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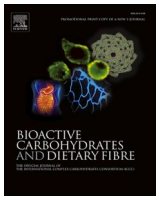

High purity galacto-oligosaccharides: Optimal process design and prebiotic effect

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ABSTRACT

Prebiotic unpurified commercial galacto-oligosaccharides (GOS) often contain significant amounts of glucose, galactose and lactose. Current purification processes remove these digestible components, especially lactose with a high risk of affecting functionality.

In the current study a high purity GOS (HP-GOS) was prepared by treating GOS with β-galactosidase to hydrolyze the remaining lactose into glucose and galactose. Subsequently mono sugars were removed by sequential simulated moving bed (SSMB) chromatography. To this end, a design of experiments (DOE) approach was applied to determine the dry matter content and β-galactosidase concentration that optimally support lactose hydrolysis in such a way that the detrimental effects on GOS composition are minimized to preserve its original oligosaccharide composition. The HP-GOS product obtained using the identified optimal settings for lactose hydrolysis contained more than 75% of the original DP2 GOS fraction while the fraction with a DP above 2 was unaffected by the purification. Using fecal fermentations, it was demonstrated that the GOS parts of the HP-GOS and the parent GOS have comparable effects on gut microbiota composition. Both GOS products equally increased the relative abundances of *Bifidobacterium*. Furthermore, there were no differences in alpha diversity and in the distribution of the different bifidobacterial species. Based on these outcomes it was concluded that the lactose hydrolysis step of the purification process did not influence gut microbiota modulating effects of GOS.

1. Introduction

β-Galacto-oligosaccharides (GOS) are enzymatically produced by transgalactosylation of a single substrate lactose by a β-galactosidase. GOS is composed of different oligosaccharides consisting of mainly β-linked galactose and glucose moieties with a degree of polymerization (DP) ranging from 2 to 9 (Van Leeuwen et al., 2016).

The prebiotic properties of GOS have been demonstrated in several clinical trials with infants and adults. GOS as single prebiotic (Ben et al., 2008; Sierra et al., 2015) or combined with long chain FOS (lcFOS) in a ratio of 9:1 (Fanaro et al., 2005; Moro et al., 2002; Boehm et al., 2002) stimulated the outgrowth of infant type bifidobacteria in these trials. A bifidogenic effect has also been demonstrated in young (Johnstone et al., 2021), middle-aged (Schoemaker et al., 2022) and older adults (Wilms et al., 2021). Bifidobacteria are beneficial bacteria that contribute to a resilient gut microbiota (Dogra, J. Dore, & Damak, 2010; Fassarella et al., 2021; O'Callaghan & van Sinderen, 2016; Sommer et al., 2017; Ladirat, Schoterman, et al., 2014). Next to bifidogenicity and subsequent stimulation of SCFA production (Burger-van Paassen et al., 2009; Hatayama et al., 2007), multiple biological functions of GOS have been demonstrated in infants and adults. GOS exert a protective effect on epithelial cells and gut barrier function (Akbari et al., 2017; Figueroa-Lozano et al., 2020; He et al., 2016; Krumbeck et al., 2018; Perdijk et al., 2019). Furthermore, GOS possess a decoy function, thus inhibiting the epithelial cell adhesion of certain pathogens (Kong et al., 2022; Laparra et al., 2013). This is a crucial step prior to invasion of host cells (Ribet & Cossart, 2015). Several other beneficial effects of GOS have been reported such as recovery after antibiotic treatment (Ladirat et al., 2014a, 2014b), weight management (Liu et al., 2020; Mistry et al., 2020), improving metabolic health (Gonai et al., 2017), increasing

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mineral absorption (Husmann et al., 2022; Jeroense et al., 2019; Whisner et al., 2013), alleviating lactose intolerance and management of lactose maldigestion (Azarate-Peril et al., 2017; Azcarate-Peril et al., 2013; Savaiano et al., 2013) and, supporting immune function (Cai et al., 2022; Vulevic et al., 2015) based on *in vitro*, animal and human studies. Emerging studies indicate that GOS can contribute to mental well-being (Johnstone et al., 2021; Burokas et al., 2017; Liu & Zhu, 2018), and supports aspects of sleep (Schaafsma et al., 2022; Colombo et al., 2021; Thompson et al., 2021; Tabrizi et al., 2019).

In the past decades, the increasing demands for GOS and its use in various types of application have driven development of new types of GOS with low residual lactose and mono sugars, so-called high purity GOS (HP-GOS) (Hong et al., 2016; Cheng et al., 2006; Li et al., 2008; Monteagudo-Mera et al., 2016). However, changes in the GOS profile due to loss of components in the purification process might affect its functionality.

Recently, it was demonstrated that infant microbiota preferentially ferment the DP2 fraction of Vivinal GOS during *in vitro* fermentation (Akkerman et al., 2022). This confirmed a previous *in vitro* single strain fermentation study that showed that of all GOS components, two lactose isoforms, namely gal-β1,2-glc and gal-β1,3-glc, are most easily digested by a broad range of *Bifidobacterium* species (Böger et al., 2019). Furthermore, it has been shown that five DP2 components that are commonly present in commercial GOS products (van Leeuwen et al., 2016), may play an important role in symbiosis of *Bifidobacterium* with *Bacteroides* (Lammerts van Bueren et al., 2017). Additionally, the GOS DP2 fraction plays an important role in protecting the gut barrier function (Akbari et al., 2017). Altogether, this emphasizes that it is very important to retain the DP2 components, especially gal-β-1,2-glc and gal-β-1,3-glc that are present in any type of commercially available GOS (van Leeuwen et al., 2016), in the purification process.

Purification of GOS entails either removing monosaccharides only or removing both monosaccharides and lactose resulting in high purity GOS (HP-GOS) with a higher GOS content. Also, when powdered GOS of low hygroscopicity is required to enable a dry blending process or to formulate a synbiotic product, removal of monosaccharides is needed because the presence of mono sugars and especially glucose makes GOS powder very hydroscopic. For regular infant milk formula (IMF), it is not necessary to remove the lactose, since lactose is the largest component in IMF. However, for other applications, removal of lactose might be preferred for instance to avoid lactose crystallization (Gänzle, 2012) and to allow its consumption by lactose intolerant people (Mattar et al., 2012). HP-GOS is also instrumental for applications that require small volumes such as supplements.

Current technology for GOS purification, aiming to remove monosaccharides is based mainly on sequential simulated moving bed (SSMB) chromatography, or nanofiltration (NF) (Córdova et al., 2017). Removing lactose in addition to monosaccharides while retaining all GOS components is very challenging with the current separation technologies. Several alternative approaches have been followed in the past, such as selective fermentation of the monosaccharides and lactose (Pázmándi et al., 2020; Tsai & Tsai, 2017; Vera et al., 2016), and hydrolyzing lactose by a suitable β-galactosidase, followed by the removal of the mono sugars. However, these strategies entail a high risk of altering the GOS profile as compared to its parent GOS (Pázmándi et al., 2020), thus potentially partially compromising GOS functionalities.

In conclusion, for the functionality of GOS it is crucial to maintain the DP2 fraction during the applied purification process to produce HP-GOS. In this regard, the objective of the current study was to develop an efficient purification process for the preparation of HP-GOS. This was done by using β-galactosidase and a SSMB process that maintained DP2 lactose isoforms at a level of at least 75% of the original levels without altering the profile and levels of other GOS components (DP > 2). In addition, the effect of the resulting HP-GOS product on gut microbiota composition was compared with that of GOS from which only mono sugars were removed using fecal batch fermentations.

2. Materials and methods

2.1. GOS

Commercially available GOS, Biotis® GOS-O (FrieslandCampina Ingredients, Amersfoort, The Netherlands), was used as starting material for the purification process. This is a GOS type specifically developed for the adult market which is made by *trans*-galactosylation of lactose solutions using the enzyme β-galactosidase derived from *Papiliotrema terrestris*. Biotis® GOS-O is composed predominantly of β 1,4 linked oligos and has the following typical composition: GOS (63% w/w); lactose including allolactose and lactulose (21%w/w); and monosaccharides including glucose and galactose (20% w/w).

2.2. GOS purification

2.2.1. Determination of optimal settings for lactose hydrolysis

A design of experiments (DOE) approach (Roy, 2001, p. 560) was used to determine the optimal dry matter content and enzyme dosing for hydrolysis of the remaining lactose in the crude GOS syrup (Table 1). GOS solutions were prepared by dissolving the required amount of GOS in a reaction mixture with end concentrations of 10 mM potassium phosphate, 10 mM KCl and 2.5 mM $MnCl_2$. The pH was adjusted to 6.5 with 1 M NaOH or HCl and the reaction mixture of 150 g in a 250 ml glass reactor was heated to the reaction temperature of 40 °C in a water bath. Subsequently, the required amount of β-galactosidase (EC 3.2.1.23) (Maxilact®LGI5000, DSM, The Netherlands) was added to initialize the hydrolysis. Maxilact®LGI5000 is a well-defined commercial β-galactosidase of *Kluveromyces lactis*. The preparation of Maxilact®LGI5000 that was used had an activity of 5000 NLU/g and enzyme dose is the amount of this complete commercial product per gram of dry matter.

2.2.2. Preparation of HP-GOS using pilot SSMB (sequential simulated moving bed)

One thousand liters of crude GOS syrup (45% w/w, pH 6.5) containing 20 mM potassium phosphate buffer and 10 mM KCl was heated to the reaction temperature of 40 °C. The required amount of β-galactosidase (Maxilact®LGI5000) per gram of lactose was added to hydrolyze residual lactose. Samples were taken at a time interval of 1 h. The reaction was stopped after 4 h by adding 1.0 M HCl to reach 1.5% (v/v) in the reaction mixture. This resulted in a pH < 3.1 which was sufficient to denature the enzyme.

The resulting partly purified GOS was subjected to a pilot scale chromatographic purification process, namely a countercurrent SSMB technology with NovaSep Applexion™ catonic exchanger resin to remove the mono sugars, thus resulting in HP-GOS. Briefly, the crude GOS syrup after Maxilact-mediated hydrolysis was directly loaded to the SSMB columns at 65 °C, in which the process water is used as eluent. The details of using SSMB technology for GOS purification have been described elsewhere (Mueller et al., 2021).

Table 1
The Taguchi-DOE setup (Roy, 2001, p. 560) for hydrolyzing lactose.

Sample code	Dry matter content (%)	Enzyme dose (mg/g dry matter) [a]
1	45	0.5
2	45	1
3	45	1.5
4	50	0.5
5	50	1
6	50	1.5
7	55	0.5
8	55	1
9	55	1.5

[a] Enzyme dose is mg Maxilact®LGI5000/gram dry matter.

2.2.3. GOS analysis

Separation and visualization of individual components of GOS preparations were achieved by HPAEC PAD mounted on a Dionex IC-3000 system. Separations were performed using a CarboPac PA1 (Thermo Scientific) analytical-anion exchange column (dimensions, 250 mm by 4 mm) with a CarboPac PA1 (Thermo Scientific) guard column (dimensions, 50 mm by 4 mm) and a detector (ED40) in the pulsed amperometric detection PAD mode (Dionex, Thermo Scientific). For the chromatographic analysis the following gradient was employed A: 100 mM NaOH, B: 600 mM NaOAc in 100 mM NaOH, C: Milli-Q water, and D: 50 mM NaOAc as previously described (van Leeuwen et al., 2014, 2016). Quantification of the individual peaks of chromatograms was performed using CHROMELEON software Ver.7 (Dionex, Thermo Scientific).

2.2.4. Statistical analysis and DOE design

The statistical analysis of the results of the DOE was performed with Minitab statistic software 16.1 version purchased from Minitab.

2.3. Fermentation study

2.3.1. Preparation of a GOS reference product low in monosaccharides for the fermentation experiment

Lab scale removal of monosaccharides from Biotis® GOS-O was performed using a SEC column: XK 50 SEC column (GE Healthcare Life Sciences) filled with Biogel P-2 solid phase (column packing), mounted on a AKTA-prime system (GE Healthcare Life Sciences, serial 1382671). The column was kept at 55 °C. Five ml of 40% (w/w) GOS syrup was loaded and eluted with Milli-Q water (Synergy UV, Millipore) of 60 °C at a speed of 1 ml/min. Aliquots of 10 ml were collected with the auto-sampler. After 24 h of elution, the samples were measured by the digital brix meter, and the plot of Brix value against tube number was used to prepare a chromatogram and thus to determine the DP separation and the position of each individual DP fraction. Mono sugars were discarded and all the DP $\geq$ 2 fractions were pooled together and concentrated to 5% (w/w) using a rotary evaporator and freeze-dried to obtain a GOS powder low in monosaccharides.

2.3.2. Fecal material and preparation of inoculum

Fecal samples were obtained from 3 healthy adults. The samples were frozen by the donors at home and kept frozen until processing in an anaerobic chamber. Thawed individual fecal samples were ten times diluted by mixing with phosphate buffer (50 mM, pH 7.2). The fecal slurries were homogenized with a stomacher and glycerol was added to a final concentration of 20%. The fecal slurries (feces concentration of 8%) were stored at −80 °C. Prior to inoculation, equal amounts of processed fecal samples of the three donors were pooled under anaerobic conditions and used for inoculation.

2.3.3. Fecal batch fermentations

The batch fermentations were carried out in a pH and temperature-controlled environment under anaerobic conditions using the micro-Matrix (Getinge Applikon, The Netherlands) (O'Donnell et al., 2018). All 24 fermentations were run simultaneously in the 24-well reactor plate. The wells were filled with standard carbohydrate-poor fermentation medium (Fooks & Gibson, 2003) adjusted to pH 6.8 The composition of the substrates reference GOS and HP-GOS are shown in Table 2. These substrates were supplemented to a final concentration of 1% pure GOS with 6 replicates per substrate. In addition, glucose was added to the wells with GOS and, galactose and lactose were supplemented to wells with HP-GOS to equal the concentrations of these sugars between GOS substrates. This was required to guarantee that potential differences in microbiota outcomes would result from differences in GOS structural composition and not from differences in levels of glucose, galactose and lactose. The final concentrations in the wells with GOS were: 1% oligosaccharides, 0.144% lactose, 0.005% glucose and 0.0123% galactose. The remainder of the reactor plate was used for the medium control without substrate (6 wells; medium control) and the control for glucose, galactose, and lactose as present in the fermentations with both GOS products (6 wells; sugar control). The wells were inoculated with 12.5% (v/v) fecal slurry, resulting in an end concentration of 1% feces. During fermentations, the pH was kept at 6.8 using ammonia- and CO_2 gas, the temperature was controlled at 37 °C and anaerobicity was maintained by nitrogen gas. A constant low flow of mixed gas (CO_2/H_2/N_2: 10/5/85) was applied to support growth of anaerobic bacteria. The reactor plate was orbitally shaken at 300 rpm. Samples of 1 ml were taken from the fecal pool and from the different wells after 7.5 and 24 h of fermentation. The samples were centrifuged for 5 min at 12 500 g at 4 °C. The cell pellets were used for DNA isolation.

Table 2
Composition of GOS substrates for fecal batch fermentation.

	HP-GOS (% of dry matter)	Reference GOS[b] (% of dry matter)
GOS[a]	95.0	86.42
Lactulose	*0.16*	*0.77*
Allo-lactose	*6.40*	*1.35*
Lactose	4.43	12.44
Glucose	0.49	0.06
Galactose	0.06	1.08

[a] GOS is including lactulose and allo-lactose.
[b] Reference GOS with reduced amount of monosaccharides.

2.3.4. DNA isolation and sequencing

DNA was extracted from the cell pellets using the QIAamp PowerFecal Pro DNA kit (QIAGEN, The Netherlands) according to the manufacturer's instructions. DNA quality and quantity were determined spectrophotometrically. A minimum of 500 ng of DNA was shipped to BaseClear (The Netherlands) for shallow shotgun metagenomics sequencing. DNA samples were subject to Illumina Nextera XT library preparation. The sequencing libraries obtained were sequenced on a NovaSeq 6000 instrument with paired-end 150 nt sequencing protocol. FASTQ read sequence files were generated using bcl2fastq2 version 2.18, which includes quality Illumina Chastity quality filtering with default settings. Subsequently, reads containing adapters and/or PhiX control signal were removed using an in-house filtering protocol. The second quality assessment was based on the remaining reads using the FASTQC quality control tool version 0.11.8.

Single-end or paired-end sequence reads were generated using the Illumina NovaSeq 6000. The sequences generated with the NovaSeq 6000 were performed under accreditation according to the scope of BaseClear B.V. (L457; NEN-EN-ISO/IEC 17025). FASTQ read sequence files were generated using bcl2fastq version 2.20 (Illumina). Initial quality assessment was based on data passing the Illumina Chastity filtering. Subsequently, reads containing PhiX control signal were removed using an inhouse filtering protocol. In addition, reads containing (partial) adapters were clipped (up to a minimum read length of 50 bp). The second quality assessment was based on the remaining reads using the FASTQC quality control tool version 0.11.8. Per sample a minimum of 500 MB paired end data was generated.

2.3.5. Processing of sequencing data

Sequencing data was further processed and analyzed via the Biomax Informatics AG microbiome pipeline (Biomax, Germany). Human reads were first removed. Kraken2 software (Wood et al., 2019) was used to map reads to the taxonomical annotated database of NCBI. Thereafter Bracken software (Lu et al., 2017) was applied, to redistribute reads which were assigned to higher or lower taxonomic levels back to species level via Bayesian re-estimation.

2.3.6. Analysis of sequencing data

Alpha diversity (Shannon and Simpson) and relative abundance data

over all taxonomic hierarchical levels were analyzed for group differences using the Wilcoxon signed rank test. Differences in overall microbiota composition (beta-diversity) were analyzed using Bray Curtis and visualized using nonmetric-multidimensional scaling (nMDS) plots. Analysis of differences in relative abundance of the 20 most abundant genera and all species of *Bifidobacterium* between groups was performed using Kruskal-Wallis tests. Differential abundance analysis (DAA) tests were applied at species level after appropriate data filtering and normalization in such a way that linear models could be applied for analysis using the Bioconductor R packages limma (Ritchie et al., 2015) and edgeR (Chen et al., 2016; McCarthy et al., 2012; Robinson et al., 2010).

For all statistical analyses, multiple testing correction was done by calculating the false discovery rate (FDR) according to Benjamini-Hochberg (Benjamini & Hochberg, 1995).

3. Results and discussion

3.1. β-Galactosidase catalyzed hydrolysis of lactose

Nine hydrolytic reactions designed according to the DOE design of Table 1 using Minitab were performed. After 4 h of hydrolysis, the obtained crude GOS preparations with reduced lactose content were analyzed using the Dionex fingerprint method to determine the levels of galactose, glucose, and lactose. The selectivity of β-galactosidase for lactose and the two lactose isoforms gal-β1,2-glc and gal-β1,3-glc (DP2 isoforms) was calculated according to the following equation:

$$\beta\text{-galactosidase selectivity} = \text{lactose hydrolyzed/isoforms hydrolyzed}$$

GOS purity was estimated based on the theoretical assumption that the GOS content is the total amount of dry matter minus glucose, galactose, and lactose.

As shown in Table 3, a high enzyme dose and a high dry matter content generally led to low selectivity, thus hydrolyzing much more of the major two lactose isoforms mentioned above. This was reflected by the decreasing β-galactosidase lactase selectivity in Table 3 and confirmed by the main effect analysis in Fig. 1.

Although deleterious for selectivity, increasing enzyme dosage and dry matter content were beneficial for achieving high GOS purity thus suggesting that higher β-galactosidase dosing is needed to overcome the inhibiting effect of the mono sugars (Boon et al., 1999), which are present before and increased after the hydrolysis of lactose (Fig. 2). Removal of lactose was found to be strongly dependent on the enzyme dose and dry matter content. High enzyme dose and lower dry matter content usually favored high lactose removal (Fig. 3) (see Fig. 4).

The interdependence of GOS purity, percentage of lactose removal and β-galactosidase selectivity on the dry matter content and β-galactosidase dose led to the conclusion that feasible conditions for preparing HP-GOS should be selected with dry matter content in the range of 45–50%, enzyme dose in the range of 0.5–1.0 mg/g dm (dry matter). Further optimization was focused on an enzyme dose from 0.5 to 1.0 mg/g dm and a dry matter content of 45% (w/w) initial GOS solution. Depending on the enzyme dosage chosen from this range mentioned above, the reaction time for the hydrolysis can be adjusted, to match factory layout and the design and integration of the overall process including the production of the crude GOS, hydrolysis and SSMB purification.

3.2. Pilot hydrolysis of crude GOS and SSMB purification

The optimal process conditions identified in the DOE were confirmed at 10 L laboratory scale with an enzyme dose of 1.0 mg Maxilact®LGI5000/gram dm and a hydrolytic reaction time of 4 h. Subsequently, the hydrolytic reaction was performed on pilot scale (1000 L) for 4 h.

As shown in Fig. 5, the resulting crude GOS mixture prior to the SSMB process contains several GOS components with increased abundance such as gal-β-1,6-gal (p3, retention time 12.5 min), allolactose (p4, retention time 14 min) and Gal-β-1,4-glc-2,1-β-gal (p9, retention time 24 min) and GOS components with reduced abundance such as lactose isoforms, namely gal-β-1,2-glc (p8a) and gal-β-1,3-glc (p8b) suggesting that β-galactosidase used can hydrolyze not only lactose and lactose-isoforms into monosaccharides, i.e. glucose and galactose but also performs intra/internal transgalactosidation of lactose resulting in the formation of additional allolactose (intra transgalactosidation), and gal-β1,4-glc-2,1-β-gal (p9) (an internal transgalactosidation product) (Huber et al., 1976). Allolactose was previously described to be hydrolyzed by human intestinal β-galactosidase at a rate of less than 5% of the lactose hydrolysis, thus 95% of allolactose is fermented as prebiotic in the colon (Burvall et al., 1980). Furthermore, it has been described that intramolecular transgalactosidation of lactose to allolactose induces the lac operon. This positive feedback loop stimulates β-galactosidase production in lactose fermenting strains such as lactobacilli and bifidobacteria in the colon (Velazco et al., 2021).

On the other hand, β-galactosidase can also catalyze intermolecular transgalactosidation of lactose to other sugar donors, especially galactose, of which the concentration increases during the lactose hydrolysis. This results in the synthesis of a galactobiose, namely gal- β-1,6-gal (p3). Most importantly, it was found that ~83% DP2 retention was obtained after the β-galactosidase hydrolysis.

After controlled β-galactosidase hydrolysis, ~75% lactose was hydrolyzed into mono sugars, namely galactose and lactose. The subsequent SSMB process resulted in 96% removal of mono sugars and only five percent of overall DP2-GOS components were lost during the SSMB

Table 3
Summary of DOE and the results.

DM (%)	Enzyme dose (mg/g DM)	DP2 isoforms retention (%)	Lactose removal (%)	HP-GOS purity (%)	Total >DP2 retention (%)	β-galactosidase selectivity*
45	0.5	83.7	49.3	90.7	100.0	3.03
45	1	72.1	66.7	93.8	100.0	2.39
45	1.5	63.3	81.5	96.3	95.0	2.22
50	0.5	83.4	38.0	89.1	100.0	2.28
50	1	71.7	62.0	93.1	100.0	2.19
50	1.5	62.4	75.8	95.5	95.9	2.01
55	0.5	84.4	37.1	88.4	99.6	2.38
55	1	69.8	51.8	91.5	95.8	1.71
55	1.5	68.2	59.7	92.6	96.0	1.88

* β-Galactosidase selectivity is the ratio between hydrolysis of lactose and hydrolysis of the lactose isoforms.
DM: dry matter.

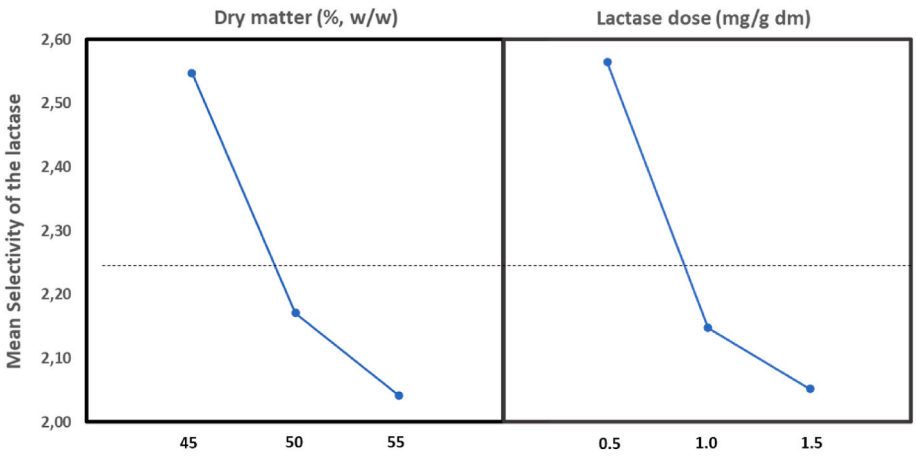

Fig. 1. Effect size analysis of enzyme dose and dry matter content.

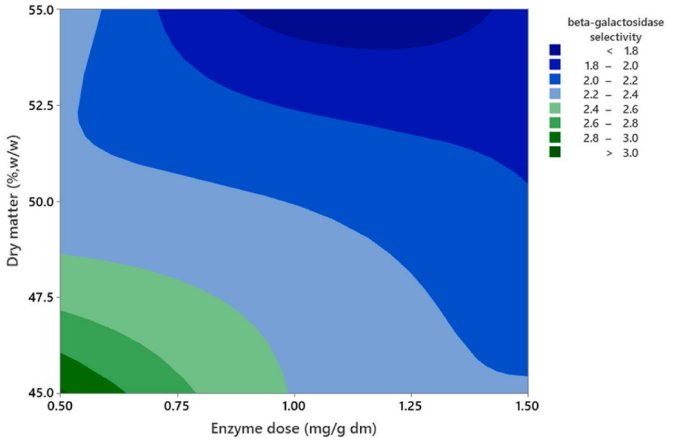

Fig. 2. Contour plot of digestion selectivity of β-Galactosidase vs dry matter (%) vs enzyme dose.

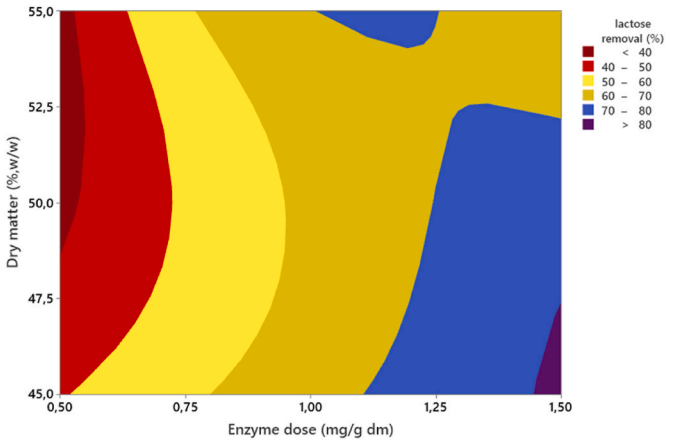

Fig. 3. Contour plot of lactose removal (%) vs dry matter (%) vs enzyme dose.

purification process. Moreover, the DP > 2 GOS composition was not affected by the SSMB process. This suggests that SSMB is indeed an efficient process for oligos purification and fractionation (Table 4). SSMB has clear benefits compared to other purification processes such as the process of making ultrapure GOS (>95% purity) which is done by the sequential microbial treatment of GOS with *Saccharomyces cerevisiae* and *Streptococcus thermophilus* (Giacomelli et al., 2011). In that process

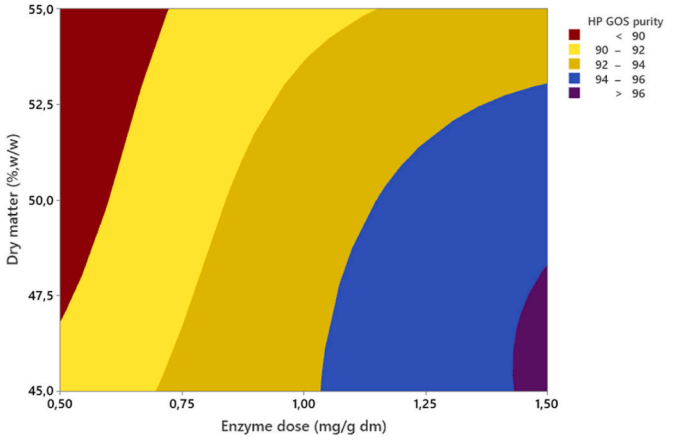

Fig. 4. Contour plot of theoretic HP-GOS purity vs Dry matter (%) vs Enzyme dose.

more than 95% of the bifidogenic DP2 GOS components are lost as well as some DP > 2 GOS components. Retaining the DP2 GOS fraction is not only beneficial because of its bifidogenicity (Akkerman et al., 2022; Böger et al., 2019) but also because it stimulates β-galactosidase in bifidobacteria by inducing the *lac* operon (Velazco et al., 2021).

3.3. *In vitro comparison of gut modulating effects of HP-GOS and its parent product*

The influence of HP-GOS and the reference product (GOS reference) on gut microbiota composition was studied using fecal batch fermentations. During the fermentations, process control (oxygen and pH) failed for 4 of the 24 wells, these wells were excluded from analysis. After 7.5 h of fermentation, fecal microbiota composition was determined using shotgun metagenomics sequencing and compared for the two GOS products. An nMDS plot of Bray-Curtis beta diversity analysis is shown in Fig. 6.

The Bray-Curtis plot further showed distinct clusters for samples derived from fermentations with GOS, the medium control and substrate control. However, samples derived from fermentations with either reference GOS or HP-GOS could not be distinguished even after applying different filtering strategies (data not shown).

After 7.5h of fermentation with either GOS reference or HP-GOS, the Shannon alpha-diversity was respectively, 1.972 and 1.977 and Simpson alpha-diversity was 0.746 and 0.754. These small differences were not significant and are physiologically not relevant.

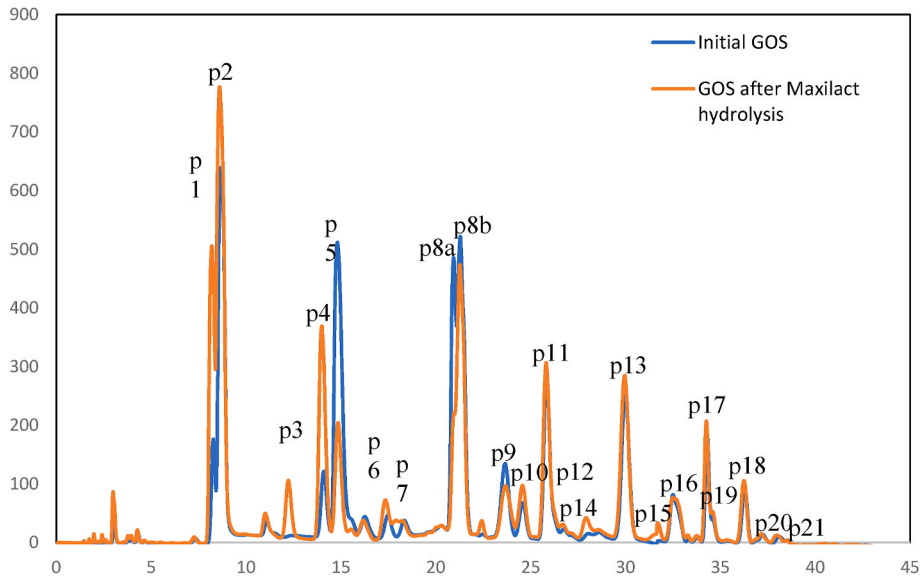

Fig. 5. Fingerprint profile of crude GOS (blue) versus β-galactosidase hydrolyzed GOS (Orange). p1: galactose, p2:glucose, p3: gal-β-1,6-gal, p4: allolactose, p5: lactose, p6: Gal-β-1,4-glc-6,1-β-gal/Gal-β-1,6-gal-1,4-β-glc, p7: gal- β-1,4-gal, p8a:Gal-β-1,2-glc, p8b: Gal-β-1,3-glc, p9: Gal-β-1,4-glc-2,1-β-gal. The peak number and the annotation refers to van Leeuwen et al., 2016. (For interpretation of the references to colour in this figure legend, the reader is referred to the Web version of this article.)

Table 4
Summary of pilot process data: sugar and DP composition.

	Impurities (% of dry matter)			GOS fractions (% of total GOS content)						DP2 Retention
	Gal	Glc	Lac	DP2-GOS	DP3	DP4	DP5	DP6	DP7+	
GOS[1]	2.20	18.30	13.20	39.90	38.20	18.60	3.10	0.33	0	100
SSMB feed[2]	4.80	30.4	3.20	33.10	40.30	21.11	4.55	0.49	0.49	83.0
HP-GOS[3]	0.10	1.10	5.20	31.30	41.50	21.70	4.60	0.50	0.50	78.4

Gal, galactose; Glc, glucose; Lac, lactose. 1 Crude GOS prior to lactose hydrolysis; [2] Composition after lactose hydrolysis; [3] Final HP-GOS syrup after SSMB.

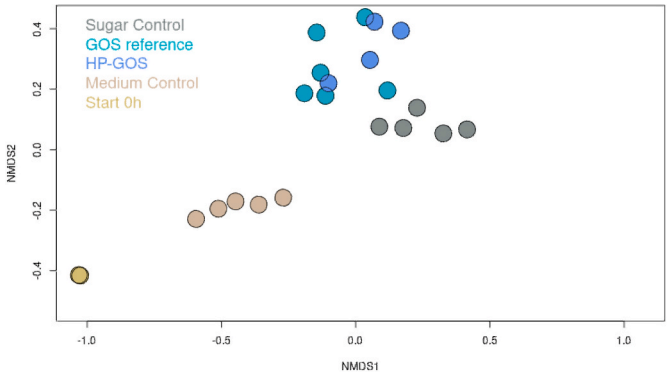

Fig. 6. Bray-Curtis nMDS plot of microbiota composition of samples at baseline and after 7.5 h of fermentation.

The average relative abundance of the top 20 most abundant genera in the samples was almost equal after fermentation of HP-GOS and GOS reference (Fig. 7). For the medium control, the average relative abundance of bifidobacteria was far lower whereas that of *Escherichia* was higher compared to fermentations with both GOS products. Comparable results were found for the sugar control although the differences compared to fermentations with GOS are less. Compared to baseline a bifidogenic effect is demonstrated for both GOS products as well as the sugar control. The observed bifidogenic effects for GOS are in line with results of numerous other *in vitro* and *in vivo* studies (e.g Bunešová et al., 2012; Johnstone et al., 2021; Schoemaker et al., 2022; Stiverson et al., 2014; Wilms et al., 2021). Bifidogenicity was equal for HP-GOS and GOS reference.

Zooming in on the different species of *Bifidobacterium* present revealed that *Bifidobacterium adolescentis* was the most dominant *Bifidobacterium* in all the samples followed by *Bifidobacterium catenulatum* and *Bifidobacterium longum* (Fig. 8) which are generally the most prevalent species of *Bifidobacterium* in the adult gut (Arboleya et al., 2016; Derrien et al., 2022). Only for *Bifidobacterium pullorum*, there was a significant difference in relative abundance (% of total number of bifidobacteria) between treatments (GOS, HP-GOS and sugar control) using Kruskal-Wallis. This is a very low abundant *Bifidobacterium* species.

More in depth comparison of microbiota species composition of samples after fermentation with GOS or HP-GOS using differential abundance analysis only showed a significant difference for *Elizabethkingia meningoseptica*. Because of the very low relative abundance of this species (<0.01%) no strong physiological effect is expected from small differences.

Overall, the effects of HP-GOS and the reference GOS product on gut microbiota were highly comparable, bifidogenicity was equal for both products. This indicates that the designed purification process did not influence GOS prebiotic functionality.

4. Conclusions

The work described in this manuscript demonstrates for the first time that a delicate balance of the reaction conditions such as β-galactosidase dose and dry matter content is required to reach a high purity GOS lower in lactose content, while maintaining its DP2 fraction above 75% and an almost identical distribution of >DP2 GOS fraction relative to the parent

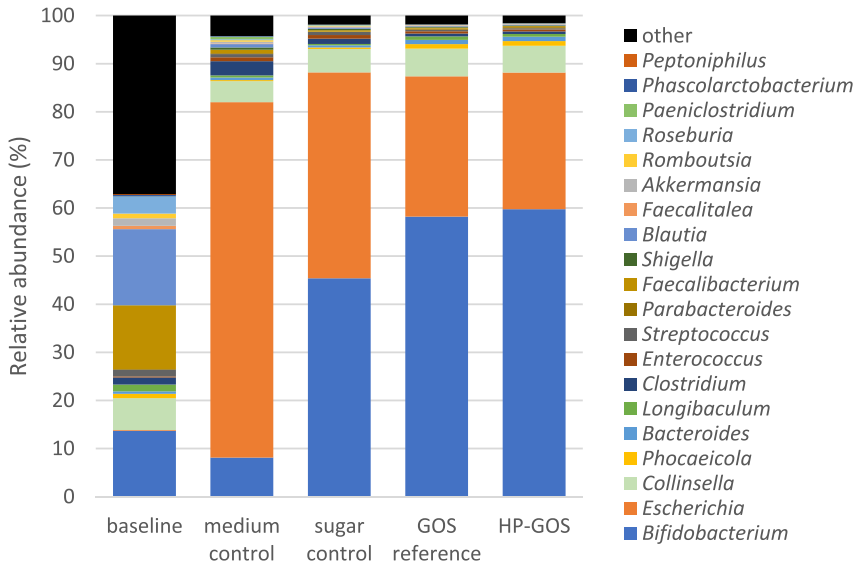

Fig. 7. Bar plots showing the relative abundance of the top 20 most abundant genera at baseline and after 7.5 h of fermentation with the indicated substrates.

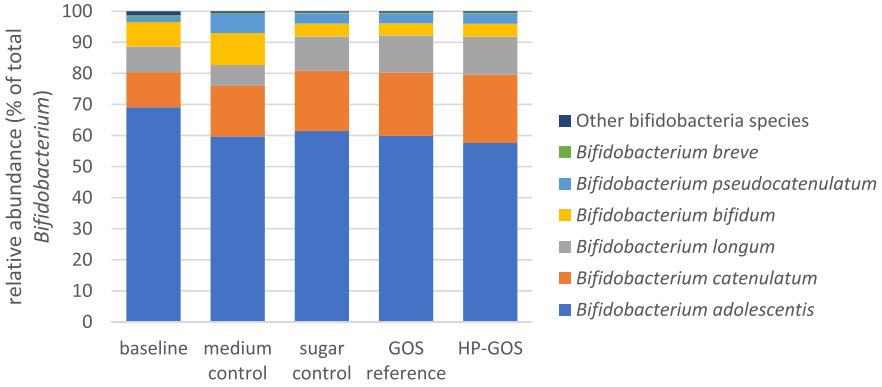

Fig. 8. Relative abundance of *Bifidobacterium* species at baseline and after 7.5 h of fermentation with the indicated substrates.

GOS. Furthermore, it was shown that the SSMB process is a highly efficient in removing the mono sugars while maintaining the DP $\geq$ 2 oligos fraction.

Additionally, it was demonstrated in an *in vitro* fermentation study that the GOS part of the obtained HP-GOS, has comparable effects on the composition of adult gut microbiota as compared to a reference GOS low in monosaccharides. Bifidogenic effects of both GOS products were equal. This eliminates the need for more complicated purification technologies, such as selective fermentation.

Author contributions

Conceptualization, M.V., L.C.; methodology, L.C., E.L., M.Z.; validation, L.C., E.L., J.W., M.B.; formal analysis, L.C., M.Z., B.G.; investigation, L.C., J.W., M.B.; data curation, B.G., M.Z.; writing—original draft preparation, E.L., L.C.; writing—review and editing, E.L., L.C.; B. G.; visualization, L.C., B.G., E.L.; project administration, M.V.

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Declaration of competing interest

Linqiu Cao, Miranda Bultsma, Jeroen Wissing, Marlies Versteeg and Ellen Looijesteijn are employees of FrieslandCampina.

Data availability

Data will be made available on request.

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