LacNAc (Murata et al., 1999). Several novel α-fucosidases were identified in a soil-derived metagenomic library and used to synthesize 2'-fucosyllactose from pNP-Fuc and lactose (Lezyk et al., 2016). pNP-Fuc is a preferred fucosyl-donor but transfucosylation from this donor releases the toxic *para*-nitrophenol. A natural fucose-containing xyloglucan was used as alternative fucosyl donor to synthesize 2'-fucosyllactose with a yield of up to 3.6% (Lezyk et al., 2016).

Bacterial N-acetylglucosaminidases belong to glycosyide hydrolase family GH20 are found in *Aspergillus oryzae, Nocardia orientalis* and some soil bacteria (Nyffenegger et al., 2015; Matsuo et al., 2003; Murata, Tashiro, Itoh, & Usui, 1997). N-acetylglucosaminidases transfer GlcNAc residues onto lactose and usually synthesize GlcNAc-β(1→3)-Lac and GlcNAc-β(1→6)-Lac from pNP-GlcNAc with low yield (Matsuo et al., 2003). The N-acetylglucosaminidases HEX1 and HEX2 originated from soil-derived metagenomic library, however, use chitin-oligosaccharides with DP2-4 to synthesize GlcNAc-β(1→3)-Lac (Nyffenegger et al., 2015). Moreover, the N-acetylglucosaminidases HEX1 and HEX2 also use glucose, galactose, sucrose and maltose as acceptor sugars (Nyffenegger et al., 2015).

Sialidases, glucansucrases, fructansucrases, α-fucosidases and N-acetylglucosaminidases are retaining glycoside hydrolases with transglycosylation activity to transfer corresponding sugar moieties to acceptor sugars such as lactose, to generate a large variety of hetero-oligosaccharides, which include several core structures of HMO, HMO-analogues. However, most of these glycoside hydrolases with weak transferase activities hindered a large-scaled synthesis of hetero-oligosaccharides. Site-directed engineering of trans-sialidase from *Trypanosoma rangeli* and α-fucosidase from *Thermotoga maritime* increased the efficiency of transglycosylation by these glycoside hydrolases (Osanjo et al., 2007; Saumonneau et al., 2016; Zeuner, Luo, Nyffenegger,

316 Aumala, Mikkelsen, & Meyer, 2014). This provides a strategy to increase transferase activities of
317 other glycoside hydrolases by mutating the amino acids located at hydrolysis-related subsites.

318 **5. Immune modulation by GOS and hetero-oligosaccharides**

319 The immunomodulation effects of GOS are indirect and mediated by shifting the composition
320 of gut microbiota and their metabolites (Oozeer et al., 2013), or are mediated through direct
321 interaction of oligosaccharides with the gut-associated immune system. Evidence for
322 immunological effects of GOS was provided by a single report of GOS triggered IgE-mediated
323 anaphylaxis in Asian atopic patients (Chiang et al., 2012). The direct effect of GOS on the gut
324 immune system and the intestinal barrier function, however, were mostly determined *in vitro*.

325 GOS are TLR4 ligands of immune cells such as intestinal epithelial cells, macrophages and
326 dendritic cells (Lehmann et al., 2015; Ortega-González et al., 2014; Searle et al., 2012). GOS
327 activate the TLR4-NFκB pathway, which also responds to LPS activation with production of pro-
328 inflammatory cytokines. Stimulation of the TLR4-NFκB by GOS produces a comparable patterns
329 of cytokines but is less efficient than stimulation by LPS, which may contribute to gut
330 homeostasis rather than an inflammatory response (Ortega-González et al., 2014). Activation of
331 the TLR4-NFκB by GOS is likely to induce naïve T cell develop to regulatory Treg cell
332 (Lehmann et al., 2015; Verheijden et al., 2016). GOS increase immune tolerance in a dose and
333 DP dependent manner (Lehmann et al., 2015; Searle et al., 2012; Vendrig, Coffeng, & Fink-
334 Gremmels, 2013). *In vitro* experiments indicated GOS that higher than DP 3 were the primary
335 stimulants to murine macrophages (Searle et al., 2012). Similar to GOS, the hetero-
336 oligosaccharides may also stimulate the immune system directly (Vendrig et al., 2013).
337 3'-Sialyllactose modulated the immune response by activating PGlyRP3 receptor on Caco-2 cell
338 (non-TLR4 cell) (Zenhom et al., 2011).

GOS strengthen the integrity of the intestinal barrier. GOS increased the expression of mucin and enterocyte-associated sucrase in the small intestine (Leforestier et al., 2009). Administration of GOS was also effective in preventing deoxynivalenol induced impairment of the epithelial barrier (Akbari et al., 2015, 2016). Modulation of intestinal barrier junction by GOS could be partially due to a direct stimulation of goblet cells (Bhatia et al., 2015), however, the mechanism is not well-defined.

6. Prevention of pathogen adhesion by GOS and lactose-derivatives

Intoxication or infection by intestinal pathogens is generally initiated by binding of toxins or pathogens to specific carbohydrate signatures on the surface of the intestinal mucosa (Shoaf-Sweeney & Hutkins, 2009; Kulkarni, Weiss, & Iyer, 2010). Glycan recognition by pathogens or toxins is highly specific for specific pathogens, and also mediates specificity for the host species. For example, due to the specific interaction of bacterial glycan binding proteins and host receptors, enterotoxigenic *Escherichia coli* are host specific and infect different hosts with different fimbriae (Grange, Mouricout, Levery, Francis, & Erickson, 2002; Li, Poole, Rasulova, McVeigh, Savarino, & Xia, 2007). Likewise, the species- and tissue specificity of the Shiga-toxins is determined by the recognition of globotriaosylceramide on the cellular surface (Johannes & Römer, 2010). Shiga-toxin Stx2e also recognises globotetraosylceramide and is the only toxin variant known to cause disease in swine (Johannes & Römer, 2010). Human milk oligosaccharides are structurally similar to glycan receptors that mediate pathogen adhesion; competitive inhibition of bacterial glycan binding proteins by HMO or analogous oligosaccharides prevents infection or intoxication (Martin-Sosa, Martin & Hueso, 2002; Shoaf-Sweeney & Hutkins, 2009, Hickey, 2012). The constant dietary intake of oligosaccharides prevents the establishment of infection or colonization; the large diversity of oligosaccharides

362 structure provides protection against a broad range of pathogens and toxins (Hickey, 2012). The
363 use of dietary glycans as receptor analogues may also prevent bacterial infections. A large
364 diversity of natural or synthetic oligosaccharides including GOS were evaluated with respect to
365 their ability to prevent pathogen adhesion *in vitro* (Shoaf-Sweeney & Hutkins, 2009; Jin & Zhao,
366 2000; Sinclair, de Slegte, Gibson, & Rastall, 2009); however, only few studies provide
367 confirmation of a protective effect *in vivo*. Because the recognition of host glycans by bacterial
368 pathogens is typically specific for the host species, the use of animal models for validation of
369 glycans for human therapy is inherently challenging. Moreover, some pathogens express multiple
370 alternative adhesins. For example, *Salmonella enterica* adheres with fimbriae-type, pilli-type,
371 auto-transporter adhesins (Wagner & Hensel, 2011) and the use of a single oligosaccharide may
372 not suffice to prevent adhesion to the intestinal mucosa.

373 Several *in vivo* studies nevertheless provide proof of concept that dietary oligosaccharides can
374 be effective tools to prevent infection or intoxication (Table 4). A majority of the successful
375 studies employed human milk oligosaccharides or lactose-derived oligosaccharides (Table 4).
376 However, many of the *in vivo* studies that reported prevention of infection by dietary
377 oligosaccharides did not confirm the mechanism of action. Therefore, protective effect of
378 oligosaccharides may result from binding to glycan-binding domains of bacterial adhesins or
379 toxins, from modulation of intestinal microbiota, or from immunomodulating effect of
380 oligosaccharides (Laparra, Hernandez-Hernandez, Moreno, & Sanz, 2013). Egg yolk derived
381 sialyloligosaccharides inhibited the binding of *S. enterica* but did not influence the production of
382 TNF-α by macrophages (Sugita-Konishi et al., 2002). Convincing evidence for the use of
383 oligosaccharides as receptor decoys with high specificities was provided for the use of lactulose
384 or IPTG to prevent intoxication with the *Clostridium botulinum* neurotoxins (Lee et al., 2013,
385 2015). The host- and tissue specificity of botulinum neurotoxins is mediated by glycan-lectin

interactions. The botulinum neurotoxin is secreted in a complex with heme agglutinins and non-heme-non-toxic proteins. The first step in toxin activity is binding of the heme agglutinins to the intestinal mucosa via glycan recognition; this binding step contributes to the specificity of the individual toxin types for the host species (Fujinaga, Sugawara, & Matsumura, 2013). Lactulose binds to the glycan-binding domain of the heme agglutinin complex and prevents toxin binding and internalization *in vitro* and *in vivo* (Lee et al., 2013, 2015).

7. Conclusion

GOS synthesized from transgalactosylation of lactose by βGal are available as a mixture of glucose, galactose, lactose, and GOS. GOS produced by different enzymes differ in their composition. Enzymatic synthesis of oligosaccharides yields structurally diverse hetero-oligosaccharides by transferring galactose from lactose to suitable acceptor carbohydrates such as fucose and GlcNAc, or by transferring sialyl-, glucosyl-, fructosyl-, or fucosyl-units to lactose. Hetero-oligosaccharides produced by transglycosylation include components of human milk oligosaccharides. The characterization of commercial and experimental GOS and other lactose-derived oligosaccharides increasingly allows the elucidation of structure function relationships, and the development of new applications.

Application development of GOS, lactulose and lactosucrose has focused on prebiotic effects. However, prebiotic effects do not differentiate GOS from other non-digestible oligosaccharides. Moreover, a majority of humans is lactose intolerant; in these individuals, lactose exerts comparable prebiotic effects. GOS and other lactose-derived oligosaccharides also exhibit potent biological activities that are highly specific to the oligosaccharide structure. These activities include the binding to glycan-binding proteins of pathogens or glycan-binding domains or toxins, and direct immunomodulation. These activities were not only demonstrated for analogues of

human milk oligosaccharides, fucosyllactose and sialyllactose, but also for other lactose-derived oligosaccharides.

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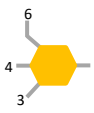
Table 1

Composition and yield of GOS produced by β-galactosidases using lactose as glycosyl donor and acceptor.

Structure [a]	Transgalactosylation of lactose reaction mixture yield (g 100 g^{-1} GOS) [b]						
	Aspergillus oryzae[c]	*Aspergillus aculeatus*[d]	*Kluyveromyces lactis*[e]	*Bacillus circulans*[f]	LacZ type[g]	LacLM type[h]	*Bifidobacterium breve*[i]
	16–30% of TS	11% of TS	26–34% of TS	15% of TS	29% of TS[j]	25–30% of TS[k]	19–33% of TS
	4–10% of TS	3% of TS	14–16% of TS	1% of TS	12% of TS[j]	12–14% of TS[k]	18% - 25% of TS
	30–59% of TS	76% of TS	7–49% of TS	43% of TS	10% of TS	15–22% of TS	5–30% of TS
	**0.2–12**	**26**	**10–15**	-	**2–15**	**14–19**	1–6
	**1–18**	**11**	**17–25**	1	**34–39**	**22–27**	**45–50**
	3	-	-	-	-	-	
	2	-	-	-	1-6	2	4-6
	7	-	-	-	6-8	1-3	2-8
	**32–77**	**35**	**34–48**	1	**19–25**	**22–37**	2-5
	5–7	6	1	**54**	-	-	1–4
	4–9	7	2	-	**11–15**	5–6	**19–32**
	-	-	-	4	-	-	
	5–6	3	4	-	-	-	
	-	-	-	**26**	-	-	
	-	-	-	4	-	-	

[a] Components are: glucose,  ; galactose,  ; structures accounting for more than 10% of the GOS preparation are printed in bold.

[b] TS, total sugars.

[c] GOS are synthesized by commercial β-galactosidase from *A. oryzae* with 400 g L^{-1} lactose in buffer solution (Urrutia et al., 2013) or in UF-skimmed milk permeate at 40 °C (Frenzel, Zerge, Clawin-Rädecker, & Lorenzen, 2015). GOS mixture yield of these two reactions was combined by authors of this review and presented in a range.

[d] GOS are synthesized by commercial β-galactosidase from *A. aculeatus* with 400 g L^{-1} lactose in UF-skimmed milk permeate at 60 °C (Frenzel et al., 2015)

[e] GOS are synthesized by commercial β-galactosidase from *K. lactis* with 400 g L^{-1} lactose in buffer solution (Rodriguez-Colinas et al., 2011) or in UF-skimmed milk permeate at 40 °C (Frenzel et al., 2015). GOS mixture yield of these two reactions was combined by authors of this review and presented in a range.

[f] GOS are synthesized by commercial β-galactosidase from *B. circulans* with 400 g L^{-1} lactose in UF-skimmed milk permeate at 40 °C (Frenzel et al., 2015)

[g] GOS are synthesized by heterologous expressed LacZ type β-galactosidases from *S. salivarius* subsp. *thermophilus* DSM 20259 (Geiger et al., in press) or *L. delbrueckii* subsp. *bulgaricus* DSM 20081 (Nguyen et al., 2012) with 205 g L^{-1} lactose in buffer solution at 30 °C or 37 °C. GOS mixture yield of these two β-galactosidases was combined by authors of this review and presented in a range.

[h] GOS are synthesized by LacLM type β-galactosidases from *L. sakei* Lb790 (Iqbal et al., 2011) or *L. plantarum* WCFS1 (Iqbal, Nguyen, Nguyen, Maischberger, & Haltrich, 2010) or *L. reuteri L103* (Splechtna et al., 2006) with 205 g L^{-1} or 215 g L^{-1} lactose in diluted whey permeate or buffer solution at 30 °C or 37 °C. GOS mixture yield of these three β-galactosidases was combined by authors of this review and presented in a range.

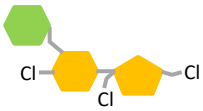
[i] GOS are synthesized by heterologous expressed β-galactosidases from *Bifidobacterium breve* DSM 20213 (Arreola et al., 2014) with 200 g L^{-1} lactose in buffer solution at 30 °C. GOS mixture yield of the β-galactosidases was combined by authors of this review and presented in a range.

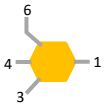
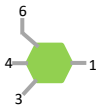
[j] The yield of glucose and galactose from β-galactosidase of *L. reuteri* L103 is not specified. The glucose and galactose yield was combined as 42% of total sugar that displayed in the original publication (Splechtna et al., 2006).

[k] The yield of glucose and galactose from β-galactosidase of *S. salivarius* subsp. *thermophilus* DSM 20259 is not specified. The glucose and galactose yield was combined as 40% of total sugar that displayed in the original publication (Geiger et al., 2016).

Table 2

Hetero-oligosaccharides (HOS) synthesized using β-galactosidases and lactose or lactulose as donor sugar. [a]

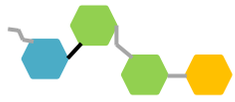
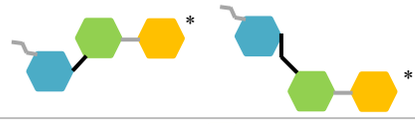
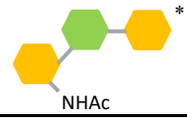
Enzyme source [b]	Donor	Acceptor	HOS structure
β-galactosidase			
A. oryzae, *K. lactis*, *P. furiosus*[1]	lactose	fructose	
A. oryzae, *A. niger*, *A. aculeatus*, *B. circulans*[2] *K. lactis*[2, 3] *K. marxianus*[3]	lactose lactulose	lactulose	
B. circulans[4]	lactose	UDP-glucose	
		UDP-glucosamine	
L. plantarum[5]	lactose	chitinbiose	
		chitintriose	
K. lactis, *B. circulans*[6] *L. bulgaricus*, *B. breve*[7] *L. plantarum*[8]	lactose	GlcNAc	
B. circulans[9]	lactose	sucrose	
L. bulgaricus[10]	lactose	sucralose	
β-galactosidase + isomerization			
K. lactis[11, 12] *K. marxianus*[12]	lactose		

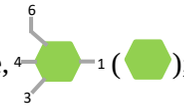
[a] HOS components are: glucose,  ( ); fructose,  ( ); galactose,  ( ); tagatose,  ( ); —— α linkage; [#] proposed structure ; * HMO core structure analogues.

[b] References (indicted by superscript numbers) are as follows: 1, Wang, Yang, Hua, Zhao, & Zhang (2013); 2, Guerrero, Vera, Conejeros, & Illanes (2015); 3, Padilla et al. (2012); 4, Kamerke, Pattky, Huhn, & Elling (2012); 5, Black et al. (2014); 6, Bridiau & Maugard (2011); 7, Arreola et al. (2016); 8, Black et al. (2012); 9, Li et al. (2009); 10, Lu et al. (2012); 11, Cardelle-Cobas, Corzo, Villamiel, & Olano (2008) and Padilla et al. (2015); 12, Padilla et al. (2015).

Table 3

Hetero-oligosaccharides synthesized using glycosyl hydrolases and lactose or GOS as acceptor sugar. [a]

Enzyme source [b]	Donor [c]	Acceptor	HOS structure
Trans-sialidase			
Trypanosoma cruzi (TcTS)[1, 2, 3]	cGMP	lactose	
	cGMP	3'Gal-lactose	
		4'Gal-lactose	
		6'Gal-lactose	
	cGMP/BPG	GOS	
Pasteurella multocida[4]	cGMP	lactose	
Glucansucrase			
Leuconostoc mesenteroides[5] *Weissella confusa*[6]	sucrose	lactose/cheese whey permeate	
Fructansucrase			
L. mesenteroides[7,8] *B. methylotrophicus*[9]	sucrose	lactose	
B. subtilis + *L. gasseri*[10]			
Fucosidase			
Alcaligenes sp.[11]	fucose-pNP	lactose	
		LacNAc	
N-Acetylglucosaminidase			
Soil bacteria	chitin-oligosaccharides	lactose	

[a] HOS components are: glucose,  ( ); fructose,  ( ); galactose,  ( ); sialic acid,  ( ); fucose,  ( ); —— α linkage; - - - - β(1→1/3/4/6) linkage; [#] proposed structure (Wilbrink et al., 2015); * HMO core structure analogues.

[b] References (indicted by superscript numbers) are as follows: 1, Holck et al. (2014); 2, Wilbrink et al. (2014); 3 Wilbrink et al. (2015); 4, Guo et al. (2014); 5, Díez-Municio et al. (2012) and Shi et al. (2016); 6, Shi et al (2016); 7,

Díez-Municio et al. (2012); 8, Li et al. (2015), Wu, Zhang, Mu, Miao, & Jiang (2015); 9, Wu et al. (2015); 10, Díez-Municio et al. (2015); 11, Murata, Morimoto, Zeng, Watanabe, & Usui (1999).

[c] Donor abbreviations are: cGMP, κ-casein-derived glycomacropeptide; BPG, bovine blood plasma glycoprotein; fucose-pNP, *p*-nitrophenyl α-L-fucopyranoside.

Table 4

In vivo studies of identified therapeutic oligosaccharides that prevent or reduce pathogen adherence by the adhesin-receptor interaction. [a]

Toxin/fimbriae	Host/ experimental host	Receptor	Inhibitory glycans	Source
E. coli				
Heat stable toxin a (STa)	Human/infants	Guanyl cyclase C [1]	2'-Fucosyllactose [2]	Human milk
K99 fimbriae	Pig, cattle/calve	Mucin glycopeptides [3]	Glycoprotein glycans [4]	Bovine plasma
K88 fimbriae	Pig/piglet	Sialoglycoproteins (IMTGP-1/2); intestinal transferrin (GP74); neolactotetraosylceramide	Casein glycomacropeptide [5]	Milk
Shiga-like toxin (Stx2, Stx2d)	Human/mice	Globotriaosylceramide (Gb_3) [6]	Gal-α(1→4)-Gal-β(1→4)-Glc [7]	Synthetic
Campylobacter jejuni				
Fibronectin -binding protein (CadF, FlpA)	Human/mice and ex vivo human intestinal mucosal cells	Fibronectin [8]	Fucosylated human milk oligosaccharide [9]	Human milk
Vibrio cholerae				
Cholera toxin	Human/ex vivo rabbit intestinal loop	Ganglioside G [10, 11, 12]	3'-Sialyllactose [13]	Human milk
Clostridium botulinum				
Neurotoxin A	Human/mice	Glycoprotein 2 [11]	Isopropyl-β-D-thiogalactopyranoside, lactulose [12]	Synthetic
Salmonella enterica				
Type 1 fimbriae FimH	Mammals including humans, cattle, poultry / mice	Glycoprotein [14]	Sialyloligosaccharides, asialo-oligosaccharides, sialylglycopeptide [15]	Chicken egg yolk
Listeria monocytogenes				
Listeria adhesion protein (LAP)	Human, rabbit/guinea pig	Stress response protein Hsp60 [16]	Xylooligosaccharides, GOS [17]	Enzymatic synthesis

[a] References (indicted by superscript numbers) are as follows: 1, Giannella & Mann (2003); 2, Morrow, Ruiz-Palacios, Jiang, & Newburg, (2005); 3, Lindahl & Carlstedt (1990); 4, Mouricout, Petit, Carias, & Julien (1990); 5, Hermes et al. (2013); 6, Karmali (2004); 7, Mulvey et al. (2003); 8, Flanagan, Neal-McKinney, Dhillon, Miller, & Konkel (2009); 9, Ruiz-Palacios, Cervantes, Ramos, Chavez-Munguia, & Newburg (2003); 10, Pan & Charych (1997); l1, Matsumura et al. (2015); 12, Lee et al. (2013, 2015); 13, Idota, Kawakami, Murakami, & Sugawara (1995); 14, Grzymajło et al. (2013); 15, Sugita-Konishi et al. (2002); 16, Burkholder & Bhunia (2010); 17, Ebersbach et al. (2010).