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In vitro batch fermentation of (un)saturated homogalacturonan oligosaccharides

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ABSTRACT

Pectin, predominantly present within plant cell walls, is a dietary fiber that potentially induces distinct health effects depending on its molecular structure. Such structure-dependent health effects of pectin-derived galacturonic acid oligosaccharides (GalA-OS) are yet largely unknown. This study describes the influence of methyl-esterification and Δ4,5-unsaturation of GalA-OS through defined sets of GalA-OS made from pectin using defined pectinases, on the fermentability by individual fecal inocula. The metabolite production, OS utilization, quantity and size, methyl-esterification and saturation of remaining GalA-OS were monitored during the fermentation of GalA-OS. Fermentation of all GalA-OS predominantly induced the production of acetate, butyrate and propionate. Metabolization of unsaturated GalA-OS (uGalA-OS) significantly increased butyrate formation compared to saturated GalA-OS (satGalA-OS), while satGalA-OS significantly increased propionate formation. Absence of methyl-esters within GalA-OS improved substrate metabolization during the first 18 h of fermentation (99 %) compared to their esterified analogues (51 %). Furthermore, HPAEC and HILIC-LC-MS revealed accumulation of specific methyl-esterified GalA-OS, confirming that methyl-esterification delays fermentation. Fermentation of structurally distinct GalA-OS results in donor specific microbiota composition with uGalA-OS specifically stimulating the butyrate-producer *Clostridium Butyricum*. This study concludes that GalA-OS fermentation induces highly structure-dependent changes in the gut microbiota, further expanding their potential use as prebiotics.

1. Introduction

The plant cell wall is a complex matrix that holds reinforcing polysaccharides such as pectins, xylans, mannans and beta-glucans that have been successfully utilized as dietary fibers in the past (Gullón, Gullón, González-Munñoz, Alonso, & Parajó, 2014; Karnaouri, Matsakas, Krikigianni, Rova, & Christakopoulos, 2019; Vigsnæs, Holck, Meyer, & Licht, 2011). Pectin, the predominant polysaccharide found in the cell wall of fruit and vegetables can be extracted from side-streams of the agro-industry with an aim to valorize otherwise low value material and potentially contributing to improved sustainability and resource efficiency. Pectin is widely applied in food ingredient formulations for its texturizing properties (Voragen, Coenen, Verhoef, & Schols, 2009).

In recent years, pectin has attracted attention due to the high production volumes, its high structural complexity and the potential application of different pectin structures as potential bioactive biomolecules (Kumari et al., 2021). Pectin predominantly consists of homogalacturonan (HG) which (depending on the source) contributes to approximately 60 % of the weight of the pectin (Ropartz & Ralet, 2020) and consists of a chain of repeating α-1,4 linked galacturonic acid (GalA) residues. These GalA residues can be methyl-esterified at C-6 and/or acetylated at O-2 and/or O-3. A pectin of which more than half of the

Abbreviations: AcA, acetic acid; ACN, acetonitrile; BCFA, branched chain fatty acids; ButA, butyric acid; DA, degree of acetylation; DM, degree of methyl-esterification; DP, degree of polymerization; FOS, fructo-oligosaccharides; GalA-OS, galacturonic acid oligosaccharides; HILIC-LC-MS, hydrophilic interaction chromatography- liquid chromatography–mass spectrometry; HPAEC-PAD, high performance anion exchange chromatography – pulsed amperometric detection; MALDI-TOF-MS, matrix assisted light desorption ionization – time of flight – mass spectrometry; NME, non-methyl-esterified; PG, polygalacturonase; PL, pectin lyase; PME, pectin methyl-esterase; ProA, propionic acid; satGalA-OS, saturated galacturonic acid oligosaccharides; satGalA-OSNME, non-methyl-esterified saturated galacturonic acid oligosaccharides; SCFA, short chain fatty acids; SHIME, simulator of Human Intestinal Microbial Ecosystem; uGalA-OS, Δ4,5 unsaturated galacturonic acid oligosaccharides; uGalA-OSNME, non-methyl-esterified Δ4,5 unsaturated galacturonic acid oligosaccharides.

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GalA residues are methyl-esterified is known as a highly methyl-esterified (HM) pectin. Likewise, a pectin of which less than half of the GalA residues are methyl-esterified is known as a low methyl-esterified (LM) pectin.

The methyl-esterified GalA residues can be distributed over the pectin backbone in a repetitive (blocky) or random pattern across the galacturonic acid backbone (Galant, Widmer, Luzio, & Cameron, 2014). The level and distribution of methyl-esterified GalA residues are important markers that determine not only the physical properties of pectin (Lara-Espinoza, Carvajal-Millán, Balandrán-Quintana, López-Franco, & Rascón-Chu, 2018), but also the effectiveness of supplemented pectinolytic enzymes (Thomassen, Larsen, Mikkelsen, & Meyer, 2011), and the bioactivity of pectin (Beukema et al., 2021; Jermendi, Beukema, van den Berg, de Vos, & Schols, 2022). The level to which 100 mol of GalA are methyl-esterified is known as the degree of methyl-esterification (DM) (Voragen, Schols, & Pilnik, 1986).

Endo-acting enzymes like pectin lyase (EC 4.2.2.10; PL) and polygalacturonase (EC 3.2.1.15; PG) are commonly used to degrade the pectin polymer to GalA monomers and galacturonic acid oligosaccharides (GalA-OS) (Christiaens et al., 2016). PG and PL result in GalA-OS that are structurally unique. PG exerts typical glycoside hydrolase activity (Massa, Clausen, Stojan, Lamba, & Campa, 2007), producing oligomers of repeating α-1,4 linked galacturonic acid. Pectin lyase catalyzes the β-elimination reaction, resulting in a Δ4,5 unsaturated bond at the non-reducing end of the resulting unsaturated galacturonic acid oligosaccharides (uGalA-OS) (Bonnin, Ralet, Thibault, & Schols, 2009; Suberkropp, 2020). The applicability of PL and PG on a pectin depends on the methyl-ester substitution of the galacturonic acids (Di et al., 2017; Mandalari et al., 2007; Zeuner et al., 2020). Pectin lyase requires neighboring galacturonic acids to be methyl-esterified to enable the β-elimination reaction (van Alebeek, Christensen, Schols, Mikkelsen, & Voragen, 2002), while PG action requires neighboring GalA residues to be non-methyl-esterified (Yang, Yu, Liang, Anderson, & Cao, 2018). Consequently, when enzymatically treating pectin with either PL or PG, unsaturated galacturonic acids released because of PL action will always contain 2 more methyl-esters than saturated galacturonic acids (satGalA-OS) that are released due to PG action. This fact highlights that pectinases should be selected carefully to be able to release a range of differently methyl-esterified GalA-OS.

Lastly, pectin-methylesterase (PME) catalyzes the cleavage of the methyl-ester from C-6 of methyl-esterified GalA units. PME is often used in tandem with PG to increase the rate and level of pectin depolymerization. Naturally, because of the removal of methyl-ester groups, PME action antagonizes the efficacy of PL (Deytieux-Belleau, Vallet, Donèche, & Geny, 2008).

After ingestion, pectin and GalA-OS are not digested in the small intestine and arrive in the colon (Agarkova, Kruchinin, Glazunova, & Fedorova, 2019) and hence bear the potential to serve as fermentation substrates for specific gut microbes and in turn might confer health benefits to the host. Previously, reports have shown that the amount and the distribution of the methyl-esters (Jermendi et al., 2022; Olano-Martin, Gibson, & Rastell, 2002), in addition to the molecular weight of pectin (Sabater, Abad-García, Delgado-Fernández, Corzo, & Montilla, 2020; Vogt et al., 2016) affected various gut related health effects, such as being a bifidogenic factor and TLR-mediated activation of the immune system upon the supplementation of pectins. Fermentation of dietary fibers including pectin by gut microbiota reportedly yield a variety of metabolites including the short chain fatty acids (SCFA) butyrate, propionate and acetate (Manderson et al., 2005; Tan et al., 2014; Topping & Clifton, 2001; Vlasova, Kandasamy, Chattha, Rajashekara, & Saif, 2016). The production of SCFA out of a given dietary fiber intake varies between individuals, as the gut microbiota composition displays high variability from individual to individual. Tian and colleagues showed that, in pigs, lower methyl-ester substitution resulted in a more efficient fermentation in later segments of the colon. This suggests that the variability in microbiota composition across the large intestine affects fermentation pathways of pectin (Rinninella et al., 2019; Tian et al., 2017). In turn, pectin and pectin-derived oligosaccharides have been shown to influence gut microbiota composition. This was reported by Chung et al. (2017) and Gómez, Gullón, Yáñez, Schols, and Alonso (2016) who observed increases in populations of *Bifidobacteria*, *Eubacteriumeligens*, *Lactobacilli*, *Faecalibacterium* and *Roseburia* during fermentation of various pectin(-derived) substrates. The fine structure and substitution of pectins, differed when extracted from varying sources and affected gut microbiota composition differently. Additionally, direct and indirect inhibition of the growth of pathogenic bacteria (Dembélé, Obdrzálek, & Votava, 1998; Servin, 2004) can be observed due to the supplementation of dietary fiber in the diet. Dietary fibers that can selectively stimulate the growth of bacteria that are beneficial to health are known as prebiotics.

Although the structure-dependent health effects of pectin have been widely reported, the structure-function relationship of pectin-derived galacturonic acid oligosaccharides (GalA-OS) is yet largely unknown. This study aims to explore the structure function relationship of GalA-OS by investigating whether the methyl-esterification and the Δ4,5-unsaturated bond at the non reducing end of GalA affect the fermentation of GalA-OS. Commercial enzymes were utilized to create defined sets of saturated and unsaturated GalA-OS that were either methyl-esterified or non-methyl-esterified, which were subsequently used as substrates for *in vitro* fermentation. As fiber fermentation starts in the proximal colon, and quick fermentation may prevent fibers from reaching more distal parts of the colon, this study focused on using proximal colon conditions. The metabolite production, GalA-OS quantity, size, methyl-ester substitution and changes in bacterial composition were monitored during a 43 h *in vitro* batch fermentation of GalA-OS. It is hypothesized that methyl-esterification of GalA-OS will suppress *in vitro* fermentation in a quantity-dependent manner.

2. Materials & methods

2.1. Production of specific GalA-OS

Commercial fungal preparations of polygalacturonase and pectin lyase from *Aspergillus Niger* (PG_{asp} and PL_{asp}) were provided by Erbslöh (Erbslöh, Geisenheim, Germany). Two different lemon pectins with a degree of methyl-esterification (DM) of 30 (DM30P) and 70 (DM70P) were obtained from CP Kelco (CP Kelco ApS., Lille Skensved, Denmark). DM30P was treated with PG_{asp} (5.7U/mg) and DM70P was treated with PL_{asp} (3.2U/mg). Enzyme treatment of 5 mg/mL DM30P & DM70P was performed at 40 °C in 1000 mL of MilliQ water (MilliporeSigma, Burlington, MA, United States), set to pH 5 using 0.1 M HCL. Incubation was performed for 18 (PG_{asp}) and 24 (PL_{asp}) h with an enzyme protein concentration of 0.031 mg/mg and 0.064 mg/mg on substrate basis, respectively. Enzyme inactivation was performed at 100 °C for 10 min. Digests were lyophilized, solubilized in 70 % EtOH (20 mg/mL) at 4 °C overnight, filtered through a 0.45 μm filter to remove ethanol insoluble polymers, flushed with nitrogen to evaporate ethanol overnight and subsequently lyophilized. Saponification to remove methyl esters from the resulting satGalA-OS and uGalA-OS was performed overnight at 4 °C using 0.1 M NaOH (20 mg/mL) followed by neutralization to pH 7 with 0.1 M HCl and subsequent lyophilization. The DM of the satGalA-OS and uGalA-OS was measured with a head-space gas chromatography (GC) method as previously described by Huisman, Oosterveld, and Schols (2004).

2.2. MALDI TOF-MS of GalA-OS

To analyze size, methyl-esterification and unsaturation of GalA-OS, matrix assisted light desorption/ionization time of flight mass spectrometry (MALDI TOF-MS) was performed in positive mode (autoflex® maX MALDI system, Bruker Daltonics, Bremen, Germany). Before

transferring the enzyme digest to the target plate, 1 spatula tip of DOWEX anion resin was added to 100 µL of 10× Milli-Q diluted (0.5 mg/mL) GalA-OS to remove cations from the solution. On the target plate, 1 µL of 25 mg/mL 2,5-dihydroxybenzoic acid in 50:50 ACN:H20, 1 µL of centrifuged (21.100*g, 10 min) GalA-OS and 1 µL of 2 mM NaCl were combined on the spot. Laser intensity was kept between 37 and 43 % and the detected *m/z* range was kept between 300 and 2000 Da. Data analysis was performed using FlexAnalysis 3.4 (Bruker Daltonics).

2.3. In vitro batch fermentation of GalA-OS

In vitro batch fermentation of methyl-esterified and saponified satGalA-OS and uGalA-OS was performed in duplo for 43 h using microbiota grown in simulated proximal colon conditions using the Simulator of Human Intestinal Microbial Ecosystem (SHIME, ProDigest, Ghent, Belgium). In short, the SHIME was built as a triple SHIME having a stomach/intestine vessel (ST/SI), proximal colon microbiota vessel (PC), pH 5,6 and a distal colon microbiota vessel (DC), pH 6,6–6,9 in triplicate, with one set for each microbiota donor. The donors were healthy, European, non-smoking adults aged 26–48 with a BMI between 19 and 27 $kg\ m^{-2}$ that did not have any health complaints and did not use antibiotics for over 6 months prior to fecal collection. All donors signed for an informed consent. The three PC and three DC vessels including feed were inoculated with frozen (−80 °C) glycerol stocks from three different donors which were stabilized in the PC or DC vessels in the SHIME for two weeks in a previous experiment (Van de Wiele, Van den Abbeele, Ossieur, Possemiers, & Marzorati, 2015). The microbiota in the SHIME received a standardized feed (140 mL, pH 2.0) three times a day containing 1.2 g/L arabinogalactan, 2 g/L pectin, 0.5 g/L xylan, 0.4 g/L glucose, 3 g/L yeast extract, 1 g/L special peptone, 3 g/L mucin, 0.5 g/L L-cysteine-HCL and 4 g/L starch (ProDigest, Ghent, Belgium). This feed was pumped into the ST/SI vessel and after 1,5 h, 60 mL of pancreatic juice (12.5 g/L sodium bicarbonate (Sigma), 6 g/L Oxgall (BD Biosciences), 0.9 g/L pancreatin (Sigma)) was added. Subsequently, the resulting 200 mL was transferred to the PC after 1,5 h incubation in the ST/SI vessel. At the same time, 200 mL PC microbiota was transferred to the distal colon and 200 mL DC microbiota was transferred to the waste (Fig. 1). After inoculating the PC and DC vessels, the SHIME was run for 4 days to achieve a defined, stable microbiota composition, representative for the proximal colon, which were subsequently used for *in vitro* batch fermentation under in proximal colon conditions. The microbiota were fed 7 h before harvest where fecal solutions from PC were transferred to sterile, anaerobic 3 mL glass bottles to be used as inoculates in the anaerobic *in vitro* batch fermentation.

A medium consisting of 2 mg/mL special peptone, 0.5 mg/mL L-cysteine, 2 mg/mL Tween 80, 2 mg/mL yeast extract, 2 mg/mL $NaHCO_3$, 1 mg/mL mucin (Sigma-Aldrich, St. Louis, Missouri, United States) and 150 mM phosphate buffer at pH 5.8 was used to simulate the proximal colon and accommodate the corresponding microbial growth. The four different substrates of GalA-OS were dissolved in an electrolyte solution (12.2 mg/mL) as described by Brodkorb et al. (2019). The stabilized inoculates from the SHIME proximal colon compartments (Van de Wiele et al., 2015), the medium and the electrolyte solution that contained the GalA-OS were combined in a ratio of 10:61:29 respectively in a total volume of 3 mL to a final concentration of 5 mg/mL of GalA-OS. The fermentation was conducted anaerobically (4%H_2, 15%CO_2 and 81%N_2) in a shaking incubator at 37 °C, 120 rpm for a total of 43 h. Sampling (400 ul) took place at t = 0, 3, 6, 18, 36 and 43 h using a sterile syringe and needle. 300 µL was boiled in safe-lock Eppendorf tubes to avoid volatile fatty acid evaporation and inhibit bacterial growth as shown by Kong et al. (2021). The remaining 100 µL was frozen immediately in a separate 2 mL Eppendorf tube at −20 °C for microbiota analysis. Substrate controls without inoculum and inoculum controls without substrate were included as negative controls. Sensus fructo-oligosaccharides (FOS) (Sensus, Roosendaal, The Netherlands) were added in separate vials as a positive control for a successful fermentation.

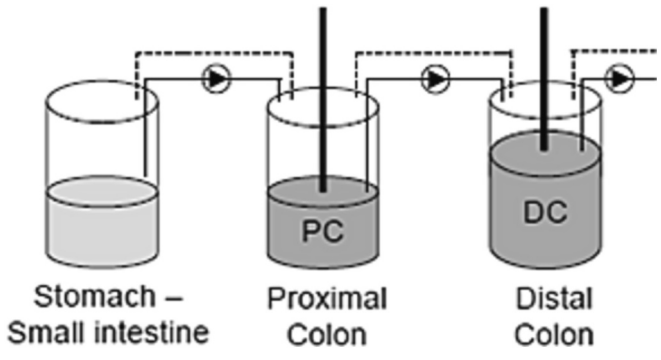

Fig. 1. SHIME (ProDigest, Ghent, Belgium) set up for one donor, three sets are built for three different donors. PC: Proximal Colon; DC: Distal Colon.

2.4. Organic acid analysis

High-performance liquid chromatography was performed according to an adapted method from Ladirat et al. (2014) to quantify short chain fatty acids (SCFAs), branched chain fatty acids (BCFAs) and organic acids (OA) throughout 43 h of fermentation on an Ultimate 3000 HPLC (Dionex, Sunnyvale, California, United States) equipped with an RI-101 refractive index detector (Shodex, Kawasaki, Japan), an autosampler and an ion-exclusion Aminex HPX – 87H column (7.8 × 300 mm) equipped with a guard column (Bio-Rad, Hercules, CA). The mobile phase consisted of 5 mM H_2SO_4 and the flow rate was 0.6 mL/min at 40 °C. Samples were centrifuged and subsequently diluted 1:1 with Milli-Q before the injection of 10 µL of sample. Standards of butyrate, succinate, lactate, acetate, propionate, isobutyrate and isovalerate (0 to 2 mg/mL) were used for quantification.

2.5. Monitoring substrate degradation using HPAEC

Degradation of GalA-OS during the 43 h fermentation period was analyzed by High Performance Anion Exchange Chromatography (HPAEC). Digests were centrifuged (5 min, 10.800*g, 20 °C) and subsequently diluted 10 times (0.5 mg/mL) in MQ. Ten µL of sample was injected to an ISC5000 HPAEC system with a CarboPac PA-1 column (250 mm × 2 mm ID), a CarboPac PA guard column (25 mm × 2 mm ID) and a ISC5000 ED detector in pulsed amperometric detector (PAD) mode (Thermo Fisher Scientific, Waltham, Massachusetts, United States). UV detection at 235 nm was used to distinguish between satGalA-OS and uGalA-OS. Mobile phase A (0.1 M NaOH) and B (1 M NaOAc in 0.1 M NaOH) were used with the following elution profile: 0–5 min, 0–20%B; 5–50 min, 20–65%B; 50–55 min, 65–100%B; 55–55.1 min, 100–0%B; 55.1–70 min, equilibration with 100%A. The flow rate was set to 0.3 mL/min. The data was analyzed using Chromeleon 7.3 (Thermo Fisher Scientific). Standards of galacturonic acid, digalacturonic acid and trigalacturonic acid were used in a range of 0 to 100 µg/mL and obtained from Sigma Aldrich (Sigma-Aldrich, St. Louis, Missouri, United States). All quantification of DP > 3 GalA-OS and unsaturated GalA-OS was performed using the calibration curve of DP3, due to a lack of purity of commercially available GalA DP > 3 standards and uGalA oligosaccharides.

2.6. Monitoring the methyl-esterification of GalA-OS using HILIC-LC-MS

Determination of the methyl-esterification of GalA-OS during the 43 h of fermentation was done according to a HILIC-LC-MS method established by Jermendi et al. (2022). Fermentation samples were analyzed using a Thermo UHPLC system in combination with electrospray ionization tandem mass spectrometry (UHPLC-ESI-IT-MS) on a Hydrophilic

Interaction Liquid Chromatography (HILIC) BEH amide column (1.7 μm, 2.1 × 150 mm). A heated ESI-IT ionized the separated oligomers from the fermentation broth in an LTQ Velos Pro Mass Spectrometer (UHPLC-ESI-IT-MS) coupled to the UHPLC. As pre-treatment, samples were centrifuged (15,000 ×*g*, 10 min, RT) and diluted (with 50 % (v/v) aqueous aceto-nitrile containing 0.1 % formic acid) to a final concentration of 1 mg/mL. The eluents used were (A) 100%ACN; (B) 99:1 % v/v water/acetonitrile (water/ACN), both containing 0.1 % formic acid with a flow rate of 400 μL/min. The following elution profile was used: 0.1 min isocratic 20%B 1–46 min, linear from 20 to 50%B; followed by column washing: 46–51 min, linear from 50 to 60 % B and column re-equilibration; 52–60 min isocratic 20 % B. The oven temperature was set at 40 °C. The injection volume was 1 μL. Mass spectra were acquired over a scan range of *m/z* 300–2000 in negative mode.

2.7. 16S rRNA sequencing of microbiota and statistical analysis

Bacterial genomic DNA was extracted from each pellet and the 16S rRNA gene sequencing and data analysis was performed as described previously by Dong, Ariëns, Tomassen, Wichers, and Govers (2020). Baseclear B.V. (Leiden, Zuid Holland, Netherlands) (L457; NEN-EN-ISO/IEC 17025) was supplied with 100 μL of frozen non-boiled fermentation solution extracted during the 43 h fermentation period, kept at −20 °C until being transported on dry ice. The microbial DNA was extracted and the V3-V4 region of the 16S rRNA gene was amplified and sequenced by using the MiSeq System (Illumina, San Diego, CA, USA). Subsequently, the raw paired-ends FASTQ files were trimmed and converted by bcl2fastq2 Conversion Software (Illumina). Analysis of the data resulting from these files was performed using CLC Genomics Workbench (Microbial Genomics toolbox version 22.0, Qiagen). 16S rRNA gene paired read sequences were used to prepare an operational taxonomic units (OTUs) table at 99 % reference of sequence similarity from Silva 16S/18S gene database (version 138.1). Statistical significance found in genomics data was evaluated using the Bonferroni *post hoc* test with a p-value of 0.05.

3. Results & discussion

3.1. Production of GalA-OS

PG_{asp} and PL_{asp} were used to de-polymerize industrially extracted DM30P and DM70P pectin into two populations that were named 'saturated galacturonic acid oligosaccharides' (satGalA-OS) and 'unsaturated galacturonic acid oligosaccharides' (uGalA-OS). The complete production of satGalA-OS and uGalA-OS resulted in 20 % and 68 % yields as based on substrate weight basis respectively (Table 1). This yield represents the amount of soluble GalA-OS obtained after precipitation of undigested polysaccharides using 70 % EtOH and subsequent filtration of insoluble material. This EtOH precipitation of undigested polysaccharides did result in substantial losses of DP6–10 satGalA-OS (73 %) compared to the DP6–10 uGalA-OS, as the satGalA-OS precipitated more readily together with the undigested polymer material. This is caused by the fact that highly charged, lower methyl-esterified galacturonic acid oligosaccharides such as the satGalA-OS (Degree of Methyl-esterification of 13) are more polar than their highly methyl-esterified uGalA-OS (Degree of Methyl-esterification of 54) counterparts, resulting in a higher solubility of DP > 5 uGalA-OS and lower solubility of the marginally methyl-esterified DP > 5 satGalA-OS in 70 % EtOH (Ibrahim, Ragab, Siliha, Labib, & El-Nemr, 2020). This resulted in a vastly increased yield of uGalA-OS (68 %) over satGalA-OS (20 %). The increased precipitation of DP6–10 satGalA-OS over DP 6–10 uGalA-OS resulted in a discrepancy in the contribution of high DP GalA-OS, with uGalA-OS consisting of 56 % DP ≥ 4 while satGalA-OS consisted of 35 % DP ≥ 4. The loss of a majority of the DP6–10 satGalA-OS was accepted due to the necessity of fully removing the undigested polymer (Supplementary Figs. S1 & S2). Purified satGalA-OS and uGalA-OS substrates would allow for accurate conclusions on the structure-function relation of low DP GalA-OS without the additional, undesired fermentation of undigested polymers.

Table 1
Galacturonic acid composition of satGalA-OS and uGalA-OS expressed as a percentage of the total weight of ethanol treated enzyme digest and yield as based on starting amount of pectin . DP: Degree of polymerization. satGalA-OS: DM30P, PG_{asp} treated saturated GalA-OS. uGalA-OS: DM70P, PL_{asp} treated unsaturated GalA-OS. S: contribution (%) of saturated GalA-OS towards total quantified uronic acid weight. U: contribution (%) of unsaturated GalA-OS towards total quantified uronic acid weight. DM: Degree of methyl-esterification.

Sample	Yield (w/w%)	DP1	DP2 + 3	≥DP4	S	U	DM
satGal-OS	20	24	41	35	100	0	13
uGal-OS	68	4	40	56	25	75	54

The satGalA-OS consist completely of saturated galacturonic acids, of which 24 % was attributed to galacturonic acid monomer due to an *exo*-PG impurity within the commercial PG_{asp} enzyme preparation (Fig. 2; Table 1). In contrast, the contribution of mono-galacturonic acid within uGalA-OS is substantially lower (4 %).

The uGalA-OS were dominated by 75 % unsaturated GalA-OS yet contained 25 % of saturated galacturonic acids because of the presence of a PG impurity within the commercial PL_{asp} enzyme preparation. Unfortunately, alternative incubation conditions did not reduce the concentration of saturated galacturonic acids within the uGalA-OS population to a significant degree without sacrificing a substantial amount of total yield. Additionally, separation of saturated and unsaturated galacturonic acids within mixtures of both was not feasible, which led to the acceptance that this composition of uGalA-OS was the best achievable, still allowing to draw conclusion on the fermentability of the various pectin-derived oligosaccharide structures.

MALDI-TOF MS (Fig. 3), confirmed the observation derived from HPAEC that the enzyme treatment successfully resulted in two separate populations of saturated and unsaturated galacturonic acid oligosaccharides. Additionally, methyl-esters were found to be present on both the satGalA-OS (3A) and uGalA-OS (3B). This confirms that the PL and PG functioned as expected. Indeed, the satGalA-OS showed a much lower methyl-esterification compared to uGalA-OS, with more than half of the satGalA-OS having <50 % of the GalA methyl-esterified and the uGalA-OS consisting mainly of OS of which >50 % were methyl-esterified. The presence of the number of methyl-esters that coincides with the theoretical minimum (PL) and maximum (PG) number of methyl-esters revealed that the commercial enzyme preparations contained no impactful PME impurities. Saponification using 0.1 M NaOH successfully removed the methyl-esters from a fraction of both satGalA-OS and uGalA-OS as can be seen in Fig. 3C and D, resulting in non-methyl-esterified fractions of satGalA-OS and uGalA-OS named satGalA-OSNME and uGalA-OSNME, respectively.

As mentioned earlier in Section 3.1, the uGalA-OS includes 25 % of saturated galacturonic acids. However, as observed in Fig. 3B, it is not possible to recognize any saturated galacturonic acids in the PL_{asp} treated material using MALDI-TOF-MS. Sodium has a higher affinity to form adducts with double bonded hydrocarbons as shown by Petrie (2001). This could reduce the ionization efficiency of saturated GalA-OS (Gass, Quintero, Hatvany, & Gallagher, 2022) and result in the observed absence of saturated galacturonic acids within the uGalA-OS material.

The analytical approach taken to inspect the composition and substitution of the different sets of enzymatically produced oligo-uronides resulted in desired mixtures of methyl-esterified GalA-OS. A controlled chemical saponification approach was employed to remove the methyl-esters from a portion of the two mixtures of GalA-OS. This saponification of a fraction of the uGalA-OS and satGalA-OS material resulted in a total of four mixtures of GalA-OS that were either saturated or unsaturated and methyl-esterified or non-methyl-esterified. These substrates were to be fermented *in vitro* with the goal of understanding the effect of methyl-

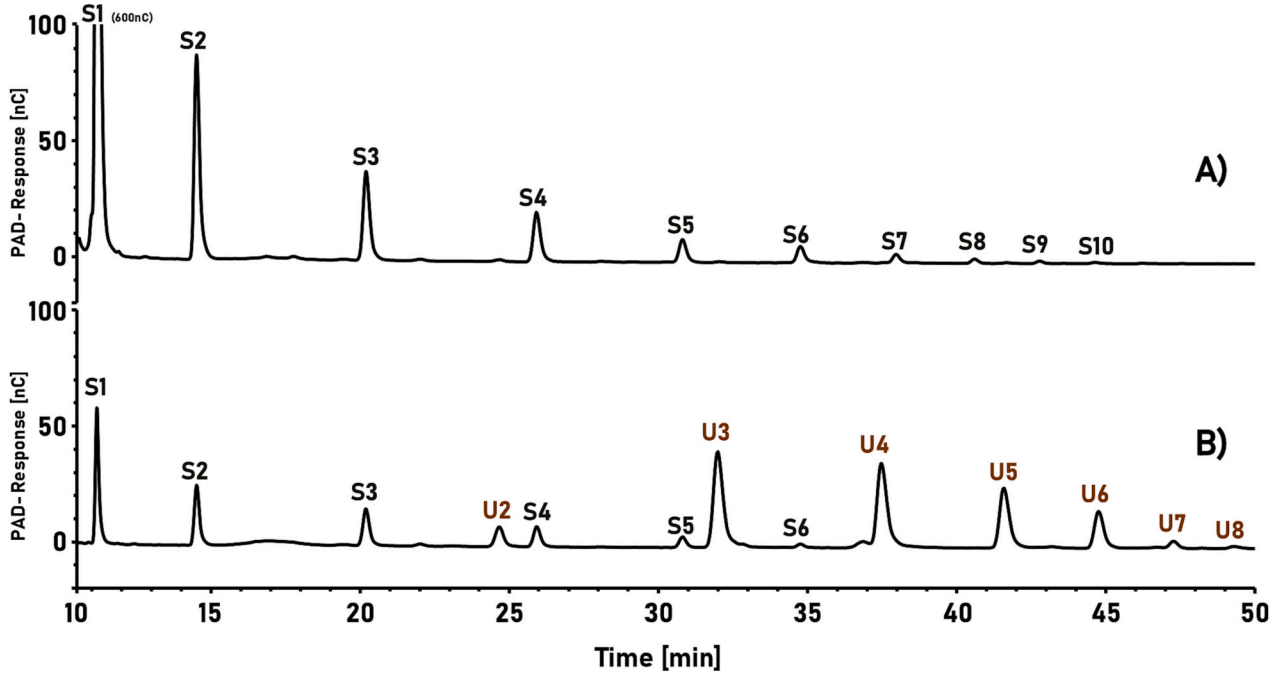

Fig. 2. HPAEC of digests that resulted in satGalA-OS (A) and uGalA-OS (B), measured after ethanol treatment, produced by the treatment of DM30P and DM70P with PG_{asp} and PL_{asp} respectively. S_n: Saturated galacturonic acid of DP = n, U_n: unsaturated galacturonic acid of DP = n.

esterification and Δ4,5-unsaturation on the fermentation of GalA-OS.

3.2. Methyl-esterification hinders fermentation of GalA-OS

The galacturonic acid substrates were subjected to 43 h of *in vitro* fermentation using inoculates that simulated the proximal colon microbiota of three healthy adult donors (Donor 1, 2 and 3). Samples were taken at t = 3,6,18,30 and 43 h. The positive control of FOS was metabolized completely before t = 18 h and already largely used at t = 6 h. FOS is known to be a readily and quickly fermentable dietary fiber as described before by Blanco-Pérez et al. (2021) and Gómez et al. (2016). As similar findings were obtained, it was confirmed that the inocula and fermentation conditions used were adequate.

To be able to follow the fate of the individual GalA-OS during the fermentation, HPAEC was performed. The *in vitro* fermentation of all four GalA-OS substrates (satGalA-OS, uGalA-OS, satGalA-OSNME & uGalA-OSNME) resulted in the reduction of the concentration of GalA-OS over time as measured through HPAEC (Fig. 4). Fermentation digesta of satGalA-OS and uGalA-OS taken at t = 3 h and t = 6 h did not show substantial GalA utilization. This indicated that the microbiota needed to adapt for a longer time than FOS before fermentation could start. Fig. 4 shows the galacturonic acid content within digests between t = 0 h and t = 18 h of methyl-esterified uGalA-OS by all donors, to clearly illustrate the difference between methyl-esterified and non-methyl-esterified fermentation of GalA-OS. Non-methyl-esterified GalA-OS were utilized much faster by the gut microbiota than the methyl-esterified GalA-OS, an effect which has been previously found for pectin polymers (Dongowski, Lorenz, & Proll, 2002) and galacturonic acid oligomers (Olano-Martin et al., 2002). This effect was more pronounced for uGalA-OS compared to satGalA-OS, which can be explained due to the higher density of methyl-esterification of uGalA-OS compared to satGalA-OS. Non-methyl-esterified uGalA-OS (uGalA-OSNME) of the same degree of polymerization (DP) as methyl-esterified uGalA-OS were largely utilized by all donors at t = 18 h (Fig. 4B). For donor 1, this utilization was quantified to account for 99 % substrate utilization of uGalA-OSNME, compared to 51 % substrate utilization of methyl-esterified uGalA-OS at t = 18 h of fermentation.

This preference for non-methyl-esterified GalA-OS was universal across the fermentations performed using the inoculates of all three donors. For donor 2, this resulted in a 94 % utilization of uGalA-OSNME compared to a 14 % utilization of methyl-esterified uGalA-OS at t = 18 h. Donor 3 utilized 0 % of the methyl-esterified uGalA-OS at t = 18 h, while utilizing 45 % of uGalA-OSNME at t = 18 h.

The saponified substrate of uGalA-OSNME contained less unsaturated dimers at t = 0 h compared to the methyl-esterified uGalA-OS, as can be seen from Fig. 4A & B. This reduction in concentration was a consequence of the saponification process and potentially caused by chemical peeling.

Concentrations of lower DP, methyl-esterified uGalA-OS (DP <4) increase during the first 18 h of fermentation, while inversely, concentrations of higher DP methyl-esterified uGalA-OS decrease in intensity. This indicated that the microbiota applied a de-polymerization mechanism to shorten the GalA-OS chain length to facilitate fermentation (Fig. 4A). Additionally, it was observed that the concentration of lower DP GalA-OS (1–3) decreased rapidly during the first 18 h for both low-, and non-methyl-esterified satGalA-OS (satGalA-OSNME) (Supplementary Fig. S4). Thus, (u)GalA-OS are de-polymerized to smaller DP GalA-OS when methyl-esterified. When methyl-esterification is (almost) absent, fermentation of smaller DP GalA-OS was prioritized.

MALDI-TOF-MS revealed an accumulation of smaller DP uGalA-OS up until t = 30 h of fermentation in addition to the fact that uGalA-OS of >DP6 were no longer detectable after 18 h, supporting this observed GalA-OS de-polymerization (Supplementary Fig. S3).

The difference between the utilization of methyl-esterified and non-methyl-esterified substrate is less clear for the fermentation of satGalA-OS (Supplementary Fig. S4). Fermentation of non-methyl-esterified satGalA-OS (satGalA-OSNME) by donor 1 only showed a slightly increased (96 %) substrate utilization over methyl-esterified satGalA-OS (90 %) during the first 18 h of fermentation. Hindrance of fermentation through the presence of methyl-esterification was more pronounced for the fermentation of satGalA-OS by both donors 2 and 3, with donor 2 showing an increased utilization of satGalA-OSNME (99 %) compared to methyl-esterified satGalA-OS (57 %). This effect was less pronounced, although still clear for slow fermenter donor 3 (57 % and 40 % respectively). Methyl-esterified satGalA-OS were utilized more readily by the three donors than methyl-esterified uGalA-OS. This can be

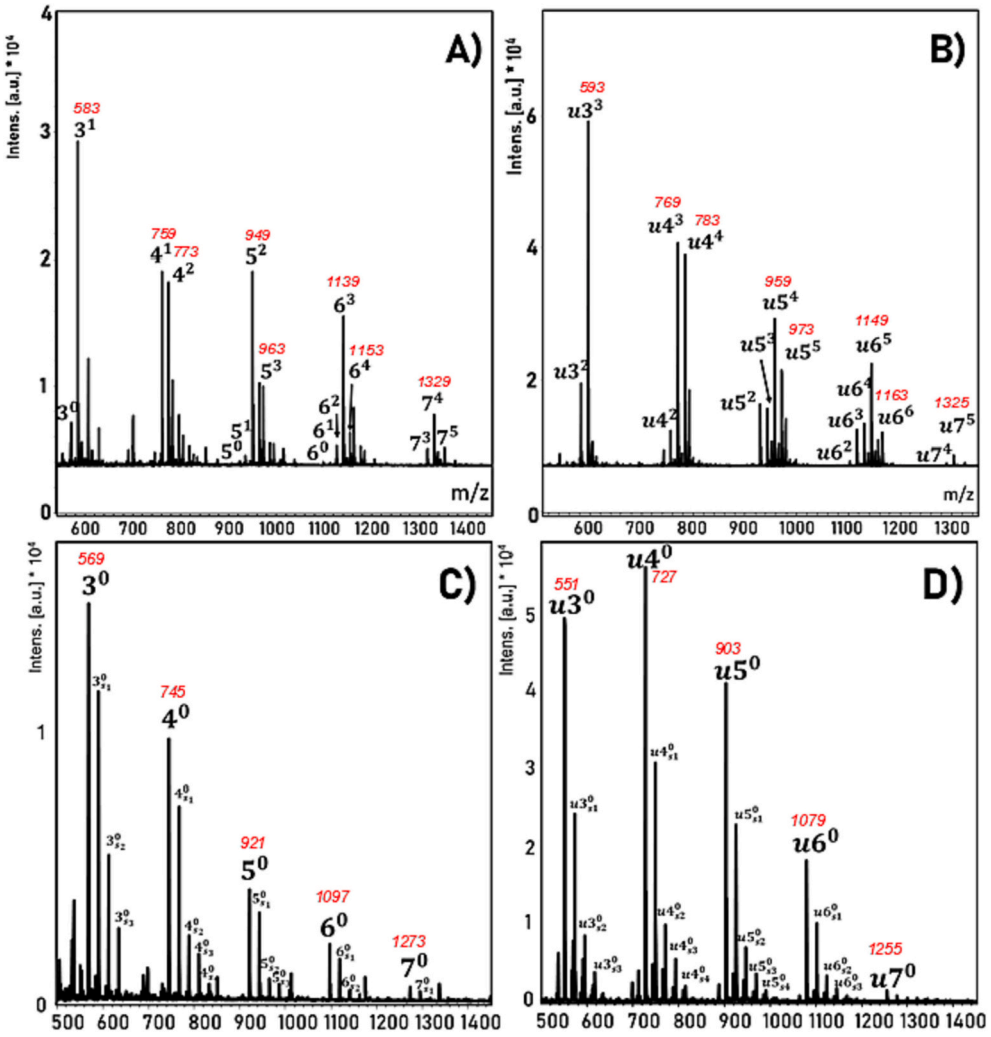

Fig. 3. MALDI TOF mass spectra of methyl-esterified satGalA-OS (A) & uGalA-OS (B) and non-methyl-esterified satGalA-OS (satGalA-OSNME) (C) & uGalA-OS (uGalA-OSNME) (D) within a mass range of 500–1450 Da. 3^1: saturated GalA trimer having one methyl-ester; $u5^3$, unsaturated GalA pentamer having 3 methyl-esters. The S_1 in the subscript of annotated GalA-OS represent the amount of sodium salts in panels C and D. Italic numbers represent the *m/z* value of the major ions.

explained by the observation that the methyl-esterified satGalA-OS inherently have less methyl-ester substitution due to mechanism of action of the polygalacturonase used to create satGalA-OS that necessitates neighboring GalA units to be non-methyl-esterified (Wakabayashi, Hoson, & Huber, 2003).

The observed variance in utilization of GalA-OS by the three individual inoculates led to the hypothesis that the fermentation of GalA-OS might depend heavily on inter-individual differences in gut microbiota composition. Different gut microbiota might have different tolerance towards methyl-esterification, potentially through the production of different levels and types of pectin-degrading enzymes which would alter the efficiency of the utilization of GalA-OS.

3.3. Gut microbiota struggle to utilize highly methyl-esterified GalA-OS

HILIC-LC-MS was performed on the two fermented, methyl-esterified uGalA-OS and satGalA-OS substrates in order to understand how the methyl-esterification of GalA-OS changes during their fermentation. It was confirmed that with the current HILIC-LC-MS method, no pre-treatment of centrifuged fermentation sample was required to accurately measure the methyl-esterification and determine the degree of polymerization of a mixture of GalA-OS after *in vitro* fermentation (Fig. 5).

Higher methyl-esterified low DP uGalA-OS ($u4^3$&$u4^2$) remain after 30 h of *in vitro* fermentation by the fecal inoculate of donor 1 (Figs. 5 & 6). In contrast, the uGalA-OS that have a relatively lower methyl-esterification ($u4^0$&$u4^1$) disappear from the spectrum, indicating that they are able to be utilized or broken down into smaller oligosaccharides by the microbiota. Identical behavior was observed for the fermentation of GalA-OS by the inoculates of both donor 2 and 3 (Supplementary Figs. S5 & S6). That highly methyl-esterified GalA-OS remain after fermentation coincides with the finding that the gut microbiota of all donors had more difficulty fermenting the highly methyl-esterified uGalA-OS compared to the less methyl-esterified satGalA-OS.

Abbott & Boraston reported in 2008 that methyl-esterification of galacturonic acid could hinders cellular transport in *Enterobacteriaceae*. If methyl-esterified GalA-OS indeed were not able to be taken up by the gut microbiota and therefore not broken down, it could potentially explain why the methyl-esterified GalA-OS remain in solution after extended fermentation. It is possible that this accumulation of highly methyl-esterified GalA-OS is due to a lack of effective PME production during fermentation.

When considering the methyl esterification of GalA-OS in more detail by HILIC-LC-MS, it was found that the heat treatment at 100 °C for 10 min of the digesta in the fermentation medium containing ~90 mM phosphate buffer to inactivate the bacteria caused some minor de-

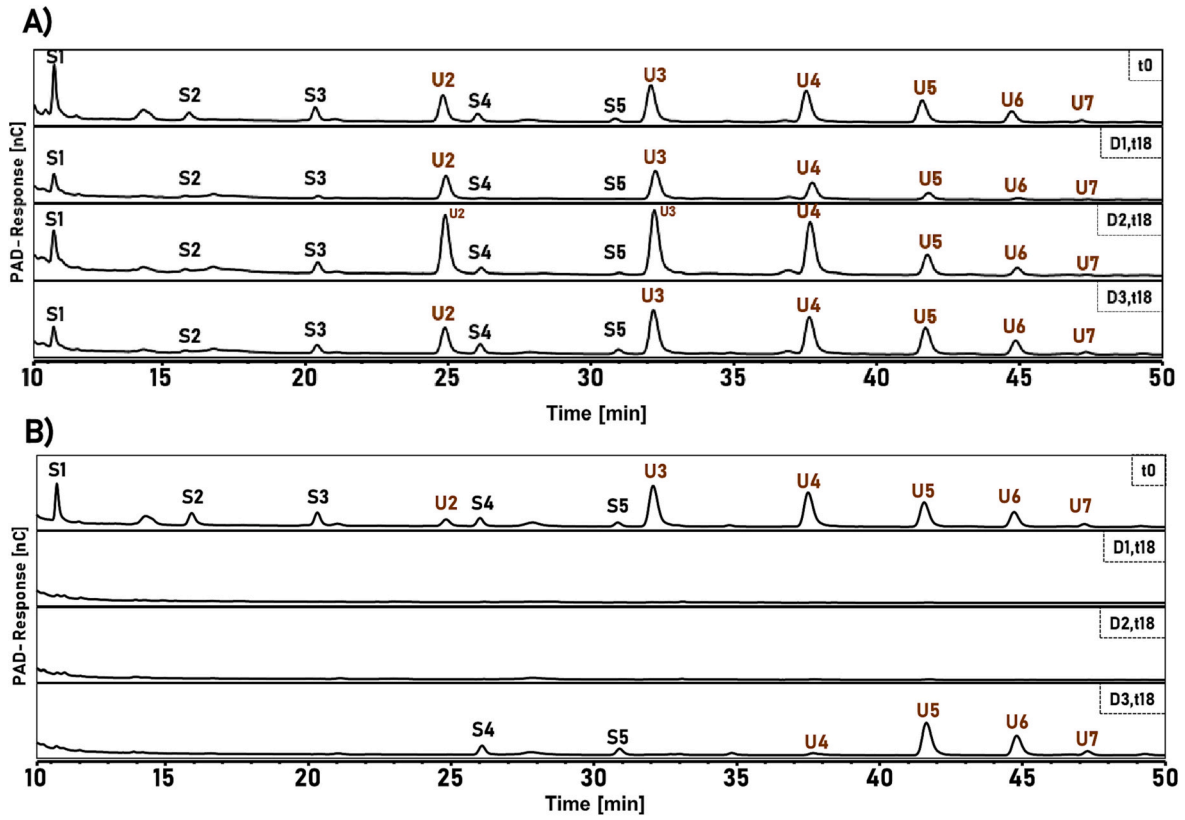

Fig. 4. HPAEC elution pattern of methyl-esterified (A) uGalA-OS and non-methyl-esterified (B) uGalA-OSNME after t = 0 h and t = 18 h of *in vitro* fermentation by fecal inoculates of human donor 1 (D1), 2 (D2) and 3 (D3). S_n: Saturated oligosaccharide of DP = n, U_n: Δ4,5 Unsaturated oligosaccharide of DP = n.

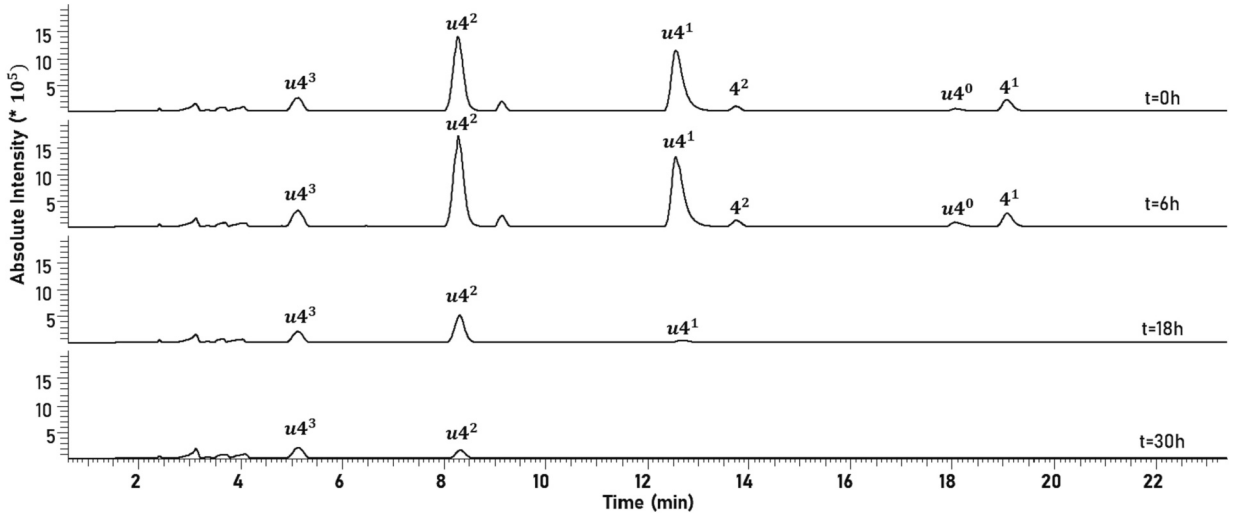

Fig. 5. HILIC-LC-MS base peak elution pattern of DP 4 uGalA-OS, simulated to only show masses from *m/z* 700–800. The chromatograms shown represent t = 0 h to t = 30 h of in vitro fermentation of methyl-esterified uGalA-OS by donor 1, measured after microbial inactivation. Peak annotation: $u4^2$, unsaturated DP4 GalA oligosaccharide having two methyl esters; 4^2, saturated DP4 GalA oligosaccharide having 2 methyl-esters. The overall elution pattern is shown in Fig. 6.

methylesterification (Fig. 6). This de-methylesterification was observed when comparing the methyl esterified uGalA-OS before fermentation (Unsat_ME_Blk) with uGalA-OS at t = 0 h but did not influence interpretation and explanation of the results. Such de-methyl-esterification due to phosphate was reported before by van Alebeek, Zabotina, Beldman, Schols, and Voragen (2000).

3.4. Δ4,5 unsaturation of GalA-OS affects SCFA formation during fermentation

The formation of organic acids, branched chain fatty acids (BCFAs) and short chain fatty acids (SCFAs) was monitored over the 43 h fermentation period to report on the metabolite production during the fermentation of all four substrates of GalA-OS *in vitro*. In general, the fermentation of GalA-OS resulted in the production of acid metabolites, of which the majority was attributed to acetate (AcA), butyrate (ButA)

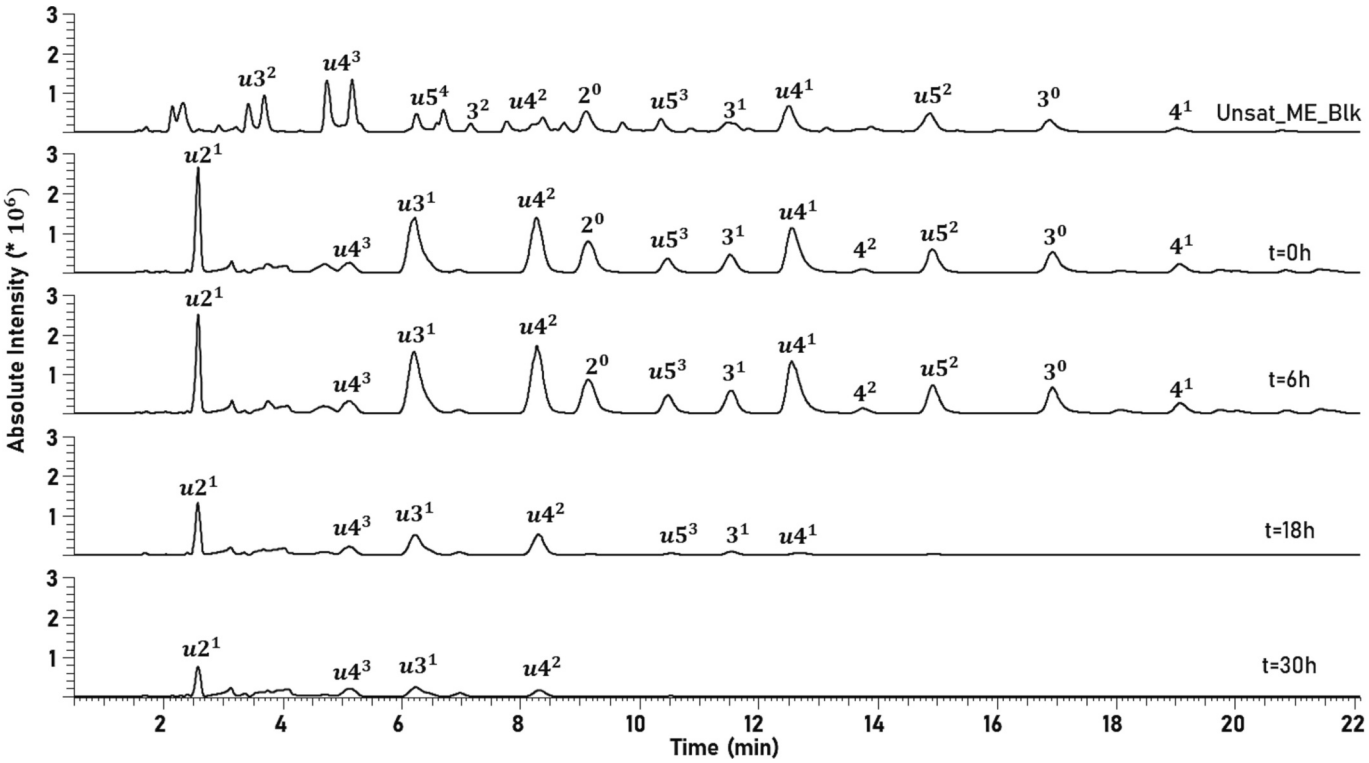

Fig. 6. HILIC-LC-MS base peak elution pattern of uGalA-OS from t = 0 h to t = 30 h of in vitro fermentation by a fecal inoculate of donor 1, measured after microbial inactivation. Unsat_ME_Blk: substrate blank of uGalA-OS, measured before fermentation. Peak annotation: $u4^2$, unsaturated DP4 GalA oligosaccharide having two methyl esters; 4^2, saturated DP4 GalA oligosaccharide having 2 methyl-esters.

and propionate (ProA) (Fig. 7A–D). During the first 6 h of fermentation, minor production of acid metabolites was observed for the inoculates to which uGalA-OS was added, as the microbiota were likely still adapting to the substrate. Comparatively, satGalA-OS fermentation led to an earlier onset of organic acid formation regardless of methyl-esterification, with an average increase of 2.6 mmol of organic acids formed after 6 h. This was likely caused by the utilization and fermentation of low DP GalA-OS (1–3) which amounted for 65 % of the satGalA-OS substrate compared to 44 % of the uGalA-OS substrate.

During the first 18 h of fermentation, it was found that the ratios of produced AcA, ProA and ButA varied depending on the saturation of GalA-OS. The fermentation of both methyl-esterified uGalA-OS and non-methyl-esterified uGalA-OS (uGalA-OSNME) led to an increased formation of ButA during the first 18 h of fermentation (Fig. 7A, B) compared to methyl-esterified satGalA-OS and non-methyl-esterified satGalA-OS (satGalA-OSNME). In contrast, satGalA-OS and satGalA-OSNME fermentation resulted in increased formation of ProA during the first 18 h of fermentation. This variance in metabolite production is exemplified by the production of an average AcA:ProA:ButA ratio of 18:1.0:5.1 and 6.6:1.0:1.9 during the first 18 h of fermentation of uGalA-OSNME and satGalA-OSNME, respectively. Yet, this difference in ButA and ProA production observed due to the presence of the Δ4,5 unsaturated bond on uGalA-OS was only significant for donors 1 and 2 ($p < 0.05$) and not for donor 3, which led to the hypothesis that the production of SCFAs due to the supplementation of uGalA-OS depends on the individual microbiota composition.

Cross feeding mechanisms were observed since lactate was formed during the fermentation of both satGalA-OS and satGalA-OSNME up to t = 6 h of fermentation and is not present at later time points anymore. Furthermore, it has been reported that lactate can be converted to ProA through a mechanism known as the acrylate pathway which might explain the significantly increased ProA production ($P < 0.05$) observed because of satGalA-OS and satGalA-OSNME metabolization compared to uGalA-OS and uGalA-OSNME metabolization (Kandasamy et al., 2013; Prabhu, Altman, & Eiteman, 2012; Reichardt et al., 2014).

Methyl-esterified uGalA-OS fermentation resulted in a higher production of SCFA per mg of fermented material than the fermentation of uGalA-OSNME. Notably, the presence of methyl-esters increased total SCFA production by the inoculates of donors 1,2 and 3 with 1.8 mmol, 0.4 mmol and 2.1 mmol per mg consumed, respectively. A potential explanation for this was that methanol, which is released during the metabolization of methyl-esterified GalA-OS (Ishaq, Moses, & Wright, 2016) could have been converted to AcA through the formation of formaldehyde due to methanol oxidation. Methanol oxidation is a mechanism exerted by gut microbiota such as *Klebsiella pneumoniae* and *Escherichia coli* (Dorokhov, Shindyapina, Sheshukova, & Komarova, 2015). The subsequent acetogenic pathway exerted by gut microbiota species such as the *Blautia hydrogenotrophica* and *Marvinbryantia formatexigensis* allow for the conversion of formaldehyde to AcA through the Wood–Ljungdahl pathway (Pietzke, Meiser, & Vazquez, 2020; Ragsdale & Pierce, 2008; Rey et al., 2010). However, such formation of methanol or formaldehyde was not observed during the fermentation of methyl-esterified uGalA-OS. Therefore, it is currently not known how the methyl-esterification of GalA-OS increased the SCFA formation per mg of substrate consumed.

Following complete depletion of GalA-OS, which was more rapid for both satGalA-OSNME and uGalA-OSNME than methyl-esterified satGalA-OS and uGalA-OS, the remaining gut microbiota likely shifted from glycosidic to proteolytic fermentation. This is exemplified by an increased pH at the final stages of the fermentation, which starts to increase at t = 18 h for both satGalA-OSNME and uGalA-OSNME and at t = 30 h for methyl-esterified satGalA-OS and uGalA-OS (Supplementary Fig. S7). The increased pH could be caused by the formation of basic metabolites such as ammonia produced during proteolytic fermentation. This theorized fermentation of protein did not result in the significant production of the measured BCFAs isovaleric and isobutyric acid, which are typically formed during protein fermentation, next to SCFAs (Diether & Willing, 2019; Yao, Muir, & Gibson, 2016). To be certain that

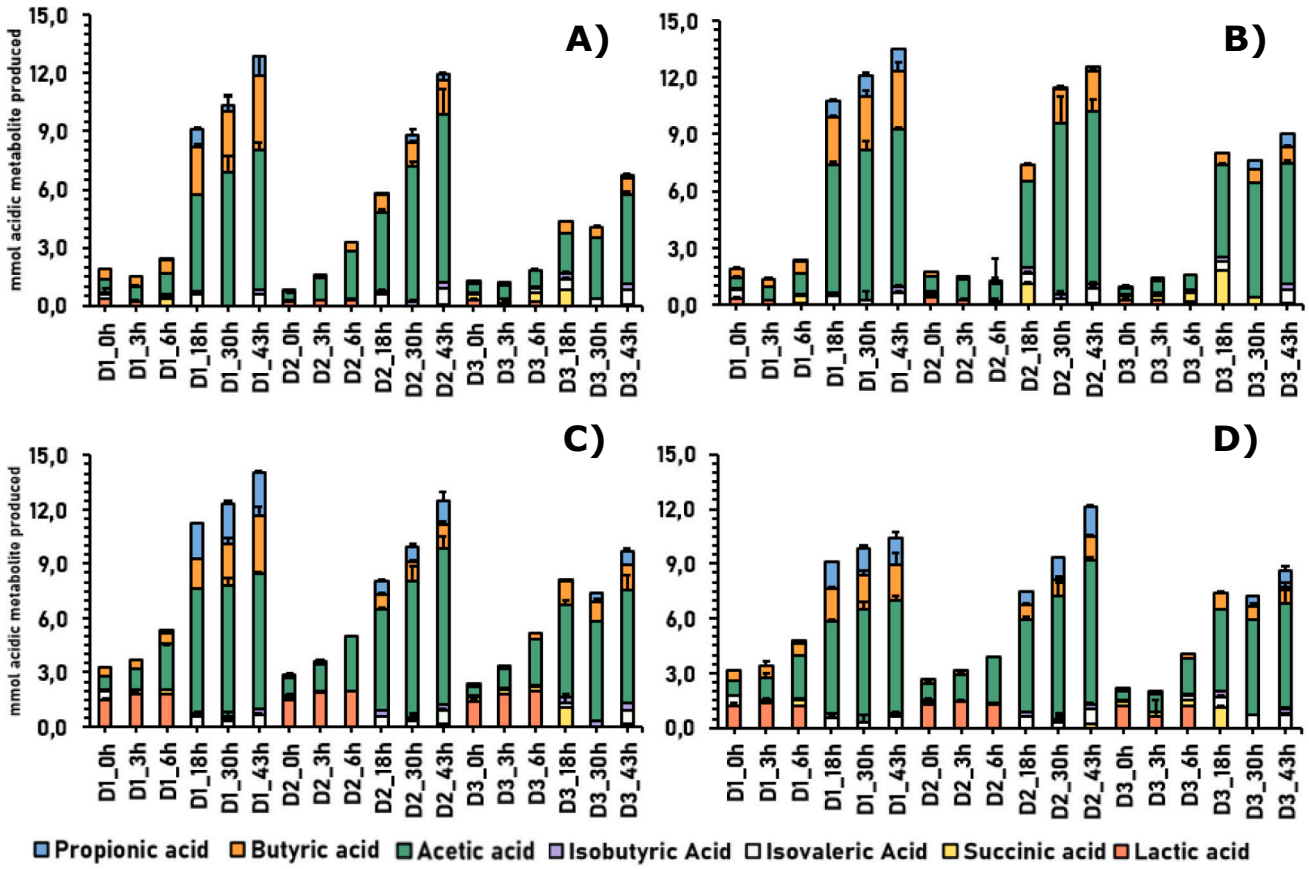

Fig. 7. SCFA, BCFA and organic acid production during the fermentation of GalA-OS by gut microbiota inocula of donors 1,2 and 3 (D1, D2, D3) at t = 0–43 h of *in vitro* fermentation. Results are shown in mmol of acid metabolites. A) Methyl-esterified uGalA-OS, B) Non-methyl-esterified uGalA-OSNME C) Methyl-esterified satGalA-OS D) Non-methyl-esterified satGalA-OSNME.

galacturonic acids were not transported into the intracellular space of the gut microbiota during the fermentation, sonication and subsequent HPAEC analysis of fermentation samples was performed. No additional galacturonic acids were released because of this treatment (data now shown) and therefore, it is likely that the metabolite production observed after t = 30 h was a result of protein fermentation. The absence of GalA-OS released after sonication of bacteria further implies that methyl-esterification could indeed hinder cellular transport by gut microbiota as shown for *Enterobacteriaceae* by Abbott and Boraston (2008).

Due to the observed accumulation and apparent slower utilization of methyl-esterified uGalA-OS and satGalA-OS and a subsequent delayed increase in pH, fermentation of methyl-esterified satGalA-OS and uGalA-OS *in vivo* might be delayed until the distal colon, which might suppress the production of undesired, potentially toxic metabolites released as a result of protein fermentation (Alhinai, Walton, & Commane, 2019; Sánchez-Alcoholado et al., 2020; Tian et al., 2017).

3.5. Fermentation of GalA-OS can alter microbiota composition in a GalA-OS structure-dependent manner

A selection of digesta that resulted from the fermentation of the four GalA-OS substrates were subjected to 16S rRNA gene sequencing to analyze the microbiota composition. As all GalA-OS were at least partly fermented for all donors at t = 18 h, it was decided to analyze the microbiota composition at both t = 0 h and t = 18 h to make sure that the analysis covered solely the changes in microbiota composition caused by the glycosidic fermentation of GalA-OS. Fig. 8 shows the relative abundance of the genera present in the fecal inoculates at t = 0 h and t = 18 h of fermentation after supplementation of non-methyl-esterified uGalA-OS (uGalA-OSNME) and satGalA-OS (satGalA-OSNME). Substantial differences were observed between the baseline t = 0 h microbiota compositions of donors 1,2 and 3 (Fig. 8). Variance was detected in the abundance of the Lactococcus genus, which was the dominant microbial genus in all donors at t = 0 h, making up [39 % ± 6.3], [46 % ± 4.8] and [36 % ± 2.1] of the total microbial abundance for donor 1 (D1), 2 (D2) and 3 (D3) respectively. The Megaspheara genus (dominated by *Lactobacillus* sp.) was a minor contributor to the total microbial abundance in D1 [+- 5 %] while being the second largest microbial genus in D2 [23% ± 2.4] and D3 [22% ± 1.9]. Other notable differences among the most dominant microbial genera found at t = 0 h included discrepancies in the abundances of the Bifidobacterium ([15 %], [6.9 %], [6.9 %]) and Bacteroides ([16 %], [0.03 %], [0.02 %]) for D1, D2 and D3, respectively. These discrepancies found within the microbiota compositions of D1, 2 and 3, supported the notion that individual gut microbiota compositions are highly variable among individuals. (Rinninella et al., 2019).

Regardless of saturation, fermentation of non-methyl-esterified GalA-OS can lead to different microbiota compositions. For donors 2 and 3, fermentation of GalA-OS promotes the growth of the Klebsiella genus, increasing their abundance in satGalA-OSNME ([+40 %], [+36 %]) and uGalA-OSNME ([+8 %], [+51 %]) at t = 18 h. For Donor 1, this effect was (almost) absent (+0.2 % for uGalA-OSNME and 0 % for satGalA-OSNME). A constant finding at t = 0 h and t = 18 h for all three donors was that the largest quantity (98 %) of the in donor 2 and 3 growing Klebsiella genus was attributed to the *Klebsiella pneumoniae* species. *Klebsiella pneumoniae* can exist as a commensal bacterium in the gut in certain individuals but can also function as an opportunistic pathogen in specific circumstances, such as when the host's immune system is compromised (Lau, Huffnagle, & Moore, 2008). This

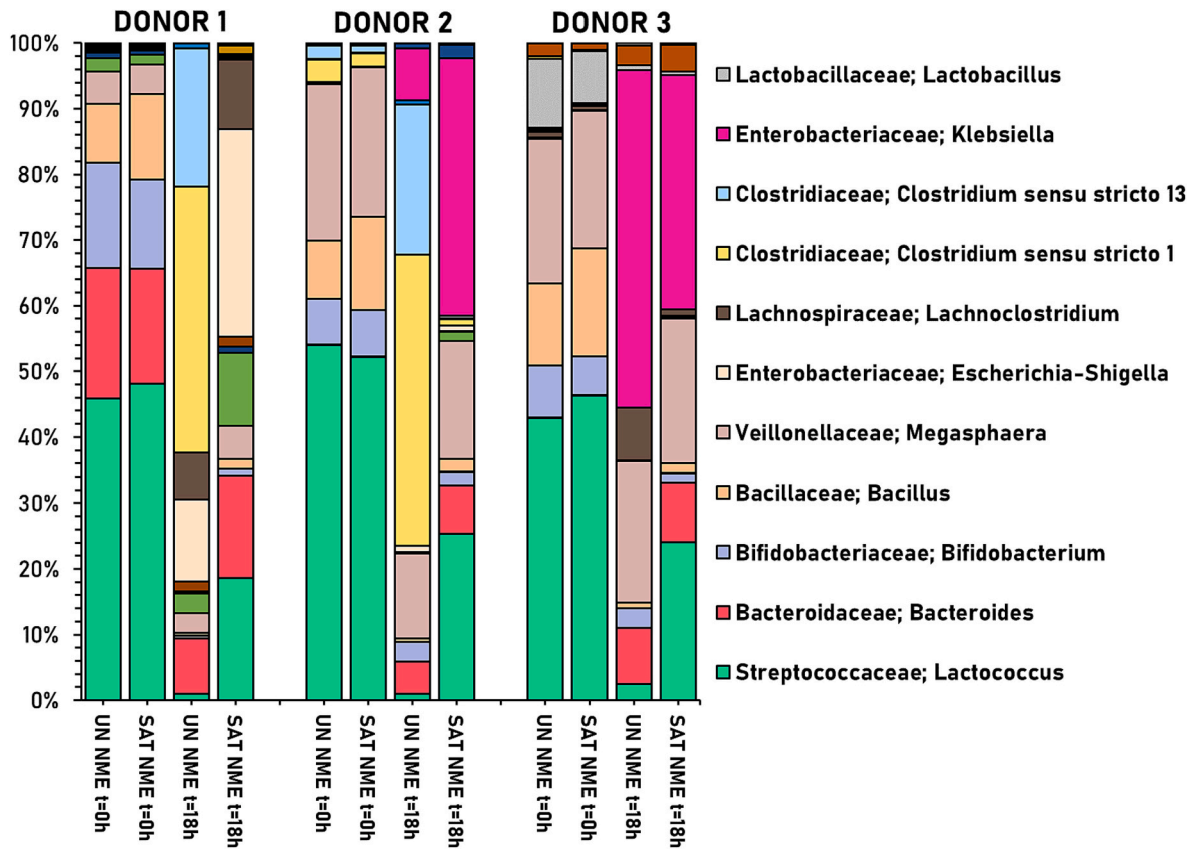

Fig. 8. Average microbiota composition of duplicate inoculates of donor 1,2 and 3 expressed as the relative abundance of microbial genera at t = 0 h and t = 18 h, after fermentation of non-methyl-esterified uGalA-OS (Un NME) and non-methyl-esterified satGalA-OS (SAT NME) substrates. Un: uGalA-OS. SAT: satGalA-OS. NME: Non-methyl-esterified.

opportunistic nature of certain micro-organisms is also true for to the *Escherichia-Shigella* genus (which comprises dominantly of *E. coli*) found only in donor 1 at t = 18 h after the fermentation of uGalA-OSNME (+13 %) and satGalA-OSNME (+32 %). The majority of *E. coli* strains are commensal and are rarely detrimental towards (human) health (Ramos et al., 2020). While it cannot be ruled out that the presence and levels of these commensals at t = 0 h may represent artifacts of the SHIME-stabilized microbiota, the opportunistic nature of (un)desired micro-organisms should be considered when GalA-OS or any other dietary fiber is supplemented *in vivo*.

Interestingly, the fermentation of both uGalA-OS and uGalA-OSNME selectively and significantly ($p < 0.0005$) promoted the growth of the Clostridiaceae family (Clostridium *sensu stricto* 1 + 13) between t = 0 h and t = 18 h of fermentation. In donors 1 and 2 this led to an increase of the relative abundance of the Clostridiaceae family from 0.2 % and 6 % at t = 0 h to 62 % and 68 % at t = 18 h upon the fermentation of uGalA-OSNME. This rapid growth of the Clostridiaceae was also observed for methyl-esterified uGalA-OS for donors 1 and 2 from t = 0 h to t = 18 h, where the abundance of Clostridiaceae increased from 0.2 % to 33 % in D1 and 6 % to 30 % in D2 (Supplementary Data Fig. S8). In the fermentation digest of donor 3, this tendency for growth of the Clostridiaceae family was not observed. The growth of the Clostridiaceae family upon supplementation of uGalA-OS and uGalA-OSNME is mainly attributed to the growth of the butyrate producing and diarrhea relieving probiotic *Clostridium butyricum* (Kanai, Mikami, & Hayashi, 2015; Seki et al., 2003; Sun et al., 2018), which comprises 40 % and 45 % of the total microbiota composition after t = 18 h of the fermentation of uGalA-OSNME and 22 % and 19 % of total microbiota composition after t = 18 h of fermentation of methyl-esterified uGalA-OS. The reduced growth of *Clostridium butyricum* during methyl-esterified uGalA-OS fermentation compared to uGalA-OSNME suggests that the lower fermentation rate of methyl-esterified uGalA-OS affects the microbiota composition. The significantly increased *Clostridium butyricum* growth supports the hypothesis that the significant increase in butyrate production observed after uGalA-OS and uGalA-OSNME fermentation by donor 1 and 2 was determined by a change in microbiota composition, as *Clostridium butyricum* is a notorious butyrate producer (Stoeva et al., 2021).

Substantial reductions of the dominant Lactococcus abundance were observed at t = 18 h for both the fermentation of uGalA-OSNME (D1,D2, D3) ([39 to 0.2 %], [47 to 0.3 %] and [36 to 0.8 %]) and satGalA-OSNME ([39 to 10 %], [45 to 13 %] and [37 to 11 %]). The results from this analysis emphasize the complex interplay of the microbiome as a response on the supplementation with dietary fibers.

That the fermentation of GalA-OS promoted the growth of *Clostridium butyricum* and *Klebsiella pneumoniae* might be related to the potential of these microbiota to secrete specific pectic enzymes that are necessary to (completely) digest GalA-OS. *Clostridium butyricum* and *Klebsiella pneumoniae* should be able to produce specific Δ-4,5-unsaturated α-galacturonidases, which are enzymes that require an unsaturated bond at the non-reducing galacturonic acid (Carbohydrate Active Enzymes database (Drula et al., 2022)). Δ-4,5-unsaturated α-galacturonidases currently belong to the GH105 family (Itoh, Ochiai, Mikami, Hashimoto, & Murata, 2006) and were previously biochemically reconstructed *in vitro* from the marine bacterium *Pseudoalteromonas* sp. (Hobbs, Hettle, Vickers, & Boraston, 2019). The potential production of these highly specific enzymes by *Clostridium butyricum* and *Klebsiella pneumoniae* might explain why these genera are able to thrive when supplemented with uGalA-OS. These 4,5-unsaturated α-galacturonidases are mechanistically like *exo*-oligogalacturonate lyases, that cleave the Δ-4,5-unsaturated residues from the nonreducing ends, resulting in the release of unsaturated galacturonic acid monomers. The

reaction catalyzed by exo-oligogalacturonate lyases was previously reported by Shevchik, Condemine, Robert-Baudouy, and Hugouvieux-Cotte-Pattat (1999). Unfortunately, the highly unstable monomeric unsaturated galacturonic acids which are products of this enzymes' reaction, are known to spontaneously convert to 5-keto-4-deoxyuronate, which is notoriously complex to detect, analyze and quantify (Kester et al., 1999).

K. pneumoniae is known to be able to produce pectinases including polygalacturonase (Latif & Sohail, 2012) and pectate lyase (Avallone, Brillouet, Guyot, Olguin, & Guiraud, 2002) to degrade pectin and GalA-OS. If the growth of *K. pneumoniae* is indeed dependent on these two pectinases, that would mean that the growth of *Klebsiella pneumoniae* is highly dependent on the presence of either (low) native methyl-esterification of the substrate or the production of enzymes by other micro-organisms that cleave the methyl-esters, making the substrate accessible. The results of this 16S analysis would partly support this hypothesis that *Klebsiella pneumoniae* growth is methyl-ester dependent, as the *Klebsiella pneumoniae* abundances found as a result of the fermentation of the highly methyl-esterified uGalA-OS (0 %, 21 %, 28 %) population at t = 18 h were not comparable to the *Klebsiella pneumoniae* abundance as a result of the fermentation of uGalA-OSNME (0 %, 8 %, 52 %) at t = 18 h of fermentation.

Many commensal colonic gut bacteria (including several found during the analysis) should be able to produce pectin-methyl-esterase (Duan et al., 2020; Lund & Brocklehurst, 1978; Schink, Ward, & Zeikus, 1981). However, the data presented clearly illustrates that methyl-esterification of GalA-OS hinders their fermentation. This led to the hypothesis that there was a lack of effective PME produced during the fermentation of GalA-OS by all three donors.

The results obtained from the 16S rRNA sequencing show that GalA-OS stimulate unique microbiota compositions, depending on the methyl-ester substitution and saturation. Potential new and exciting research is made possible due to the novel finding presented in this report that the unsaturation of pectin is a determinant of the fermentation of pectin-derived galacturonic acid oligosaccharides as the field of pectin structure-function becomes more detailed. Of course, human trials are necessary to prove clinical relevance of these results.

4. Conclusion

This study showed that diverse types of galacturonic acid oligosaccharides (GalA-OS) can be easily produced from commercial pectins using commercially available pectinolytic enzyme preparations, yielding up to ~68 % GalA-OS. The fine structure of GalA-OS, including methyl-esterification and the presence of an unsaturated bond at the non-reducing end of galacturonic acid oligosaccharides was investigated as a variable for the proximal colon simulated *in vitro* fermentation of GalA-OS. As a result, it was shown that diverse types of GalA-OS were differently fermentable, with methyl-esterified GalA-OS fermenting slower than non-methyl-esterified GalA-OS, showing that distinct GalA-OS could have unique applications. Highly methyl-esterified low DP GalA-OS accumulated up until t = 30 h of fermentation as observed using HILIC-LC-MS, indicating a reduced ability of gut microbiota to utilize highly methyl-esterified GalA-OS compared to non-methyl-esterified GalA-OS. Furthermore, metabolite production and microbiota composition both were directly affected by the presence of a Δ4,5 unsaturated bond at the non-reducing end of GalA-OS. Although GalA-OS fermentation yielded predominantly acetate, propionate and butyrate during the 43 h fermentation experiment, significantly increased butyrate production was measured as a result of the fermentation by the fecal inoculates of donors 1 and 2 of both methyl-esterified and non-methyl-esterified Δ4,5 unsaturated GalA-OS. Specific growth of beneficial microbiota such as *Clostridium Butyricum* after supplementation of distinct, pectin-derived dietary fibers may confer health benefits to the host. This could lead to the development of specific functional or therapeutic applications using different, unique structures of GalA-OS.

CRediT authorship contribution statement

J.W. Zwolschen: Conceptualization, Data curation, Formal analysis, Investigation, Methodology, Visualization, Writing – original draft. **A.P. Vos:** Conceptualization, Supervision, Writing – review & editing. **R.M. C. Ariëns:** Methodology, Writing - review & editing. **H.A. Schols:** Conceptualization, Funding acquisition, Investigation, Project administration, Resources, Supervision, Validation, Writing – review & editing.

Declaration of competing interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

Data availability

Data will be made available on request.

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Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.carbpol.2024.121789.

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