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In Vitro Fermentation of Porcine Milk Oligosaccharides and Galacto-oligosaccharides Using Piglet Fecal Inoculum

Elisabetta Difilippo,[†] Feipeng Pan,[†] Madelon Logtenberg,[†] Rianne (H.A.M.) Willems,[†] Saskia Braber,[§] Johanna Fink-Gremmels,[§] Henk A. Schols,[*,†] and Harry Gruppen[†]

[†]Laboratory of Food Chemistry, Wageningen University, Wageningen, The Netherlands

[§]Faculty of Veterinary Medicine, Institute for Risk Assessment Sciences, Subdivision of Veterinary Pharmacology, Pharmacotherapy, and Toxicology, Utrecht University, Utrecht, The Netherlands

ABSTRACT: In this study, the in vitro fermentation by piglet fecal inoculum of galacto-oligosaccharides (GOS) and porcine milk oligosaccharides (PMOs) was investigated to identify possible preferences for individual oligosaccharide structures by piglet microbiota. First, acidic PMOs and GOS with degrees of polymerization 4−7 were depleted within 12 h of fermentation, whereas fucosylated and phosphorylated PMOs were partially resistant to fermentation. GOS structures containing β1−3 and β1−2 linkages were preferably fermented over GOS containing β1−4 and β1−6 linkages. Upon in vitro fermentation, acetate and butyrate were produced as the main organic acids. GOS fermentation by piglet inoculum showed a unique fermentation pattern with respect to preference of GOS size and organic acids production.

KEYWORDS: *human fermentation, fibers, nondigestible carbohydrates, gas chromatography, liquid chromatography, short-chain fatty acids, sugars, organic acids*

INTRODUCTION

Milk is the first food source for infants, providing not only energy but also protection against pathogens.[1] The third largest class of compounds present in human milk is oligosaccharides (HMOs).[1] Numerous health-related effects of HMOs, involving direct interactions with pathogen and/or indirect effects upon their fermentation by the intestinal microbiota, have been reported.[2] When human milk is lacking during the early stage of an infant's life, cow's milk based infant formula is used.[3,4] However, cow's milk has a low content of milk oligosaccharides; therefore, in many cases infant formulas are fortified with prebiotics such as galacto-oligosaccharides (GOS).[5,6] Prebiotics are defined as nondigestible food ingredients that can influence the intestinal microbiota composition, resulting in beneficial effects for the host.[5,7,8] GOS are produced from lactose using β-galactosidases of microbial or yeast source.[9] The enzymatic reaction results in a GOS mixture containing oligosaccharides varying in degree of polymerization (DP), mostly from 2 to 8, and in types of glycosidic linkages present.[5] Commonly, GOS have a glucose monomer at their reducing end to which multiple galactose monomers with different linkages (β1−2, β1−3, β1−4, and β1−6) are attached.[10] GOS exhibit less structural complexity than HMOs. The major part GOS resist degradation in the upper intestinal tract, consequently reaching the colon, as is the case for HMOs.[8,11−13] In the colon, GOS enhance Bifidobacteria growth, potentially protecting the infant against infections and pathogen development.[2,14] Moreover, upon GOS fermentation, short-chain fatty acids (SCFAs) are produced, including butyrate.[15] Butyrate has been shown to be anti-inflammatory and anticarcinogenic, also enhancing (in vitro) the intestinal barrier function.[5,16,17] A recent study showed that in vivo GOS fermentation in young pigs (piglets) was extensive, not being conclusive on microbiota utilization toward individual GOS structures.[18] The understanding of the preference of microbiota toward specific oligosaccharide structures is important for the understanding of bacterial metabolization by the intestinal microbiota including enzyme activity involved and to target specific oligosaccharides to be more beneficial for the gut microbiota for animal health. In the present study, in vitro fermentation by piglet fecal inoculum was used to control different stages of fermentation, thereby focusing on the fate of individual oligosaccharides and organic acids produced. Oligosaccharides investigated were from porcine milk as present as primary feed source at the early stage of piglet life and GOS.

MATERIALS AND METHODS

Acetic acid, acetonitrile (ACN), ammonium formate, formic acid, chloroform and methanol were purchased from Biosolve BV (Valkenswaard, The Netherlands). Butyric acid, lactic acid, succinic acid, maltotriose, 2-(*N*-morpholino)ethanesulfonic acid, 2-ethylbutyric acid, sodium chloride, 2-picoline borane complex, and sodium hydroxide were purchased from Sigma-Aldrich (Steinheim, Germany). Propionic acid, oxalic acid, and 2-aminobenzamide were purchased from VWR International (Amsterdam, The Netherlands). β3′-, β4′-, and β6′-galactosyllactose were purchased from Carbosynth (Compton, UK), whereas 3′- and 6′-sialyl-*N*-acetylactosamine, 3′-fucosyllactose, lacto-*N*-(neo)tetraose, and lacto-*N*-(neo)hexaose were purchased from Dextra (Reading, UK). 3′- and 6′-sialyllactose and lacto-*N*-fucosylpentaose were purchased from Sigma-Aldrich. Water was filtered using a Milli-Q water purification system (Millipore, Darmstadt, Germany), which is referred to as "water" in the text. Vivinal GOS powder (FrieslandCampina Domo, Borculo, The Netherlands), dry matter (DM) 97%, with GOS 69%, lactose 23%,

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glucose and galactose together 5% on DM, is referred to in the text as "GOS". Compositions of GOS-DPs are 31% for GOS-DP2 (different from lactose), 38% for DP3, 18% for DP4, 8% for DP5, and 5% w/w for GOS-DP ≥5, as reported by the manufacturer.

Oligosaccharide Extraction and Separation. Porcine milk oligosaccharides (PMOs) from porcine colostrum (37 mL) (Pro-efaccommodatie de Tolakker, Utrecht University, Utrecht, The Netherlands) were extracted, freeze-dried, and dissolved in water (70 mg/mL), and subsequently separated using size exclusion chromatography (SEC) as reported elsewhere.[19] Collection of 103 fractions (7 mL each) was performed as described before.[19] In total, four runs were performed with an injection volume of 2.86 mL each injection. Afterward, the fractions were pooled and freeze-dried: pool 1, fraction 290−500 mL; pool 2, fraction 501−697 mL; pool 3, fraction 698−719 mL; pool 4, fraction 720−741 mL; pool 5, fraction 742−770 mL. The freeze-dried pools were dissolved in 36 mL of water. For each pool, 1 mL was used for oligosaccharide characterization, whereas the remaining volumes were mixed and freeze-dried again to obtain PMOs with a reduced amount of lactose and PMOs with DP2.

Fermentation of GOS and PMOs by Pig Fecal Inoculum. *Culture Medium.* The culture medium was based on a modified simulated ileal environment medium (SIEM), not containing carbohydrates prepared as reported elsewhere.[20] The pH was set at 6.0 using MES buffer. All of the culture medium components were provided by Tritium Microbiology (Veldhoven, The Netherlands).

Fecal Inoculum. Inoculum for the in vitro fermentation was obtained from fecal material from three Dutch Landrace piglets (age of 3 weeks). The piglets were fed porcine milk over the feeding period, combined with pig formula (de Heus, Ede, The Netherlands) from the second day to the eighth day of life. From the ninth day of life until the end of the 3 weeks the piglets received pellets (de Heus). Immediately after defecation, fecal material from each piglet was collected in eppendorf tubes, which were immediately put into an insulated box with crushed ice and afterward stored at −80 °C. The pig fecal inoculum was prepared as described elsewhere, with some minor modifications.[21] Feces (±100 mg) of three piglets were pooled after defrosting in an anaerobic cabinet (gas phase: 96% N_2 and 4% H_2). The pooled feces were diluted 6 times (w/v) with sterilized 0.9% (w/v) NaCl solution and subsequently homogenized using a vortex. The homogenization was facilitated by the addition of sterile glass beads. The fecal solution was added to the culture medium in a 20 mL flask at a 5:82 (v/v) ratio to obtain the final inoculum. Afterward, the bottle was closed with a rubber stopper and an aluminum cap to maintain anaerobic conditions. Next, the inoculum was kept overnight in an incubator shaker (Innova 40, New Brunswick Scientific, Nijmegen, The Netherlands) (39 °C, 100 rpm) to stabilize the bacterial population.

In Vitro Fermentation. GOS and PMOs, obtained as described above, were used as substrates for the fermentation by pig fecal inoculum. The in vitro fermentation experiment was performed as described elsewhere with minor modifications.[21] GOS or PMOs were added to the culture medium with a final substrate concentration of 11.1 mg/mL. The fecal inoculum was added to the solution containing substrate and medium in a 1:10 ratio (v/v) and a final volume of 10 mL for the fermentation flasks. Control samples consisting of culture medium without substrate were used to monitor the background fermentation. All procedures were performed in an anaerobic cabinet. Flasks were closed with rubber stoppers and aluminum caps, and they were put into an incubator shaker (Innova 40) (100 rpm, 39 °C). After 9, 10, 12, 24, and 48 h of fermentation, two volumes of 70 μL were taken from the fermentation bottles with a syringe and transferred to eppendorf tubes. To inactivate fecal enzymes, eppendorf tubes were put into a boiling water bath for 5 min and stored afterward at −20 °C.

Characterization and Quantification of GOS and PMOs. Samples after fermentation were cleaned prior to analysis by hydrophilic interaction liquid chromatography with mass spectrometry (HILIC-ESI-MS^n). The samples after fermentation were diluted 10 times in water, and 150 μL was placed into a polyvinylidene fluoride centrifugal filter unit (0.22 μm, Merck Millipore, Amsterdam, The Netherlands) and centrifuged (5 min, 20000*g*, 20 °C). Afterward, ACN was added to the filtrate in a ratio of 1:1 (v/v), and the samples were centrifuged (5 min, 20000*g*, 20 °C). Supernatants were analyzed on a Thermo Accela Ultra High Liquid Chromatography (UHPLC) system (Thermo Scientific, Waltham, MA, USA) as described elsewhere.[22] Briefly, the mobile phases used were A + water + 1% (v/v) acetonitrile (ACN), B = ACN, and C = 200 mM ammonium formate buffer (pH 4.5) with a flow rate of 300 μL/min. The elution profile was as follows: isocratic 85% B (0−1 min); 85−60% B (1−31 min); from 60 to 40% B (31−35 min); isocratic 40% B (35−36 min); from 40 to 85% B (36−36.1 min); isocratic 85% B (36.1−45 min). Mobile phase C was kept at 5% during the entire gradient, with autosampler and column oven temperatures of 15 and 35 °C, respectively.

To recognize GOS linkage type, HPAEC-PAD was used and the HPAEC profiles were compared with profiles as reported elsewhere.[10,15] Oligosaccharide standards were used for PMO characterization and quantification by HILIC-MS^n. Calibration curves of the corresponding milk oligosaccharide standards and GOS (0.002−0.5 mg/mL respectively) were used to quantify PMOs and GOS by mass spectrometry. Area and concentration of individual GOS-DP were obtained by selecting the mass range for each GOS-DP and taking into account the relative amount of a given GOS-DP in the total GOS sample. If no corresponding standards for neutral PMOs were available, GOS with a comparable DP were used for the calibration curve. The curves fitting the PMO standards showed a linear correlation with R^2 of 0.90−0.99, whereas the curves fitting the GOS-DPs showed a linear correlations with R^2 of 0.98−0.99.

Organic Acid Analysis. To quantify acetate, propionate, and butyrate produced during the fermentation, GC analysis was performed as described elsewhere with minor modifications.[15] Samples after fermentation were diluted 10 times with water. Aliquots (50 μL) and SCFA standards (0.01−3 mg/mL) were mixed with 0.15 M oxalic acid (50 μL). The solutions were kept for 30 min before the addition of 0.025 mg/mL 2-ethylbutyric acid as internal standard in water (143 μL). To analyze succinic and lactic acid, high-performance liquid chromatography with refractive index detection was performed as described elsewhere.[15] Briefly, a Dionex system (Ultimate 3000 HPLC) equipped with an Aminex HPX-87H column with a guard column (Bio-Rad, Hercules, CA, USA) was used, with a H_2SO_4 (5 mM) mobile phase with a flow rate of 0.6 mL/min at 65 °C.

RESULTS AND DISCUSSION

Fermentation of Porcine Milk Oligosaccharides. As a result of rapid colonic bacterial colonization, piglets can extensively ferment dietary fibers.[22−26] GOS and PMOs present in the diet of piglets are almost completely fermented in the colon, resulting in trace amounts of oligosaccharides present in the feces other than intact GOS or PMO structures.[18,22] Similarly as found for dietary fibers, piglets' intestinal fermentation of GOS is suggested to start in the small intestine and to continue extensively in the cecum and colon.[24,25] To study any preference and differentiation in rate of fermentation among GOS and PMO structures, in vitro fermentation was performed using pig fecal inocula. Prior to monitoring their degradation profiles, the main PMOs were identified and quantified by HILIC-MS^n.[19] Through single ion monitoring, in total 13 main PMOs representing about 77% of the total peak area were identified and monitored during the in vitro fermentation. Using previously gained knowledge,[22] PMOs identified were the neutral β3′- and β6′-galactosyllactose (β3′- and β6′-GL), lacto-*N*-neotetraose (LNnT), lacto-*N*-pentaose-I (LNP-I), lacto-*N*-neohexaose (LNnH), and 2′- and 3′-fucosyllactose (2′- and 3′-FL) and the acidic 3′- and 6′-sialyllactose (3′- and 6′-SL), 3′- and 6′-sialyl-*N*-acetyllactosamine (3′- and 6′-SLN), phosphorylated lactose (P+ lactose), and Neu5Ac(α2−6)GlcNAc(β1−3)Gal(β1−4)Glc. The degradation of individual PMOs during fermentation was indirectly

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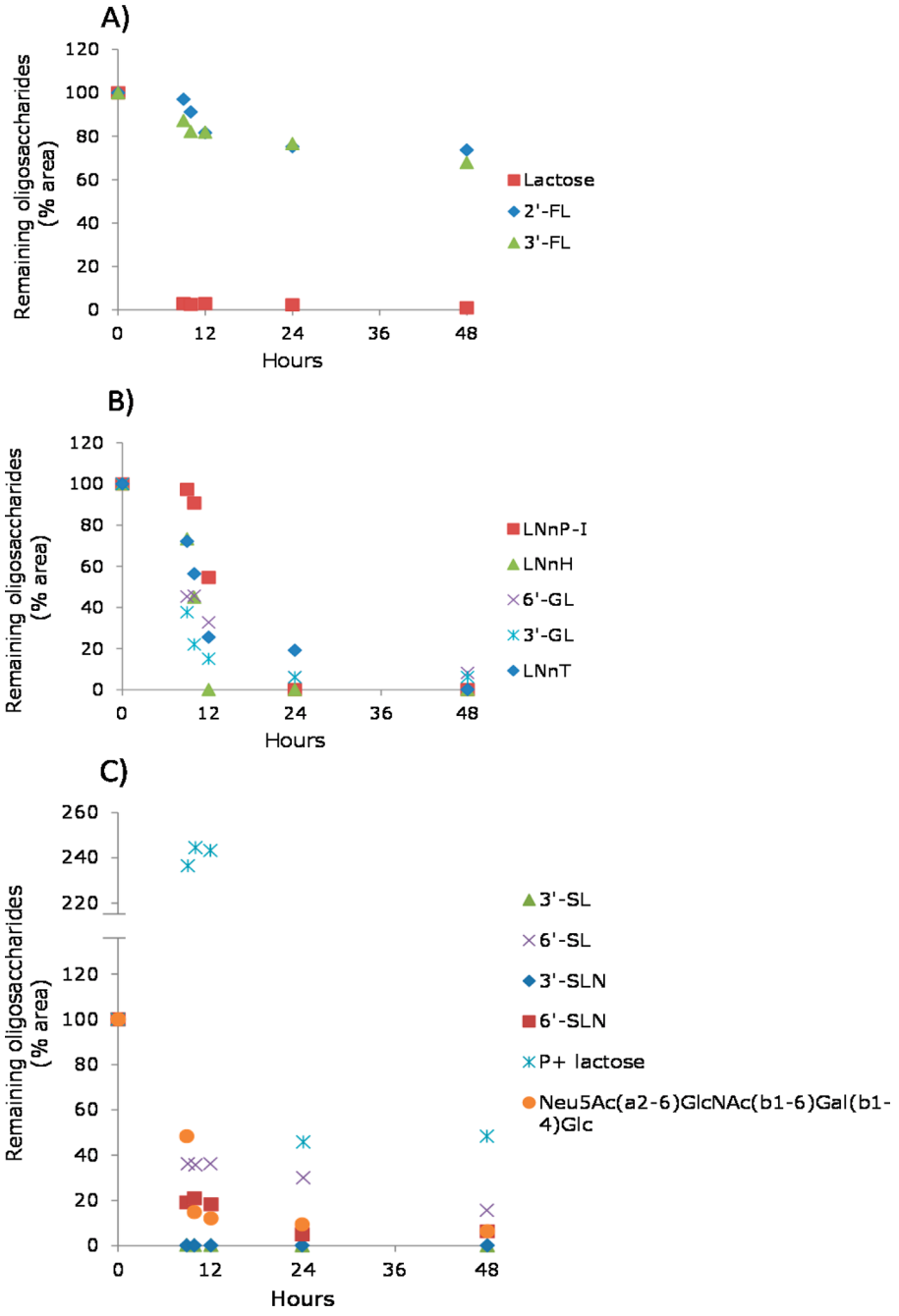

Figure 1. Proportion (%) of remaining PMOs during in vitro batch fermentation by piglet fecal inocula: (A) lactose and 2′- and 3′-fucosyllactose (2′- and 3′-FL); (B) β3′- and 6′-galactosyllactose (β3′- and β6′-GL), lacto-*N*-neotetraose (LNnT), lacto-*N*-pentaose-I (LNP-I), and lacto-*N*-neohexaose (LNnH); (C) 3′- and 6′-sialyllactose (3′- and 6′-SL), 3′- and 6′-sialyl-*N*-acetyllactosamine (3′- and 6′-SLN), phosphorylated lactose (P+ lactose), and Neu5Ac(α2−6)GlcNAc(β1−3)Gal(β1−4)Glc. Concentration per individual PMO at t = 0 was set to 100%.

expressed via the abundance of remaining oligosaccharides present (Figure 1). Overall, a decrease in concentration upon fermentation was observed for each PMO, with individual differences in degradation rate (Figure 1). One exception was phosphorylated lactose, which increased in concentration during the first 12 h of fermentation, but was partially depleted afterward (Figure 1C). The lactose present was attributed partially to the small amount of lactose still present after purification with SEC and partially to lactose released by bacterial degradation of PMOs. Lactose was the first carbohydrate to be fully fermented by the porcine microbiota, decreasing approximately 98% during the first 9 h of fermentation. Trimers, such as β3′-GL, β6′-GL, 2′-FL, and 3′-FL, were all fermented with different rates.

The trimers β3′-GL and β6′-GL diminished about 50% of their initial concentrations in the first 9 h of fermentation, whereby β3′-GL is more quickly fermented than β6′-GL (Figure 1B). LNnH is fermented more rapidly than LNnT and LNnP-I, decreasing 80% of its original concentration during 12 h of fermentation. All of the neutral PMOs are almost completely fermented within 24 h, whereas LNnT still was not consumed completely. Most probably, part of the LNnT present at 24 h is released by the degradation of larger PMOs.[27] LNnT is totally consumed in the subsequent 24 h by the piglet

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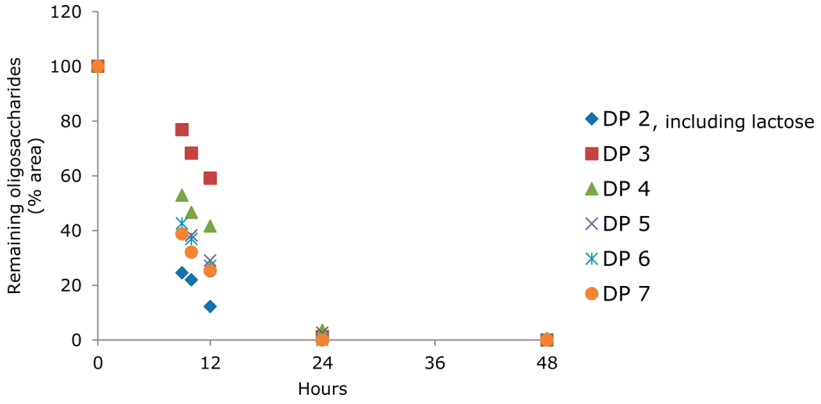

Figure 2. Proportion (area %) of remaining GOS during in vitro fermentation by piglet fecal inoculum as measured by HILIC-MS^n. Concentrations per PMOs at t = 0 were set to 100%. DP, degree of polymerization.

Peak	Compound
Glc/Gal	Glucose/Galactose
2.1	β-D-Gal-(1↔1)-α-D-Glc
	+ β-D-Gal-(1↔1)-β-D-Glc
2.2 (L)	β-D-Gal-(1→4)-D-Glc (Lactose)
2.3	β-D-Gal-(1→3)-D-Gal
2.4	β-D-Gal-(1→4)-D-Gal
2.5	β-D-Gal-(1→3)-D-Glc
2.6	β-D-Gal-(1→2)-D-Glc
3.4	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
3.5	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
3.6 + 3.7	β-D-Gal-(1→4)-β-D-Gal-(1→4)-D-Glc + β-D-Gal-(1→4)-β-D-Gal-(1→4)-Fru
3.8	β-D-Gal-(1→4)-β-D-Gal-(1→3)-D-Glc
3.9	β-D-Gal-(1→4)-β-D-Gal-(1→2)-D-Glc

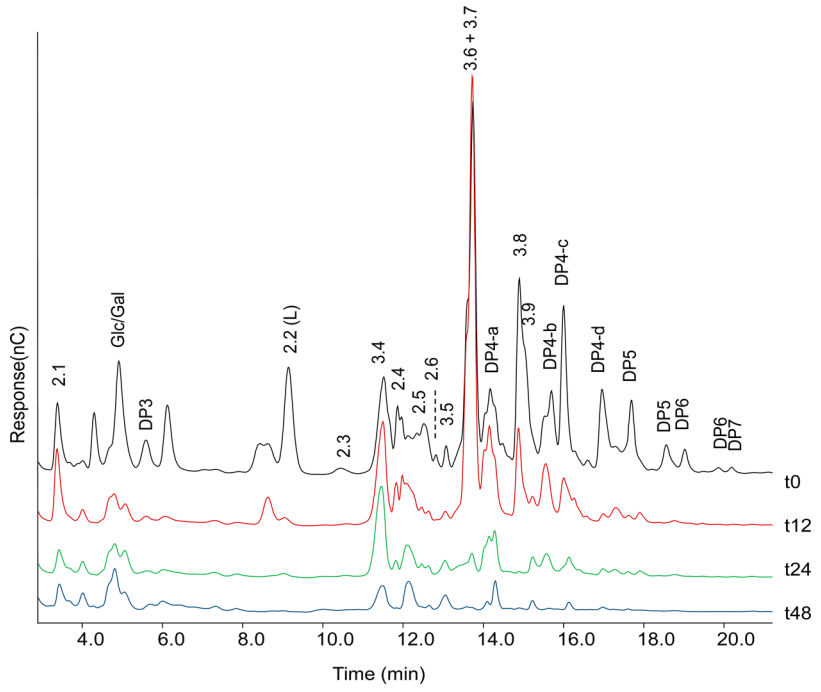

Figure 3. HPAEC-PAD chromatograms of GOS fermentation with piglet fecal inoculum, with structures of oligosaccharides as present in the GOS mixture (2.1−3.9) (according to Ladirat[30]). DP, degree of polymerization.

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colonic microbiota. Acidic PMOs are fermented for 50% during the first 12 h and are fermented more quickly than neutral PMOs (Figure 1C). Surprisingly, the P+ lactose more than doubled in concentration during the first 12 h of fermentation, with a subsequent decrease to about 50% of its starting concentration. Hypothetically, the increase of phosphorylated lactose could be derived from fermentation of larger phosphorylated PMOs present, although they were not reported so far to be present in domestic animal milk samples.[22,27] Overall, fermentation of acidic and neutral PMOs was almost complete in 48 h, with the exception of fucosylated PMOs and phosphorylated lactose. The resistance of fucosylated structures during the in vitro fermentation might be an indication of a relevant physiological role by preventing microbial pathogens for binding to intestinal cells in the piglets, as suggested for human studies.[28,29] In conclusion, in vitro fermentation with piglet microbiota showed a rapid consumption of PMOs, with different fermentation rates detected for individual PMOs. Piglet microbiota first ferment small PMOs, such as neutral and acidic trimers, and afterward neutral larger PMOs, each with its own specific rate.

Fermentation of Galacto-oligosaccharides. Although GOS are not naturally occurring in the piglet diet, the in vitro fermentation of GOS by piglet fecal microbiota showed that GOS is a quite easily fermentable substrate, as, on average, 36% of residual GOS was present after 12 h of fermentation. However, a difference in fermentation rate was noted concerning the different GOS-DPs, being present at 59, 42, 29, 27, and 25% for DP3, DP4, DP5, DP6, and DP7, respectively, after 12 h of fermentation. GOS-DP2 were fermented first, as expected from a previous investigation on GOS fermented by human fecal inoculum.[15] After 24 h of fermentation, almost all GOS were metabolized. Lactose as present in the PMO mixture was totally consumed within the first 12 h of fermentation (Figure 1A), whereas GOS-DP2, including lactose, was still present for about 12% after 12 h of fermentation. By comparing DP2 oligosaccharides in PMOs and GOS (at time point 12 h), it can be concluded that lactose was fermented much more rapidly than non-lactose GOS-DP2 structures. Lactose was introduced in the pig diet not only by nursing but also by the cow's milk based pig formula, presumably leading to adaptation of the pig microbiota to lactose. On the contrary, GOS-DP2 were not present at any phase of the pig diet. Hence, a slower adaptation of the microbiota than for lactose was expected. For GOS-DP3−7 (Figure 2), longer fermentation times compared with fermentation times found for dimers were observed (9−12 h), with different utilization rates depending on the DP. In the first 12 h of in vitro fermentation by piglet fecal inoculum, GOS-DP5−7 were mostly degraded (about 30% remaining oligosaccharides), whereas GOS-DP3 was still present in relatively high abundance (60% remaining oligosaccharides) (Figure 2). This was not observed during 12 h of in vitro fermentation of GOS by human fecal inoculum, when about 70% of remaining GOS-DP4−6 and about 50% of remaining GOS-DP3 were observed.[15] After 24 h of fermentation, all GOS-DPs showed to be fully fermented without DP discrimination. As expected from GOS fermentation by human fecal inoculum, also piglet fecal microbiota showed preference regarding specific GOS structures.[15] Using HPAEC-PAD analysis, not only the decrease during fermentation time of GOS-DP but also the decrease of individual GOS isomers was monitored. In Figure 3, GOS peaks are assigned with numbers as reported previously.[30] Isomeric GOS structures were degraded differently depending on their structures, as can be seen for lactose and for GOS-DP2 such as Gal(1−3)Gal, Gal(1−3)Glc, and Gal(1−2)Glc (Figure 3, numbers 2.3, 2.5, and 2.6, respectively). This observation was similar to that observed in a previous study, reporting that β1−2 and β1−3 linkages were more easily degradable than β1−4 linkages during GOS-DP2 fermentation by human fecal inoculum.[15] As expected from the HILIC-MS^n profile, GOS-DP3 were harder to degrade than GOS-DP2. The trimers indicated by numbers 3.8 and 3.9 were easier to ferment by pig inoculum than trimers 3.4, 3.5, 3.6, and 3.7 (Figure 3), as expected from GOS fermentation by human fecal inoculum.[30]

A relevant difference between piglet and human inocula was noted for Gal(β1−4)Gal(β1−4)Glc, and Gal(β1−4)Gal(β1−4)Fru (numbers 3.6 + 3.7, Figure 3), which accumulated with an increase of 17% during the first 12 h of fermentation by piglet fecal inocula, whereas they were easily fermentable substrates for the human microbiota.[15] A similar preference toward the degradation of specific linkages was noted when piglet and human in vitro fermentations were compared: β1−3/β1−2 linkages were preferred over β1−4/β1−6 linkages for GOS-DP2−3. The literature describes human and pig microbiota compositions to be similar, mainly consisting of Firmicutes and Bacteriodes.[31] On the other hand, differences were reported to occur in *Bifidobacterium* species, relevant for GOS degradation.[31] Studies reported extremely low amounts of *Bifidobacterium* species present in the gastrointestinal tracts of piglets (<1% of the total bacteria present).[31−33] Certain *Bifidobacterium* species present in piglet intestine could differ from the species reported in human intestinal tract.[31,34] Our results indicate a difference in microbiota composition based on the fermentation characteristics of oligosaccharides.

Organic Acid Production upon PMOs and GOS Fermentation by Piglet Fecal Inoculum. The degradation of PMOs and GOS by piglet fecal inoculum resulted in the production of organic acids: acetate (A), succinate (S), butyrate (B), lactate (L), and propionate (P) as shown in Figure 4. The molar ratios A:S:B:L:P observed after 24 h of fermentation were 49:1:20:2:27 and 45:1:21:4:29 for PMOs and GOS, respectively. Acetate was the most abundant organic acid detected after 24 h of PMO and GOS fermentation by piglet fecal inoculum. Similarly, acetate was the most abundant organic acid produced for GOS during in vitro fermentation by human fecal inoculum.[35] Next to acetate, also propionate and butyrate (Figure 4) were produced for both GOS and PMO fermentations by piglet fecal inoculum, whereas mainly succinate and propionate were coproduced for GOS fermentation by human fecal inoculum.[15,16] For 24 h of in vitro fermentation, GOS fermentation with piglet and human inoculum showed differences in A:S:B:L:P molar ratios: 45:1:21:4:29 and 75:10:6:2:8 for piglet and human inoculum, respectively.[15,16] Overall, after fermentation for 24 h, butyrate was produced in higher abundance upon GOS fermentation by piglet microbiota than by human microbiota.

The absolute amounts of butyrate were 1.4 and 0.7 μmol/mg substrate for GOS fermented by piglet and human inoculum, respectively.[15,16] During fermentation for 24 h by piglet inoculum, about 7 and 4 μmol of organic acids per milligram of substrate were produced for GOS and PMOs, respectively. Fermentation of GOS by human fecal inocula resulted in about 12 μmol/mg organic acids production in 24 h of fermentation.[15] Overall, in the intestinal tract, 10^{10-11} and

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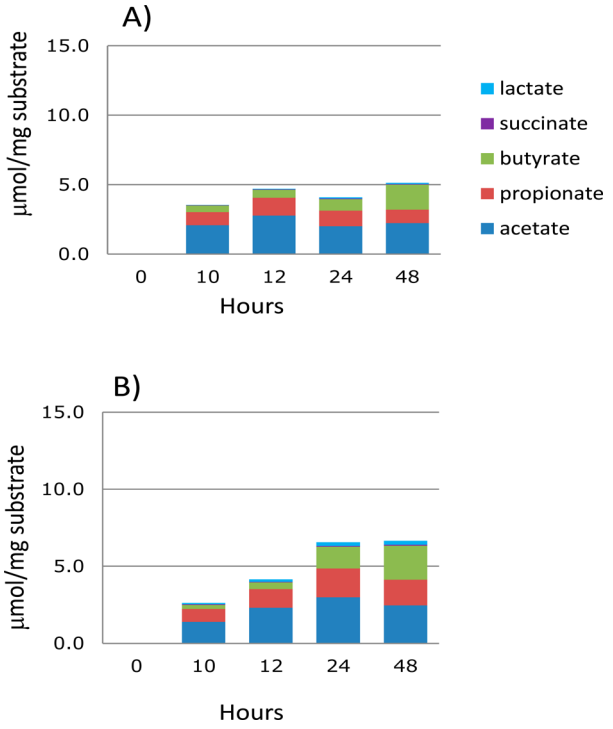

Figure 4. Organic acid amount and relative concentration during in vitro fermentation of GOS (A) and PMOs (B) by piglet fecal inoculum and of GOS by human fecal inocula.

10^{14} bacteria per gram of intestinal content for piglets and human are present, respectively.[31,36]

In summary, different from in vivo piglet fermentation, in vitro fermentation provides the opportunity to observe the preference of piglet microbiota on fermentation of individual oligosaccharides. During in vitro fermentation, acidic PMOs and neutral PMO trimers were the first to be degraded followed by larger neutral PMOs. Fucosylated and phosphorylated PMO were the most resistant to fermentation. Interestingly, whereas PMO trimers were quickly degraded, GOS trimers showed a slow apparent degradation rate for the first 12 h of fermentation. The GOS trimer degradation rate was hypothesized to be influenced not only by utilization by the microbiota but also by the release of trimeric degradation products upon fermentation of larger GOS. In addition, GOS containing β1−3 and β1−2 linkages were more quickly fermented than GOS containing β1−4 and β1−6 linkages. Major products upon PMO and GOS fermentation by piglet microbiota were acetate, propionate, and butyrate.

AUTHOR INFORMATION

Corresponding Author

*(H.A.S.) Phone: +31 317 482239. E-mail: henk.schols@wur.nl.

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Notes

The authors declare no competing financial interest.

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