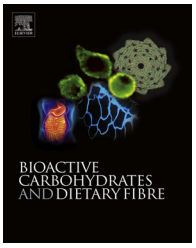
Available online at www.sciencedirect.com

ScienceDirect

www.elsevier.com/locate/bcdf



In vitro fermentation of galacto-oligosaccharides and its specific size-fractions using non-treated and amoxicillin-treated human inoculum

S.E. Ladirat[a], H.A. Schols[a,*], A. Nauta[b], M.H.C. Schoterman[b], F.H.J. Schuren[c], H. Gruppen[a]

[a]Laboratory of Food Chemistry, Wageningen University, Bornse Weilanden 9, 6700 AA Wageningen, The Netherlands
[b]FrieslandCampina, Stationsplein 4, 3818 LE Amersfoort, The Netherlands
[c]TNO Microbiology & Systems Biology, Utrechtseweg 48, 3704 HE Zeist, The Netherlands

ARTICLE INFO

Article history:
Received 20 January 2014
Received in revised form
14 February 2014
Accepted 14 February 2014

Keywords:
Prebiotic
Galacto-oligosaccharides
Antibiotic
Amoxicillin
Intestinal microbiota
Degree of polymerization

ABSTRACT

In order to elaborate on the impact of amoxicillin treatment on the *in vitro* fermentation of specific structures of galacto-oligosaccharides (GOS), GOS was fractionated based on its degree of polymerization (DP) and the fermentation of individual DPs was studied. Different DP fractions of GOS and different isomeric structure within a DP fraction were preferentially degraded depending on the treatment applied to the microbiota. For the non-treated microbiota, the small DP fractions (dimers and trimers) were preferentially degraded as compared to the large DP fractions (tetramers till hexamers). β-D-Gal-(1→4/6)-D-Glc and β-D-Gal-(1→4)-β-D-Gal-(1→4)-D-Glc were the isomeric structures preferentially degraded within the DP2 and DP3 fraction, respectively. The fermentation of each size-fraction induced the production of various short chain fatty acids and the growth of several species of bifidobacteria. For amoxicillin-treated microbiota, the large size-fractions of GOS were preferentially degraded as compared to the small fractions. β-D-Gal-(1→4)-D-Gal and β-D-Gal-(1→4)-β-D-Gal-(1→3)-D-Glc were the isomeric structures preferentially degraded within the DP2 and DP3 fraction, respectively. Butyrate was only produced upon the fermentation of the large size-fractions. The differences in metabolic pattern of GOS depending on the treatment applied correlated with the changes in the microbiota composition, especially the growth of bifidobacteria. These results suggest that GOS, especially its large size-fractions, support the recovery of bifidobacteria and butyrate-producing bacteria after a treatment of the microbiota with amoxicillin.

1. Introduction

Galacto-oligosaccharides (GOS) are well accepted as a prebiotic and contribute to human health (Gibson, Probert, Van Loo, Rastall, & Roberfroid, 2004). GOS are produced by trans-galactosylation of lactose by β-galactosidases from yeast, fungi or bacteria, resulting in complex mixtures of oligosaccharides with different degrees of polymerization (DP) and glycosidic linkages (Macfarlane, Steed, & Macfarlane, 2008). The DP varies from DP2 to DP8. Possible linkages are β

*Corresponding author. Tel.: +31 317 482 239.
E-mail address: henk.schols@wur.nl (H.A. Schols).

http://dx.doi.org/10.1016/j.bcdf.2014.02.002
2212-6198

(1–1), β(1–2), β(1–3), β(1–4) and β(1–6), the latter two being the most common ones (Coulier et al., 2009). Due to the diversity of possible glycosidic linkages, various isomeric oligosaccharides are present in each DP fraction (Coulier et al., 2009). The β-glycosidic linkages between the saccharide units can specifically be hydrolysed in the colon by dedicated enzym es from *Bifidobacterium* and *Lactobacillus* species. In pure cultures, different *Bifidobacterium* species have shown to have preferential utilization of selected DP of GOS. For instance, *Bifidobacterium longum* subsp. *infantis* ATCC 15697 preferred GOS of DP4, whereas *Bifidobacterium adolescentis* ATCC 15703 utilized preferentially GOS of DP3 (Barboza et al. 2009). In the same study, *B. longum* subsp. *longum* DJ010A was reported to partly utilize larger GOS (DP5 to DP6). Furthermore, differences in terms of oligosaccharides composition (type of glycosidic linkage and the monomer composition) can also influence the stimulation of specific species. *Bifidobacterium breve* 26M2 and *Bifidobacterium lactis* BB12 were shown to have preference for β(1–6) and β(1-1) linkages over β(1–4) linkages (Cardelle-Cobas et al., 2011), while *Bifidobacterium animalis* in the microbiota from growing rats was shown to grow only upon the addition of GOS derived from lactulose and not on GOS derived from lactose (Marín-Manzano et al., 2013).

Prebiotics, such as GOS, have been suggested to help balancing the microbiota composition during antibiotic treatment and, thus, to limit antibiotic-associated disease, such as diarrhoea (Preidis & Versalovic, 2009; Saulnier, Kolida, & Gibson, 2009). Amoxicillin is one of the mostly used antibiotic in Europe. This bactericidal antibiotic belongs to the penicillin class of antibiotics and has a broad spectrum of action, including an impact on bifidobacteria. It also has one of the highest incidence (5–35%) of antibiotic-associated diarrhoea (McFarland, 2008). In an *in-vitro* fermentation, it was shown that specific species of bifidobacteria recovered upon GOS addition depending on the antibiotic used (Ladirat et al., 2013b). For an amoxicillin-treated microbiota, the growth of mainly *B. longum* was observed upon addition of GOS. Considering the fact that specific species of bifidobacteria grow preferentially depending on the available substrate (Barboza et al., 2009; Marín-Manzano et al., 2013), it was hypothesized that different DPs of GOS may be preferentially degraded depending on the treatment applied to the microbiota and may, therefore, contribute to a different extend to the recovery of the microbiota. In the present study, the metabolic pattern of GOS and its DP fractions as well as the changes in the composition of the microbiota, especially the level of bifidobacteria, were investigated during *in vitro* fermentation using a non-treated microbiota (NT-MB) and an amoxicillin-treated microbiota (AMX-MB).

2. Materials and methods

2.1. Antibiotic

Amoxicillin (AMX) (≥ 90%) was obtained from Sigma-Aldrich (St Louis, MO, USA).

2.2. Prebiotic

Vivinal® GOS (FrieslandCampina Domo, Borculo, The Netherlands) was fractionated to obtain a purified Galacto-oligosaccharides (GOS) with a low amount of monomers and lactose (see Section 3). For the purification, Vivinal® GOS was treated with a beta-galactosidase to hydrolyse the lactose into glucose and galactose. The beta-galactosidase used was very specific for lactose. After the treatment, the monosaccharides were removed by nanofiltration. The degree of polymerization (DP) of the purified GOS was the same as the commercial available Vivinal® GOS, ranging from 2 to 8. Furthermore, based on HPAEC analysis, the composition of the GOS in the Vivinal® GOS and the purified GOS was highly similar (data not shown). It was therefore concluded that the enzyme treatment hardly affected the GOS composition.

2.3. Fractionation of GOS

The oligosaccharides present in the purified GOS were fractionated by size exclusion chromatography (SEC). An Äkta purifier (GE Healthcare, Uppsala, Sweden) was equipped with three serially connected HiLoad 26/60 Superdex 30 prep-grade columns (GE Healthcare). The columns were maintained at 35 °C. GOS (180 mg dissolved in 1.5 mL Milli-Q water) was applied onto the columns and was eluted with 0.5% (v/v) EtOH in Milli-Q water at a flow rate of 1 mL/min. A refractive index RI-detector (RI-72, Showa Denko, Tokyo, Japan) was used to monitor the eluate. The system was controlled using Unicorn software. Fractions of 1.9 mL were collected, pooled according to the SEC-RI profile (data not shown) and subsequently freeze-dried.

2.4. Experimental set up

Using a recently developed fermentation screening-platform (96 wells of 1.5 mL), the antibiotic amoxicillin (1 μg mL^{-1}) and the purified GOS or individual DP fractions of GOS (4.2 mg mL^{-1}) were simultaneously added to the Standard Ileal Efflux Medium (SIEM) and *in-vitro* fermented (37 °C; pH 5.8) using pooled faecal inoculum (0.1% v/v) of healthy adults under anaerobic conditions (Ladirat et al., 2013a). Selection of volunteers ($n=8$) and methodology to obtain, pool and store the faecal samples were performed as described previously (Ladirat et al., 2013a). To investigate the microbiota metabolic activity, samples (70 μL) were collected at 6 time points during fermentation. On basis of test experiments that determined the course of GOS degradation, the time range chosen was 8 h and 24 h fermentation for the non-treated microbiota and between 16 h and 32 h for the fermentations using 1 μg mL^{-1} amoxicillin (data not shown). The samples collected were boiled (5 min) and stored at −20 °C. To investigate the microbiota composition, additional samples (70 μL) were collected from the same well at 24 h and 48 h, and directly stored at −20 °C. Unfortunately, the experimental set-up and the limited amount of material did not allow to have replicates.

2.5. Monitoring GOS degradation

High performance Anion Exchange Chromatography (HPAEC) was used to quantify GOS degradation. The samples collected were 10× diluted with Millipore water (final concentration: 0.42 mg mL^{-1}) and analysed using an ICS5000 HPLC system (Dionex, Sunnyvale, CA, USA), equipped with a CarboPac PA-1 column (2 mm ID × 250 mm; Dionex) in combination with a CarboPac PA guard column (2 mm ID × 25 mm) and a ICS5000 ED detector (Dionex) in the PAD mode. A flow rate of 0.3 mL min^{-1} was used with the following gradient of 0.1 M sodium hydroxide (solution A) and 1 M sodium acetate in 0.1 M sodium hydroxide (solution B): 0–20 min, 0–20% B; 20–26 min washing step with 100% B; 26–41 min, equilibration with 100% A. Ten μL of sample was injected each time. The degradation rate of GOS and its DP fractions in time was calculated using the peak area of the HPAEC chromatograms. Initial concentrations per DP present in the DP fractions and in the GOS mixture were set to 100%. The degradation rate of the isomeric structures within DP2 and DP3 fractions was estimated using the peak area of each identified isomer.

2.6. Organic acids analysis

The SCFA production during fermentation were quantified using a TRACE™ GC Ultra Gas Chromatography system coupled to a FID detector (Interscience, Breda, The Netherlands) (Pierce et al., 2006): 50 μL of diluted sample (0.42 mg mL^{-1}) or standard (1 mg mL^{-1} to 0.125 mg mL^{-1}) mixed with 50 μL of 0.15 M oxalic acid stood for 30 min before addition of 150 μL of water. Next, samples (1 μL) were injected to a CP-FFAP CB column (25 m × 0.53 mm × 1.00 μm, Agilent, Santa Clara, CA, USA). The temperature profile was as follow: start at 100 °C, increase to 155 °C at 5 °C min^{-1}, and held at this temperature 1 min. GC data was processed using the Xcalibur® software (Thermo Scientific). Concentration of acids was expressed as μmol mg^{-1} of GOS.

Lactate and succinate were quantified on a Dionex Ultimate 3000 HPLC (Dionex) equipped with an ion-exclusion Aminex HPX—87H column (7.8 × 300 mm) combined with a guard column (Bio-Rad, Hercules, CA, USA), and a RI-101 refractive index detector (Shodex, Kawasaki, Japan). The mobile phase was 5 mM H_2SO_4, the flow rate was 0.6 mL min^{-1} and the elution temperature was 65 °C. Samples (10 μL–0.42 mg mL^{-1}) were injected onto the column.

2.7. Microbiota composition analysis

The Intestinal (I)-Chip, developed at TNO (Zeist, The Netherland), was used to investigate the composition of the microbiota. This DNA based microarray enabled the detection of over 400 bacterial targets from the human large intestinal microbiota. Total faecal DNA from samples collected was isolated, amplified, purified and hybridized as described previously (Ladirat et al., 2013a). The hybridization took place on a microarray constructed and validated as described before (Crielaard et al., 2011), using intestinal bacteria primers instead of oral primers. Imagene 5.6 software (BioDiscovery, Marina del Rey, CA, USA) was used to analyse the results. Genes with a signal intensity higher than 3 ($>10^5$ bacteria) were used to describe the bacterial fingerprint.

2.8. Total bacteria and Bifidobacterium spp. quantification

Quantitative PCR (qPCR) was performed to investigate the variation in the number of total bacteria and of *Bifidobacterium* spp. during fermentation. Primers used to measure the number of total bacteria were the universal primers 16S-uni-II-R [10 pmol μL^{-1}] (GGA CTA CCA GGG TAT CTA ATC CTG TT) and 16S-uni-II-F [10 pmol μL^{-1}] (TCC TAC GGG AGG CAG CAG T), and probe 16S-uni-II [5 μM] (6FAM-CGTAT-TACCGCGGCTGCTGGCAC-TAMRA). The primers to measure the number of *Bifidobacterium* spp. were 16S-Bif-F [10 pmol μL^{-1}] (GGA GCA TGC GGA TTA ATT CG), 16S-Bif-R [10 pmol μL^{-1}] (GAC CAT GCA CCA CCT GTG AAC), 16S-Bifspec (6FAM-CTG GGC TTG ACA TGT T) (Applied Biosystems, Bleiswijk, The Netherlands). The amplification was performed with 5 μL DNA sample and 25 μL q-PCR mixture (Ladirat et al., 2013b). Total microbial faecal DNA was diluted 1:10 to quantify the number of total bacteria and 1:100 to quantify the number of *Bifidobacterium* spp. The experiment was performed using the 7500 Fast Real Time PCR system (Applied Biosystems) at settings previously described.(Ladirat et al., 2013b) DNA of the microbiota from the control and *B. longum* was used as quantitative standards for total bacteria and *Bifidobacterium* spp., respectively (1 fg μL^{-1} to 1 ng μL^{-1}).

Numbers of total bacteria and *Bifidobacterium* spp. obtained upon the addition of the individual DP fractions were compared with that of obtained upon the addition of the original GOS and a control (no addition of GOS). Differences larger than 0.5log_{10} were emphasized.

3. Results & discussion

3.1. Fractionation of GOS and oligosaccharide identification

GOS was fractionated based on the degree of polymerization (DP) using SEC. Each pool consists majorly of oligosaccharides of one DP with minor contamination of the preceding or following DP as determined by MALDI-TOF-MS (data not shown). The composition of GOS (weight percentage) was 3% DP1, 12% DP2, 49% DP3, 20% DP4, 11% DP5, 4% DP6, <1% DP>6. Monosaccharides and oligosaccharides of DP>6 were excluded as monosaccharides are absorbed in the upper part of the gastro-intestinal tract and are not relevant for the gut fermentation, while the abundance of oligosaccharides of DP>6 was too low to obtain sufficient amounts for the fermentation experiments. The fractions DP2 to DP6 were further analysed with HPAEC (Fig. 1A). As it can be seen, the complexity of the elution pattern increases with increasing DP, which confirms the presence of high numbers of isomeric structures present in GOS (Coulier et al., 2009). The pattern of peaks obtained per DP was comparable to previous data (Coulier et al., 2009) (Fig. 1B). By comparing the two patterns, the oligosaccharides from the DP2 fraction and most oligosaccharides from the DP3 fraction could be identified (Table 1). For the DP2 fraction, β(1–2), β(1–3), β(1–4), β(1–6) as

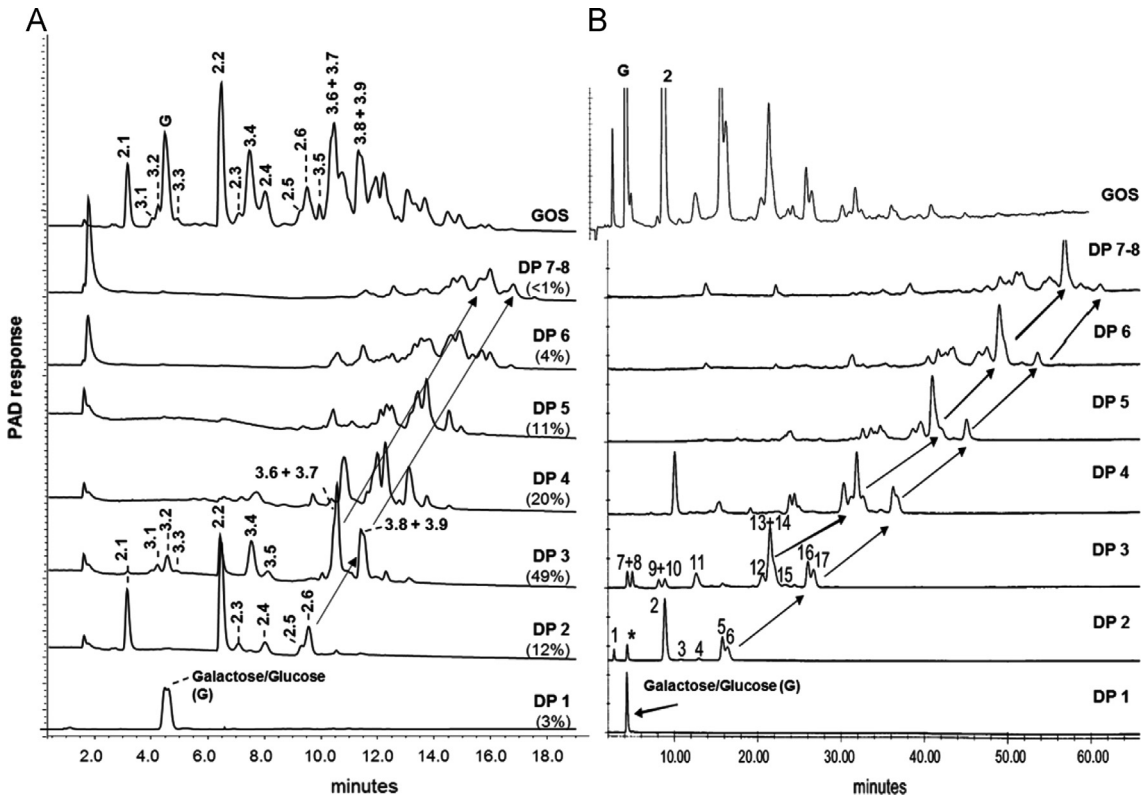


Fig. 1 – HPAEC-PAD chromatograms of GOS and DP fractions of GOS obtained by SEC from this study (A) and from Coulier et al. (2009) (B). Peak identification can be seen in Table 1. Ratio of each DP fraction in the GOS complex mixture is indicated in figure A (weight %).

Table 1 – Identities and levels of DP2 and DP3 compounds in GOS (adapted from Coulier et al. (2009)).

Peak in this study	Peak from Coulier et al. (Coulier et al., 2009)	Compound	wt% in DP2
2.1	1	β-D-Gal-(1↔1)-α-D-Glc +β-D-Gal-(1↔1)-β-D-Glc	7
2.2	2	β-D-Gal-(1→4)-D-Glc (lactose)	27
2.2	2a	β-D-Gal-(1→6)-D-Glc (*allo*-lactose)	15
2.2	2b	β-D-Gal-(1→4)-D-Fru (lactulose)	5
2.3	3	β-D-Gal-(1→3)-D-Gal	1
2.4	4	β-D-Gal-(1→4)-D-Gal	3
2.5	5	β-D-Gal-(1→3)-D-Glc	26
2.6	6	β-D-Gal-(1→2)-D-Glc	16
3.1, 3.2, 3.3	7+8	?[a]	
–	9+10	?[b]	
3.4	11	β-D-Gal-(1→4)-β-D-Gal-(1→6)-D-Glc or β-D-Gal-(1→6)-β-D-Gal-(1→4/6)-D-Glc[c]	
3.5	12	β-D-Gal-(1→4)-β-D-Gal-(1→6)-D-Glc or β-D-Gal-(1→6)-β-D-Gal-(1→4/6)-D-Glc[d]	
3.6–3.7	13+14	β-D-Gal-(1→4)-β-D-Gal-(1→4)-D-Glc + β-D-Gal-(1→4)-β-D-Gal-(1→4)-Fru[e]	
	15	β-D-Gal-(1→6)-β-D-Gal-(1→2)-D-Glc	
3.8	16	β-D-Gal-(1→4)-β-D-Gal-(1→3)-D-Glc	
3.9	17	β-D-Gal-(1→4)-β-D-Gal-(1→2)-D-Glc	

[a] Total of four small peaks, all containing (1↔1) linkages in their structure.
[b] Total of two small peaks, all containing (1↔1) linkages in their structure.
[c] Coelution with one small unknown peak.
[d] Coelution with two small unknown peaks.
[e] Coeluting peaks.

well as α- or β(1–1) linkages were identified. For the DP3 fraction, identified structures were structures present in the DP2 fraction having an additional β-linked Gal, mostly at the O-4 position, added to the non-reducing terminal residue. For the fractions of DP4 to DP6, it was more difficult to fully identify individual structures due to the complexity of the pattern. The different DP fractions obtained were further *in-vitro* fermented using a non-treated and amoxicillin inoculum from healthy adults. The metabolic pattern of GOS and its specific DP fractions were investigated as well as the changes in the composition of the microbiota.

3.2. *Metabolic pattern of GOS*

Metabolic pattern of GOS was studied by monitoring the degradation of GOS and its DP fractions as well as by monitoring the levels of organic acids during *in-vitro* fermentation.

3.2.1. *Degradation of GOS and its specific DP fractions*

The degradation of GOS and its DP fractions during fermentation with and without AMX treatment were monitored using HPAEC (Fig. 2). Considering the individual DP fractions of GOS, the oligosaccharides preferably degraded after 18 h of fermentation for NT-MB were the oligosaccharides of DP2 (95% degraded), followed by the oligosaccharides of DP3 (80% degraded), and at last the oligosaccharides of DP4 to 6 (50% degraded). A similar order of preferential degradation was observed for the GOS mixture, except for oligosaccharides of DP6 that were already 80% degraded. After 24 h of fermentation, the original GOS was completely degraded whereas, for the individual DP fractions, 20% of oligosaccharides of DP3 and 10% of oligosaccharides of DP4 were still present. Overall, these results indicate a preferred use of small DP oligosaccharides by the non-treated microbiota. This has also been seen for fructans, with FOS being faster degraded than Inulin (Rossi et al., 2005).

For AMX-MB, the individual DP fractions were degraded at a same speed within 24 h of fermentation except for the oligosaccharides of DP3 that remained until 32 h of fermentation. However, a clear preference for specific size of oligosaccharides was observed for the mixture of GOS. Oligosaccharides of DP4 to DP6 were already completely degraded within 24 h of fermentation, while about 10% of oligosaccharides of DP3 could still be detected after 32 h of fermentation. Oligosaccharides of DP2 accumulated till 24 h of fermentation before being degraded within 32 h of fermentation. The results of DP2 as fraction and as present in GOS are contradictive. The accumulation of DP2 upon the fermentation of GOS mixture indicates that more DP2 are produced from the degradation of larger oligosaccharides than the disrupted microbiota is able to consumed. Overall, the degradation pattern of GOS mixture indicates that DP4 to DP6 fractions were preferentially degraded by AMX-MB independently from the presence of smaller structures. Hence, oligosaccharides of DP4 to DP6 appear to be a potential substrate to stimulate a higher recovery of certain species of bifidobacteria in AMX-MB.

Beside a preference for certain DP fractions, a preference for certain isomeric oligosaccharides within a specific DP fraction was also observed depending on the treatment applied to the microbiota (Fig. 3). The DP2 structure of β-D-Gal-(1→4/6)-D-Glc and β-D-Gal-(1→4)-D-Fru were completely degraded within 8 h of fermentation for NT-MB, whereas they were degraded last by AMX-MB. Another major difference concerned β-D-Gal-(1→4)-D-Gal that remained one of the last structure to be degraded by

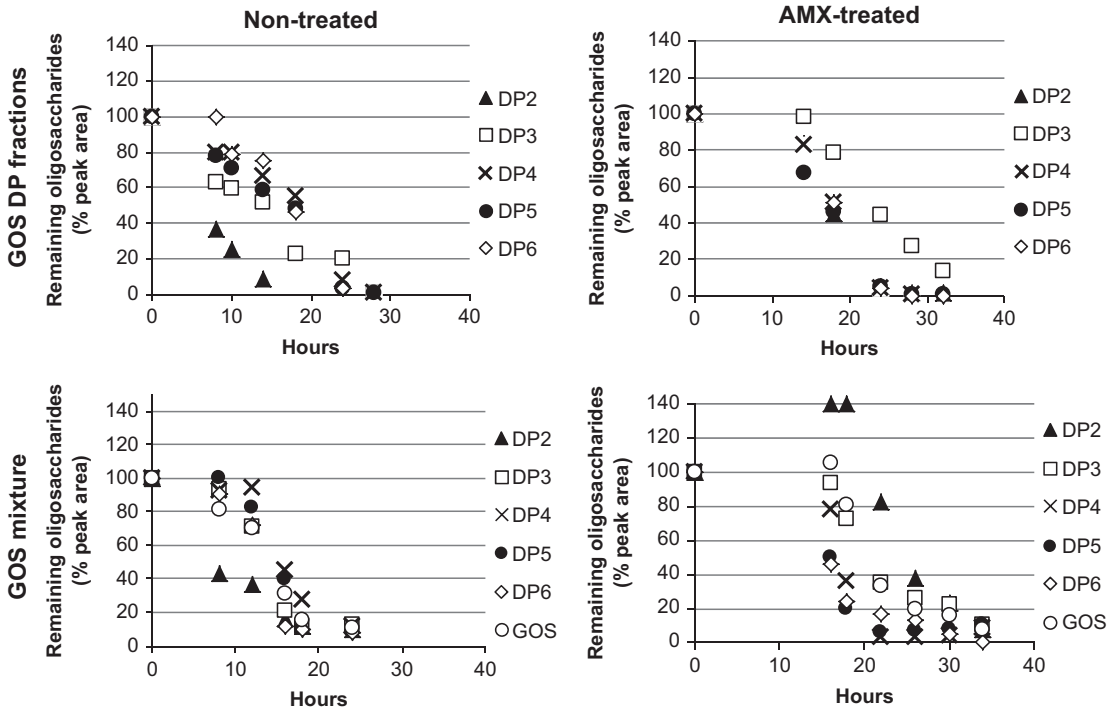


Fig. 2 – Proportion (%) of remaining oligosaccharides from GOS mixture and individual DP fractions of GOS during *in-vitro* fermentation using a non-treated and AMX-treated (1 μg mL^{-1}) microbiota. Concentrations per DP present in the DP fractions or in the GOS mixture were set to 100%.

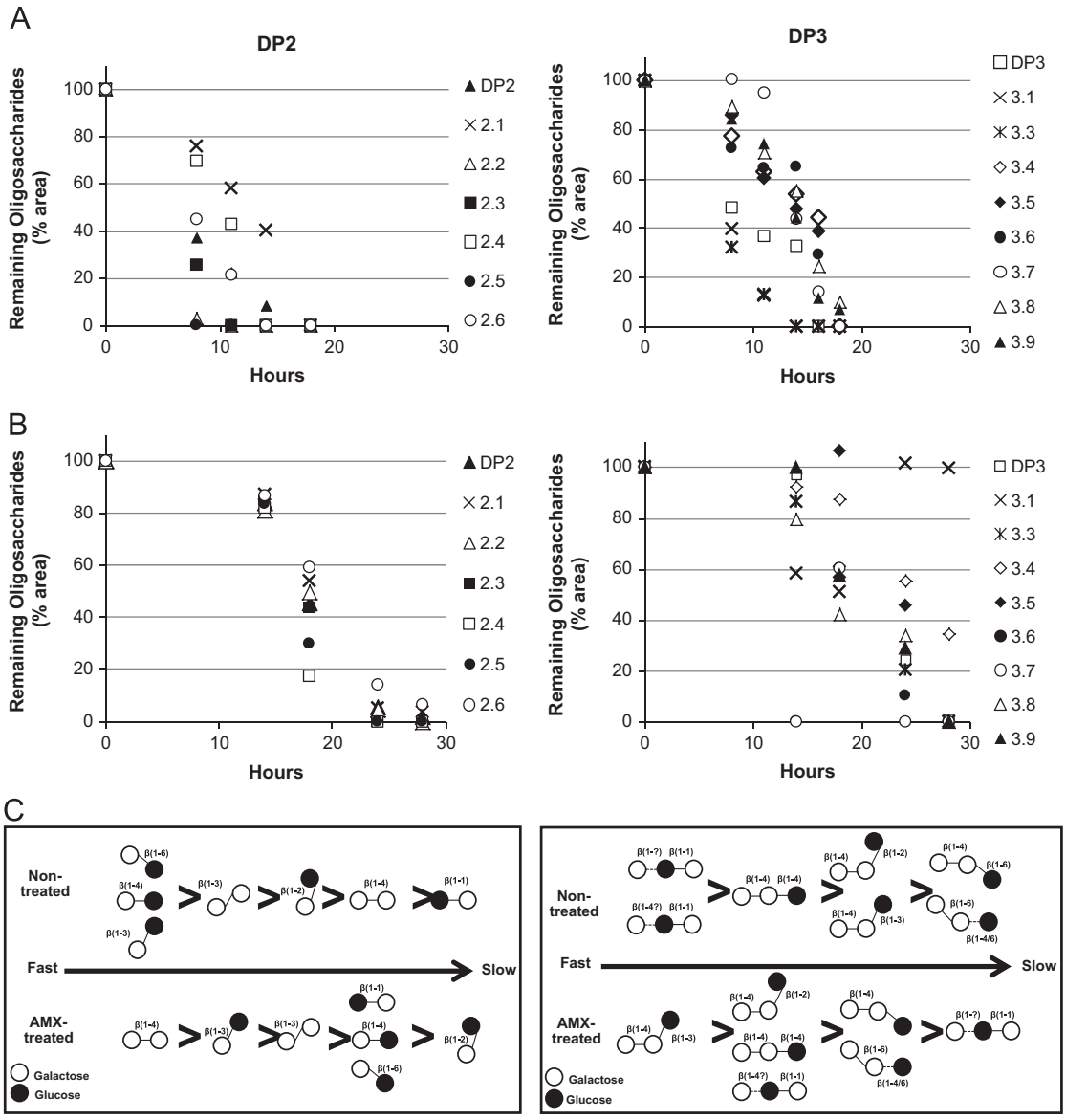


Fig. 3 – Proportion (%) of remaining oligosaccharides of DP2 and DP3 during *in-vitro* fermentation using a non-treated microbiota (A) and 1 μg mL^{-1} AMX-treated microbiota (B), and schematic representation of the preferred hydrolysed linkages (C). Identification of the isomeric structures in DP2 and DP3 fractions can be seen in Table 1.

NT-MB, whereas it was degraded first by AMX-MB. With respect to the DP3 fraction of GOS, one of the major differences concerned β-D-Gal-(1→4)-β-D-Gal-(1→3)-D-Glc that remained one of the last to be degraded by NT-MB whereas it was degraded first by AMX-MB.

The oligosaccharides with an α- or β[1–1] linkage in the DP2 fraction were difficult to be degraded by both NT-MB and AMX-MB. This result indicates that enzymes able to hydrolyse β[1–1] linkage were produced by certain bifidobacteria species (Van Laere, Abee, Schols, Beldman, & Voragen, 2000). These enzymes were, however, present in a low amount (van den Broek, Hinz, Beldman, Vincken, & Voragen, 2008) or had a low activity. In the DP3 fraction, the oligosaccharides with an α- or β[1–1] linkage were easily degraded by NT-MB, whereas some of them were last to be degraded by AMX-MB. In pure cultures of *Bifidobacterium* spp., previous literature reported that the [1–1] linkage feature might help the action of β-galactosidases (Cardelle-Cobas et al., 2011). An accumulation of the oligosaccharides of DP2 with α- or β(1–1) linkage was, however, observed in our study (data not shown). This observation suggests that only the β(1–4) linkage between the two galactoses next to the β (1–1) linkage was easily cleaved.

Overall, different DP fractions of GOS and different isomeric structure within a DP fraction were preferentially degraded depending on the treatment applied to the microbiota. The large oligosaccharides (DP4 to DP6) were preferentially degraded by AMX-MB with a preference for β-D-Gal-(1→4)-D-Gal and β-D-Gal-(1→4)-β-D-Gal-(1→3)-D-Glc in the DP2 and DP3 fractions respectively. The small oligosaccharides (DP2 and DP3) were preferentially degraded by NT-MB with a

preference for β-D-Gal-(1→4/6)-D-Glc and β-D-Gal-(1→4)-β-D-Gal-(1→4)-D-Glc in the DP2 and DP3 fractions respectively.

3.2.2. *Levels of organic acids*

The total level of organic acids (OA), being intermediate acids, such as lactate (L) and succinate (S), and SCFA, such as acetate (A), propionate (P) and butyrate (B), was increasing (Fig. 4) as the substrate was degraded (Fig. 3). In most samples, the maximum level of OA was approximately 10 to 14 μmol mg substrate^{-1}. For both NT-MB and AMX-MB, the level of OA upon the fermentation of oligosaccharides of DP2 and DP3 increased until 16 h and 18 h of fermentation, respectively, but subsequently decreased. Most likely, the OA were utilized by the microbiota as the substrate was depleted or not easily degradable.

At the maximum level of OA, the molar ratio for NT-MB (A:P:B:S:L=75:6:5:11:2) was similar for GOS and its individual DP fractions. One exception was that, for the DP2 fraction, more acetate and no propionate was detected. Overall, a small amount of lactate and succinate was detected for NT-MB. This is in line with previous results reporting that the fermentation of GOS results in the production of SCFA (Hernot et al., 2009; Hopkins & Macfarlane, 2003). Most likely the intermediate OA were converted by the healthy microbiota to SCFA, such as butyrate (Bourriaud et al., 2005). This is illustrative for the synergic action of the microbiota to degrade and use intermediate metabolic products is indicative of the good functioning of the gut microbiota.

The proportion of OA changed upon amoxicillin treatment. Succinate accumulated (about 3.5 times more than for NT-MB) while less acetate was produced (about 1.5 times less than for NT-MB).This indicates a disruption of the metabolic activity of the microbiota upon antibiotic treatment, as previously reported (Antunes et al., 2011; Woodmansey, McMurdo, Macfarlane, & Macfarlane, 2004). In addition, it can be noticed that the proportion of OA for AMX-MB was influenced by the DP: Butyrate was higher (around 5% present) upon addition of oligosaccharides of DP4 to DP6 after 36 h of fermentation for AMX-MB, while lactate was higher (around 7% present) upon addition of oligosaccharides of DP2. It is known that oligosaccharides of DP2 are easily incorporated into the bifidobacteria cell via specific transporters (van den Broek et al., 2008), which results in a high lactate and acetate production. The accumulation of lactate, therefore, indicates the disruption of the lactate-utilizing bacteria microbiota. The production of butyrate upon the addition of large DP fractions is an indication that the butyrate-producing bacteria recovered their activity. As bifidobacteria is expected to be specifically stimulated by the addition of GOS and as bifidobacteria do not produce this SCFA (Palframan, Gibson, & Rastall, 2003), the butyrate production occurred most likely through cross-feeding. An increase of the production of butyrate is of interest as this SCFA is the main energy source for epithelial cells and has been reported to have beneficial effect for human health, such as lowering the risk for colon cancer and chronic intestinal inflammations (Wong, De Souza, Kendall, Emam, & Jenkins, 2006).

Overall, the proportion of OA was more influenced by the antibiotic treatment than by the DP fractions of GOS present. Nevertheless, it was clear for AMX-MB that lactate was produced upon the addition of small DP fractions (DP2-DP3) and butyrate was produced upon the addition of larger DP fractions (DP4-DP6). Large DP fraction appear to be a potential substrate to stimulate the recovery of other butyrate-producing bacteria.

3.3. *Changes in the microbiota composition*

GOS is a prebiotic known to have a bifidogenic effect in *in vitro* and *in vivo* experiments (Maathuis, van den Heuvel, Schoterman, & Venema, 2012; Walton et al., 2012). As the degradation of GOS was observed and SCFA were produced, changes in the microbiota composition, especially in the level of bifidobacteria, were expected. In order to investigate whether different DP fractions contribute to a different extend to the recovery of the microbiota, the changes in the microbiota composition were studied by interpreting the microbiota fingerprints obtained with the I-chip and by quantifying the levels of total bacteria and *Bifidobacterium* spp.

3.3.1. *Microbiota fingerprinting using the I-chip*

The impact of GOS and its individual DP fractions on the overall microbiota composition of NT-MB and AB-MB was determined using the I-chip (Fig. 5). The impact was observed mainly within the bifidobacteria population for both NT-MB and AMX-MB. The growth of butyrate-producing bacteria, such as *Eubacterium*, *Roseburia* (Louis & Flint, 2009), was not detected in our experiments despite the observed production of butyrate. Most likely, the changes in relative abundance of the targeted species were not detected with the microarray due to the low amount of these species (<1% total bacteria) (Louis & Flint, 2009; Rajilić-Stojanović et al., 2009). Quantification by qPCR of these bacteria is recommended to conclude about the indirect effect of GOS on butyrate-producing bacteria.

For NT-MB, several *Bifidobacterium* spp. were stimulated, mainly *B. longum* and *B. adolescentis/angulatum* (Fig. 5). These species are quite common in the healthy adult microbiota (Hopkins & Macfarlane, 2002). The same *Bifidobacterium* spp. were stimulated by the different DP fractions of GOS. This finding contradicts previous studies reporting that specific DP fractions are preferentially degraded by specific *Bifidobacterium* spp (Barboza et al., 2009). However, those results were based on pure *Bifidobacterium* cultures. In a complex ecosystem as used in the present study, the different *Bifidobacterium* spp. present degrade synergically and simultaneously the substrate. Hence, no specific species is stimulated by a certain DP fraction for NT-MB.

The AMX treatment clearly disrupted the microbiota composition. The main bacterial groups that were affected constituted of *Enterobacteriaceae, Clostridium, Bifidobacterium and Lactobacilli*, as reported before (Ladirat et al., 2013a). Addition of GOS and specific DP fractions mainly stimulated the recovery of *Bifidobacterium* spp. The recovery of *Bifidobacterium* spp. within 48 h of fermentation was mainly due to the increase of *B. longum*. This species was shown not to be affected by an amoxicillin treatment in infants (Mangin, Suau, Gotteland, Brunser, & Pochart, 2010). *B. longum*, therefore, had a potential to use available substrate to grow in the absence of competitors.

For AMX-MB, the impact of the addition of GOS and its individual DP fractions was also observed on *Lactobacillus* and *Enterobacteriaceae*. The abundance of *Lactobacillus gasseri* moderately increased upon the addition of GOS and DP2 fraction

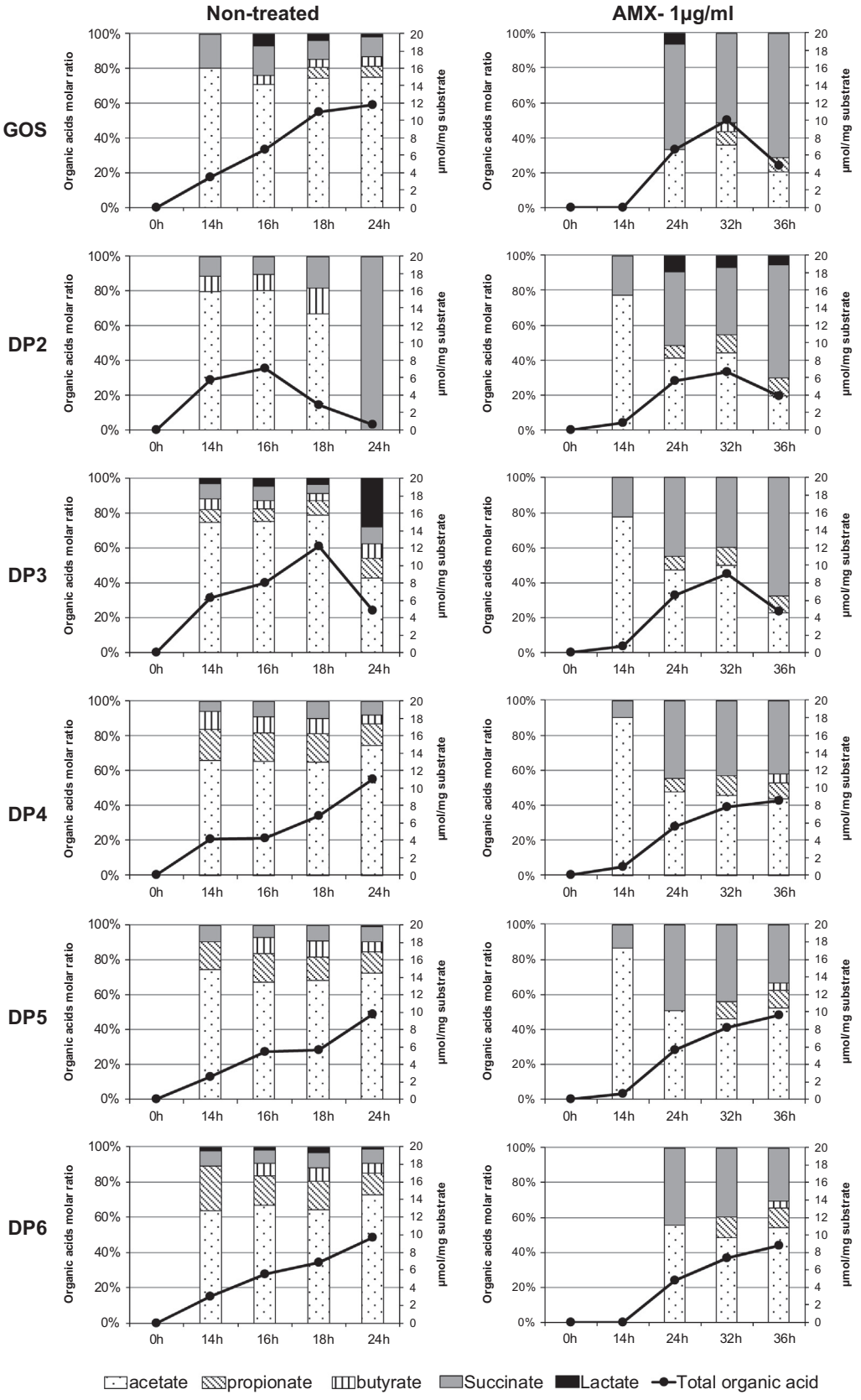


Fig. 4 – SCFA and organic acid levels during fermentation of GOS and DP fractions of GOS using a non-treated and AMX-treated microbiota (1 μg mL^{-1}).

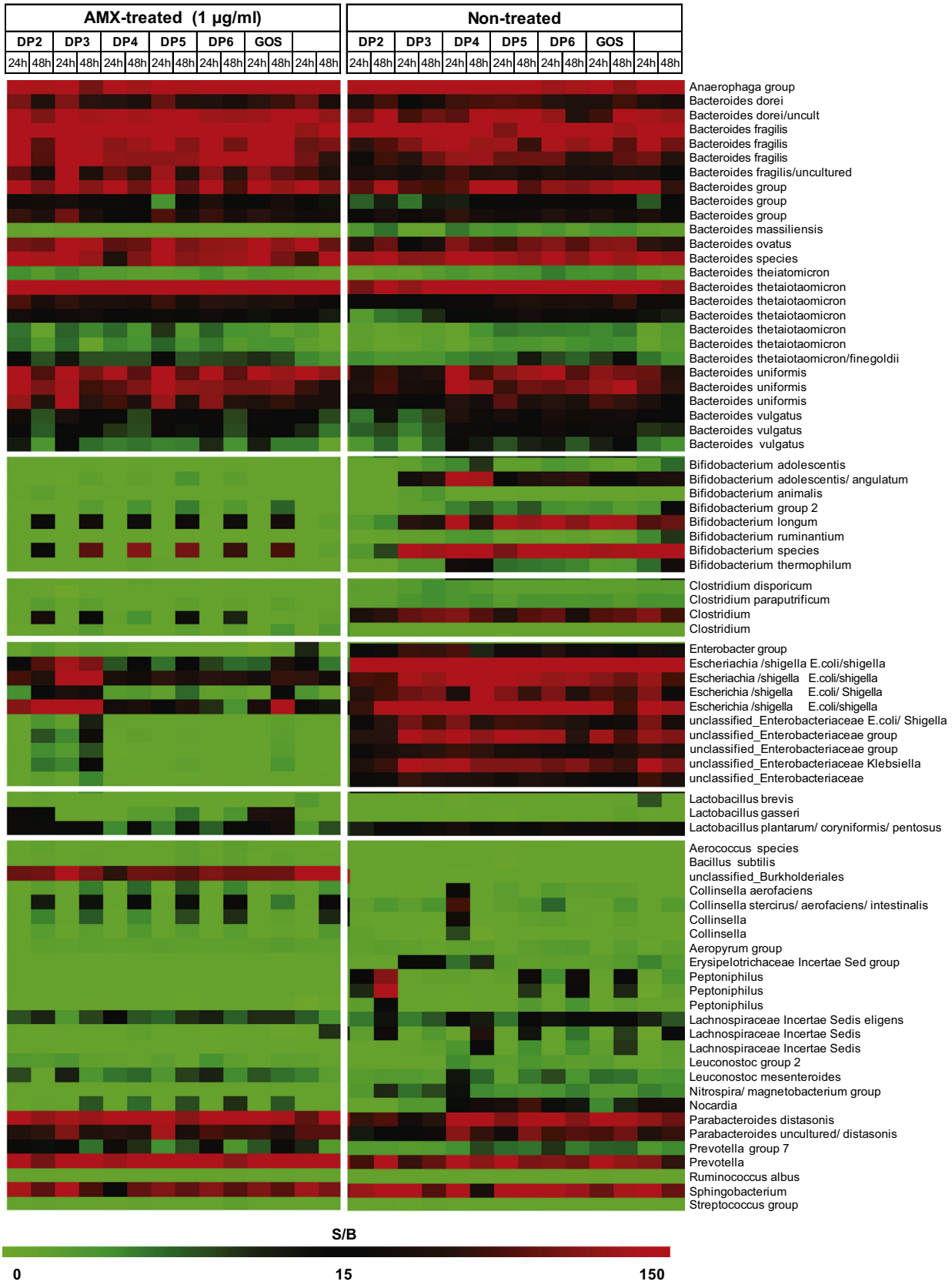


Fig. 5 – Bacterial fingerprints obtained with the I-chip revealing the impact of addition of GOS and DP fraction of GOS on non-treated and AMX-treated (1 µg mL^{-1}) microbiota from healthy adults. Signal compared to the background (S/B): green: below detectable level, black: medium abundance, red: high abundance. (For interpretation of the references to color in this figure legend, the reader is referred to the web version of this article.)

for AMX-MB as compared to NT-MB. The abundance of some of the *Enterobacteriaceae,* which were observed to decrease upon AMX treatment, remain equal to the non-treated groups upon addition of DP2 and DP3 fractions of GOS. The growth of *Lactobacillus* could be beneficial for the host as it is a reported beneficial group of bacteria (Gibson & Roberfroid, 1995). However, limiting the decrease of *Enterobacteriaceae* by AMX could be a risk for pathogen development and gut diseases (Gibson, McCartney, & Rastall, 2005). Limiting the decrease of *Enterobacteriaceae* upon addition of small DP fractions might be an indication that (1) these bacteria can utilize the substrate themselves, (2) they can cross-feed on the degradation products released by the bifidobacteria, (3) oligosaccharides that bind to the bacterial cell wall (Gibson et al., 2005) may limit AMX action. In future research, it would be of interest to investigate whether and which specific GOS oligosaccharides can bind to pathogens in more details.

3.3.2. *Total bacteria and Bifidobacterium spp. quantification*

For the NT-MB, the numbers of total bacteria and of *Bifidobacterium* spp. after 24 h of fermentation were $10^{9.1}$ and $10^{6.2}$ copies g faeces^{-1}, respectively (Table 2). Upon GOS addition, the numbers of total bacteria and of *Bifidobacterium* spp. after 24 h of fermentation increased of 0.9 $\log_{10}$ and of 3.7 $\log_{10}$, respectively, as compared to the control (no GOS added). These results confirm the bifidogenic effect of GOS observed in previous studies (Hernot et al., 2009; Maathuis et al., 2012). A similar increase was observed upon the addition of DP2 and DP3 fractions. An increase of the numbers of total bacteria and *Bifidobacterium spp*. was also observed upon the addition of DP4 to 6 fractions. The log values found were 0.5 to 0.7 log lower than the values found upon the addition of the original GOS. After 48 h fermentation, the difference in the number of *Bifidobacterium* spp. depending on the DP fractions added was still evident. These results are in line with the preferential degradation of small DP fractions by NT-MB as compared to large DP fractions. Since the degradation rate of GOS and the DP3 fraction were similar and the highest recovery of bifidobacteria was also found upon the addition of the DP3 fraction, the current mixture of GOS, mostly consists of oligosaccharides of DP3 (49% w/w), is, therefore, well adapted to support bifidobacteria growth in non-treated microbiota.

For AMX-MB, the number of total bacteria was $10^{7.6}$ copies g faeces^{-1}, so 1.5 $\log_{10}$ lower than in NT-MB due to the action of AMX during 24 h of fermentation. A recovery of the number of total bacteria was observed after 48 h of fermentation, reaching $10^{8.1}$ copies g faeces^{-1}, which was about 0.9 $\log_{10}$ lower than the number of total bacteria for NT-MB after the same time of fermentation. Addition of GOS and its individual DP fractions to the AMX-MB had no influence on the total number of bacteria during the fermentation time studied as compared to the control. With respect to the number of *Bifidobacterium* spp., the action of AMX during 24 h of fermentation decreased their number of 1.7 $\log_{10}$ as compared to NT-MB, reaching $10^{4.5}$ copies g faeces^{-1}. The addition of GOS to the AMX-MB induced an increase of 1.0 $\log_{10}$ in the level of *Bifidobacterium* spp. after 24 h of fermentation and of 1.6 $\log_{10}$ after 48 h fermentation as compared to the control. A similar increase was observed upon the addition of the individual DP fractions after 24 h of fermentation. After 48 h of fermentation, the increase of the number of *Bifidobacterium* spp. occurring upon the addition of DP2 and DP3 fractions was similar to that of occurring upon the addition of GOS. Interestingly, the increase of the number of *Bifidobacterium* spp. was 0.8 log and 0.6 log larger upon the addition of DP4 and DP5 fractions, respectively, than upon the addition of GOS. These results correlate with the preferred degradation of large oligosaccharides by AMX-MB. Furthermore, *B. longum* was shown to be the main bifidobacteria species to recover upon the addition of GOS and its DP fractions. *B. longum* has already been reported to grow on DP5 and DP6 in a monoculture (Barboza et al., 2009). Furthermore, *B. longum* is one of the few bifidobacteria species that

Table 2 – Numbers of total bacteria and *Bifidobacterium* ($\log_{10}$ copies g faeces^{-1}) during *in-vitro* fermentation using adult inoculum treated with 1 μg mL^{-1} (1) AMX with and without addition of GOS and DP fractions of GOS as measured with qPCR.

		Total bacteria		*Bifidobacteria*	
		Non-treated	AMX 1	Non-treated	AMX 1
No GOS	24 h	9.1	7.6	6.2	4.5
	48 h	9.0	8.1	6.5	5.9
GOS	24 h	10.0[a]	7.3	9.9[a]	5.5[a]
	48 h	9.1	8.3	9.5[a]	7.5[a]
DP2	24 h	10.3[a]	7.4	9.5[a]	5.4[a]
	48 h	9.4	8.3	9.2[a]	7.2[a]
DP3	24 h	9.8[a]	7.6	9.9[a]	5.5[a]
	48 h	9.1	8.5	9.4[a]	7.6[a]
DP4	24 h	9.3[b]	7.2	9.2[a,b]	5.3[a]
	48 h	9.4	8.2	8.9[a,b]	8.3[a,b]
DP5	24 h	9.5[b]	7.2	9.2[a,b]	5.1[a]
	48 h	9.0	8.5	9.0[a,b]	8.1[a,b]
DP6	24 h	9.5[b]	7.1	9.4[a,b]	5.5[a]
	48 h	9.1	8.3	8.6[a,b]	7.9[a]

[a] Difference larger than 0.5 $\log_{10}$ as compared to the values of 'no GOS' at their respective time.
[b] Difference larger than 0.5 $\log_{10}$ as compared to the values of 'GOS' at their respective time.

has a membrane bound endo-galactanase (van den Broek et al., 2008). The fast degradation of the large fractions of GOS for AMX-MB and the fast degradation of the specific type of linkages β-D-Gal-(1→4)-D-Gal suggest that this enzyme was active. This support the hypothesis that *B. longum* has a mechanism to incorporate galacto-oligosaccharides (van den Broek et al., 2008) and has most potential to grow in AMX-MB. Hence, we hypothesize that a faster recovery of *B. longum* upon AMX treatment would be possible upon addition of a GOS mixture enriched in oligosaccharides of DP4-DP5 and DP6 with a linear structure.

Overall, a growth of *Bifidobacterium* spp., mainly *B. longum*, occurred upon the addition of GOS and all its individual DP fractions to an amoxicillin-treated microbiota. The growth rate slightly higher upon addition of large DP fractions, but this has to be confirmed with statistical analysis. Nevertheless, the results on the changes in the microbiota composition corroborates the results on the metabolic activity, which indicates that the results are sound. The metabolic pattern of GOS changed upon a treatment of the gut microbiota with amoxicillin. The large DP fractions of GOS were degraded preferentially in amoxicillin-treated microbiota and induced production of the beneficial butyrate as compared to the small DP fractions. Hence, we conclude that in particular the presence of large oligosaccharides within GOS is responsible for their beneficial effect to counter the negative impact of amoxicillin on the microbiota. It is, therefore, suggested to investigate the effect of a GOS enriched with large oligosaccharides on an amoxicillin treated microbiota.

Acknowledgements

This project is jointly financed by the European Union, European Regional Development Fund and The Ministry of Economic Affairs, Agriculture and Innovation, Peaks in the Delta, the Municipality of Groningen, the Province of Groningen as well as the Dutch Carbohydrate Competence Center (CCC-WP2), and by FrieslandCampina. The authors thank Hakim Rahaoui for the technical support on the I-chip.

REFERENCES

Antunes, L. C. M., Han, J., Ferreira, R. B. R., Lolić, P., Borchers, C. H., & Finlay, B. B. (2011). Effect of antibiotic treatment on the intestinal metabolome. *Antimicrobial Agents and Chemotherapy*, 55, 1494–1503.

Barboza, M., Sela, D. A., Pirim, C., LoCascio, R. G., Freeman, S. L., German, J. B., et al. (2009). Glycoprofiling bifidobacterial consumption of galacto-oligosaccharides by mass spectrometry reveals strain-specific, preferential consumption of glycans. *Applied and Environmental Microbiology*, 75, 7319–7325.

Bourriaud, C., Robins, R. J., Martin, L., Kozlowski, F., Tenailleau, E., Cherbut, C., et al. (2005). Lactate is mainly fermented to butyrate by human intestinal microfloras but inter-individual variation is evident. *Journal of Applied Microbiology*, 99, 201–212.

Cardelle-Cobas, A., Corzo, N., Olano, A., Peláez, C., Requena, T., & Ávila, M. (2011). Galactooligosaccharides derived from lactose and lactulose: Influence of structure on Lactobacillus, Streptococcus and Bifidobacterium growth. *International Journal of Food Microbiology*, 149, 81–87.

Coulier, L., Timmermans, J., Richard, B., Van Den Dool, R., Haaksman, I., Klarenbeek, B., et al. (2009). In-depth characterization of prebiotic galactooligosaccharides by a combination of analytical techniques. *Journal of Agricultural and Food Chemistry*, 57, 8488–8495.

Crielaard, W., Zaura, E., Schuller, A. A., Huse, S. M., Montijn, R. C., & Keijser, B. J. F. (2011). Exploring the oral microbiota of children at various developmental stages of their dentition in the relation to their oral health. *BMC Med Genomics*, 4.

Gibson, G. R., & Roberfroid, M. B. (1995). Dietary modulation of the human colonic microbiota: Introducing the concept of prebiotics. *Journal of Nutrition*, 125, 1401–1412.

Gibson, G. R., Probert, H. M., Van Loo, J., Rastall, R. A., & Roberfroid, M. B. (2004). Dietary modulation of the human colonic microbiota: Updating the concept of prebiotics. *Nutrition Research Reviews*, 17, 259–275.

Gibson, G. R., McCartney, A. L., & Rastall, R. A. (2005). Prebiotics and resistance to gastrointestinal infections. *British Journal of Nutrition*, 93, S31–S34.

Hernot, D. C., Boileau, T. W., Bauer, L. L., Middelbos, I. S., Murphy, M. R., Swanson, K. S., et al. (2009). In vitro fermentation profiles, gas production rates, and microbiota modulation as affected by certain fructans, galactooligosaccharides, and polydextrose. *Journal of Agricultural and Food Chemistry*, 57, 1354–1361.

Hopkins, M. J., & Macfarlane, G. T. (2002). Changes in predominant bacterial populations in human faeces with age and with *Clostridium difficile* infection. *Journal of Medical Microbiology*, 51, 448–454.

Hopkins, M. J., & Macfarlane, G. T. (2003). Non-digestible oligosaccharides enhance bacterial colonization resistance against *Clostridium difficile* in-vitro. *Applied and Environmental Microbiology*, 69, 1920–1927.

Ladirat, S. E., Schols, H. A., Nauta, A., Schoterman, M. H. C., Keijser, B. J. F., Montijn, R. C., et al. (2013a). High-throughput analysis of the impact of antibiotics on the human intestinal microbiota composition. *Journal of Microbiological Methods*, 92, 387–397.

Ladirat, S. E., Schuren, F. H., Schoterman, M. H., Nauta, A., Gruppen, H., & Schols, H. A. (2013b). Impact of galacto-oligosaccharides on the gut microbiota composition and metabolic activity upon antibiotic treatment during in vitro fermentation. *FEMS Microbiology Ecology*

Louis, P., & Flint, H. J. (2009). Diversity, metabolism and microbial ecology of butyrate-producing bacteria from the human large intestine. *FEMS Microbiology Letters*, 294, 1–8.

Maathuis, A. J. H., van den Heuvel, E. G., Schoterman, M. H. C., & Venema, K. (2012). Galacto-oligosaccharides have prebiotic activity in a dynamic in vitro colon model using a 13C-labeling technique. *Journal of Nutrition*, 142, 1205–1212.

Macfarlane, G. T., Steed, H., & Macfarlane, S. (2008). Bacterial metabolism and health-related effects of galacto-oligosaccharides and other prebiotics. *Journal of Applied Microbiology*, 104, 305–344.

Mangin, I., Suau, A., Gotteland, M., Brunser, O., & Pochart, P. (2010). Amoxicillin treatment modifies the composition of Bifidobacterium species in infant intestinal microbiota. *Anaerobe*, 16, 433–438.

Marín-Manzano, M. C., Abecia, L., Hernández-Hernández, O., Sanz, M. L., Montilla, A., Olano, A., et al. (2013). Galacto-oligosaccharides derived from lactulose exert a selective stimulation on the growth of bifidobacterium animalis in the large intestine of growing rats. *Journal of Agricultural and Food Chemistry*, 61, 7560–7567.

McFarland, L. V. (2008). Antibiotic-associated diarrhea: Epidemiology, trends and treatment. *Future Microbiology*, *3*, 563–578.

Palframan, R. J., Gibson, G. R., & Rastall, R. A. (2003). Carbohydrate preferences of Bifidobacterium species isolated from the human gut. *Current Issues in Intestinal Microbiology*, *4*, 71–75.

Pierce, K. M., Sweeney, T., Callan, J. J., Byrne, C., McCarthy, P., & O'Doherty, J. V. (2006). The effect of inclusion of a high lactose supplement in finishing diets on nutrient digestibility, nitrogen excretion, volatile fatty acid concentrations and ammonia emission from boars. *Animal Feed Science and Technology*, *125*, 45–60.

Preidis, G. A., & Versalovic, J. (2009). Targeting the human microbiome with antibiotics, probiotics, and prebiotics: Gastroenterology enters the metagenomics era. *Gastroenterology*, *136*, 2015–2031.

Rajilić-Stojanović, M., Heilig, H. G. H. J., Molenaar, D., Kajander, K., Surakka, A., Smidt, H., et al. (2009). Development and application of the human intestinal tract chip, a phylogenetic microarray: Analysis of universally conserved phylotypes in the abundant microbiota of young and elderly adults. *Environmental Microbiology*, *11*, 1736–1751.

Rossi, M., Corradini, C., Amaretti, A., Nicolini, M., Pompei, A., Zanoni, S., et al. (2005). Fermentation of fructooligosaccharides and inulin by bifidobacteria: A comparative study of pure and fecal cultures. *Applied and Environmental Microbiology*, *71*, 6150–6158.

Saulnier, D. M., Kolida, S., & Gibson, G. R. (2009). Microbiology of the human intestinal tract and approaches for its dietary modulation. *Current Pharmaceutical Design*, *15*, 1403–1414.

van den Broek, L. A. M., Hinz, S. W. A., Beldman, G., Vincken, J.-P., & Voragen, A. G. J. (2008). Bifidobacterium carbohydrases-their role in breakdown and synthesis of (potential) prebiotics. *Molecular Nutrition & Food Research*, *52*, 146–163.

Van Laere, K. M. J., Abee, T., Schols, H. A., Beldman, G., & Voragen, A. G. J. (2000). Characterization of a novel β-galactosidase from Bifidobacterium adolescentis DSM 20083 active towards transgalactooligosaccharides. *Applied and Environmental Microbiology*, *66*, 1379–1384.

Walton, G. E., van den Heuvel, E. G., Kosters, M. H., Rastall, R. A., Tuohy, K. M., & Gibson, G. R. (2012). A randomised crossover study investigating the effects of galacto-oligosaccharides on the faecal microbiota in men and women over 50 years of age. *British Journal of Nutrition*, *107*, 1466–1475.

Wong, J. M. W., De Souza, R., Kendall, C. W. C., Emam, A., & Jenkins, D. J. A. (2006). Colonic health: Fermentation and short chain fatty acids. *Journal of Clinical Gastroenterology*, *40*, 235–243.

Woodmansey, E. J., McMurdo, M. E. T., Macfarlane, G. T., & Macfarlane, S. (2004). Comparison of compositions and metabolic activities of fecal microbiotas in young adults and in antibiotic-treated and non-antibiotic-treated elderly subjects. *Applied and Environmental Microbiology*, *70*, 6113–6122.