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RESEARCH ARTICLE

Effects of pectin supplementation on the fermentation patterns of different structural carbohydrates in rats

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Scope: We aimed to investigate and compare the effects of four types of pectins on dietary fiber (DF) fermentation, microbiota composition, and short chain fatty acid (SCFA) production throughout the large intestine in rats.

Methods and results: Male Wistar rats were given diets supplemented with or without 3% structurally different pectins for 7 weeks. Different fermentation patterns of pectins and different location of fermentation of pectin and diet arabinoxylans (AXs) in the large intestine were observed. During cecal fermentation, sugar beet pectin significantly stimulated *Lactobacillus* ($p < 0.01$) and Lachnospiraceae ($p < 0.05$). The stimulating effects of sugar beet pectin on these two groups of microbes are stronger than both other pectins. In the cecum, low-methyl esterified citrus pectin and complex soy pectin increased ($p < 0.05$) the production of total SCFAs, propionate and butyrate, whereas high-methyl esterified pectin and sugar beet pectin did not. The fermentation patterns of cereal AXs in the cecum were significantly different upon supplementation of different pectins. These differences, however, became smaller in the colon due to an enhanced fermentation of the remaining DFs.

Conclusion: Dietary supplementation of pectin is a potential strategy to modulate the location of fermentation of DFs, and consequently microbiota composition and SCFA production for health-promoting effects.

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1 Introduction

Obesity and related disorders, such as type 2 diabetes and cardiovascular diseases, are nowadays prime public health

concerns [1]. Consumption of dietary fibers (DFs) is believed to reduce the risk of such diseases through influencing the location of intestinal DF fermentation, and promoting growth and activity of beneficial intestinal bacteria [2, 3].

Pectin, on a dry matter basis highly present in fruits and vegetables, is one of the most important DFs in human nutrition. Isolated pectins are widely used as emulsifier, stabilizer, and thickening agent by the food industry. Pectins are extracted from food by-products, like citrus peel, sugar beet pulp and soybean meal, yielding pectins with different galacturonic acid (GalA) contents, and levels of esterification [4]. Pectins are complex heteropolysaccharides, containing GalA residues in their backbones. GalA residues are organized in “smooth” homogalacturonan (HG) and “hairy” rhamnogalacturonan I (RG-I) regions. In the latter neutral sugars are present as side chains [4, 5]. HG are linear

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Abbreviations: DF, dietary fiber; SCFA, short chain fatty acid; AXs, arabinoxylans; GalA, galacturonic acid; HG, homogalacturonan; RG-I, rhamnogalacturonan I; CONT, control diet chow; LMP, low-methyl esterified pectin enriched diet; HMP, high-methyl esterified pectin enriched diet; SBP, sugar beet pectin enriched diet; SSPP, soy pectin enriched diet; NSP, nonstarch polysaccharides; OTUs, operational taxonomic units; RDA, redundancy analysis; DM, degree of methyl esterification; DA, degree of acetylation; A/X, arabinose to xylose

(1-4)- α -D-GalpA polymers, which are partially methyl esterified at the carboxyl group and O-acetyl esterified on O-2 and/or O-3 [4]. RG-I are composed of the repeating disaccharide [-2)- α -L-Rhap-(1-4)- α -D-GalpA-(1-)] as the backbone. Depending on the plant source, 20–80% of the rhamnose residues are substituted at O-4 with arabinan, galactan, and/or arabinogalactan side chains. The GalA residues in the RG-I backbone may be O-acetyl esterified at the O-2 and/or O-3 positions [4]. Thus, the source and manner of processing can influence the chemical, functional and nutritional properties of these fibers.

In the human intestine, pectins are fermented by the resident microbiota in the cecum and colon. The presence of pectins affects microbial composition and activity, and thereby the production of SCFAs by the microbes in the colon [6, 7]. SCFA production as a result of pectin consumption provides the most direct link between pectin intake and associated health benefits [8]. During in vitro fermentation using human fecal microbiota, pectin from sugar beet and soybean significantly stimulated growth and activity of the genera *Bifidobacterium* and *Lactobacillus* [7], members of which have shown the potential to protect enterocytes from an acute inflammatory response [9]. In rats, citrus pectins tended to increase not only the total SCFA concentration, but also the proportions of propionate and butyrate [6]. Propionate has the potential to reduce blood cholesterol concentrations, while butyrate is an important energy source for intestinal epithelial cells and plays a role in the maintenance of colonic homeostasis [8].

Most of the studies on fermentation of pectins were performed using in vitro and/or in vivo rat models with pectin as the sole DF source [6, 7]. However, the intake of pectin is usually accompanied by the consumption of other DFs. Up to now, data are lacking on the potential effects of pectin on the degradation of other specific DFs. Consequently, modulation of SCFA patterns and microbiota composition by pectins in the presence of other DFs are unknown.

The aim of the present study was to monitor the fate of different types of pectin in the large intestine of rats, and evaluate the effects of pectin supplementation on the fermentation patterns of other DFs. To achieve this aim, high- and low-methyl esterified citrus pectins and more complex (acetylated and branched) sugar beet pectin and soy pectin were added as soluble fibers to the diets of rats.

2 Materials and methods

2.1 Materials and diets

Standard rat diet chow (RMH-B) was purchased from AB diets (Woerden, The Netherlands). The four experimental diets based on the control diet RMH-B (CONT) are LMP containing 3% (w/w) low-methyl esterified citrus pectin (Classic CU-L 020/13, Herbstreith & Fox, Neuenbürg, Germany), HMP containing 3% (w/w) high-methyl esterified citrus

pectin (Classic CU-L 021/13, Herbstreith & Fox), SBP containing 3% (w/w) sugar beet pectin (GENU[®] BETA pectin, CP Kelco, Grossenbrode, Germany), and SSPS containing 3% (w/w) soy pectin (soyafibe, Fuji Oil, Osaka, Japan). The added pectin fiber of 3% is equivalent to human dosage of 20 g fiber per day [10]. 0.25% (w/w) titanium dioxide (TiO₂; Sigma-Aldrich, St. Louis, MO, USA), as an indigestible marker for calculation of nutrient digestibility was added to control and experimental diets. All diets were characterized (Supporting Information Table 1) with respect to chemical composition.

2.2 Animals and sampling

Forty male Wistar rats, weight 300 ± 10 g were purchased from Harlan Laboratories (Horst, The Netherlands) and housed individually for the duration of this study. Temperature was 21°C with a light/dark cycle of 12h/12h. After acclimatization to diet CONT for 1 week, animals were divided into five experimental groups of eight rats each. The diets of four groups were changed to pectin supplemented diets for 7 weeks to ensure the adaptation of microbiota to the different diets [11], while one group was still fed with CONT. Food and water were available ad libitum. All experiments were accepted by the Animal Ethics Committee of Groningen University (DEC 6375, Groningen, The Netherlands).

Animals were sacrificed after 7 weeks of diet treatment. After 3 h of fasting following the meal ad libitum, animals were anesthetized using isoflurane and subsequently decapitated. Cecal and colonic digesta were collected separately. Approximately 200 mg of cecal or colonic content from animals belonging to the same treatment group were pooled and freeze-dried. The rest of cecal and colonic digesta were frozen at -80°C for SCFA and microbiota analysis. Dry digesta and defatted diets were milled using a ball-milling apparatus MM2000 (Retsch, Haan, Germany).

2.3 Extraction of nonstarch polysaccharides

Gelatinization and enzymatic degradation of starch in diets, followed by ethanol precipitation and washing of the nonstarch polysaccharides (NSPs) were performed as described previously [12].

2.4 DNA extraction and quality control

Microbial DNA was extracted from 250 mg of digesta using a Repeated Bead Beating protocol plus column purification [13]. DNA was quantified using a NanoDrop 2000 spectrophotometer (Thermo Scientific, Wilmington, DE, USA). DNA extracts were diluted to 20 ng/ μL in nuclease-free water and used in PCR.

2.5 Analytical methods

2.5.1 Chemical analysis

Dry matter was determined by drying samples overnight in an oven at 103°C. Crude ash was measured by incineration at 550°C [14]. Titanium dioxide in diets and digesta was analyzed using a UV spectrophotometer at 410 nm as described previously [15]. Protein content was determined according to the Dumas method of the AOAC [16] on a Thermo Quest NA 2100 Nitrogen and Protein Analyzer (Troy, NY, USA). Starch and mixed linkage (1-3),(1-4)- β -D-glucan (β -glucan) contents were analyzed using the total starch and β -glucan kits from Megazyme (Bray, Ireland), respectively. The constituent monosaccharide composition of pectins and DF fractions present in the digesta was determined as described previously [14]. Cellulose content was calculated from the difference between glucose present in NSP and glucose present in β -glucan. SCFAs in digesta were analyzed on the basis of a method described before [17] with minor modifications. Duplicates of 250–300 mg samples in 1.5 mL distilled water, or 1.5 mL standards (0.06–0.6 mg/mL) were mixed with 1.5 mL of 0.3 mg/mL 2-ethylbutyric acid (internal standard) in 0.2 M HCl. After centrifugation (5 min, 30 000 $\times$ g, 20°C), 200 μ L of the supernatant of either a sample or the standard was mixed with 50 μ L of 0.15 M oxalic acid. After 30 min, the mixture was centrifuged again and supernatant was transferred into a vial for analysis. Analysis was done on a TRACE GC Ultra system [17]. Samples (1 μ L) were injected onto a CP-FFAP CB column (25 m $\times$ 0.53 mm $\times$ 1.00 μ m, Agilent, Santa Clara, CA, USA). Helium was supplied as carrier gas at a flow rate of 10 mL/min. The flow rates of air, H₂ and N₂ as makeup gas were 260, 30, and 30 mL/min, respectively. The initial oven temperature was 100°C, increased to 160°C at 5°C/min, and held at this temperature for 4 min. Data were processed using the Xcalibur[®] software (Thermo Scientific, Waltham, MA, USA). Lactate was analyzed using HPLC as described elsewhere [18].

2.5.2 16S rRNA gene amplification and MiSeq sequencing

Barcoded amplicons from the V1-V2 region of 16S rRNA genes were generated using a two-step PCR method [19]. First, the forward 27F-DegS primer and an equimolar mix of two reverse primers (338R I and II) were used [20,21]. The forward primer (UniTag1-27F-DegS) included UniTag1 (5'-GAG CCGTAGCCAGTCTGC-3') at the 5'-end, and reverse primers (UniTag2-338R-I + II) had UniTag2 (5'-GCCGTGACCGTGA CATCG-3') at the 5'-end. UniTags were selected with Primer-BLAST (<http://www.ncbi.nlm.nih.gov/tools/primer-blast/>) to avoid close matches to any 16S rRNA sequence in the database (July 8th, 2014), and IDTDNA Oligoanalyzer 3.1 (Integrated DNA Technologies) was used to predict secondary structures. PCRs were performed using a SensoQuest

Labcycler (Göttingen, Germany). Twenty nanograms of template DNA was used in the first PCR, including 10 μ L 5 $\times$ HF buffer (Finnzymes, Vantaa, Finland), 1 μ L PCR Grade Nucleotide Mix (Roche Diagnostics, Mannheim, Germany), 1U Phusion[®] Hot Start II High-Fidelity DNA polymerase (2U/ μ L), 32.5 μ L nuclease free water, and 2.5 μ L Primer UniTag1-27F-DegS (10 μ M) and 2.5 μ L of Primer UniTag2-338R-I + II (10 μ M). Amplification included an initial denaturation at 98°C for 30 s; 25 cycles of denaturation at 98°C for 10 s, annealing at 56°C for 20 s, and elongation at 72°C for 20 s; and a final extension at 72°C for 10 min. The presence of PCR products was confirmed by agarose gel electrophoresis containing 1 $\times$ SYBR Safe (Invitrogen, Carlsbad, CA, USA). A second PCR was employed to add an 8 nucleotide sample-specific barcode to the 5'- and 3'-end of PCR products.

Each PCR reaction with a final volume of 100 μ L contained 5 μ L PCR product, 5 μ L each of barcoded forward and reverse primers (10 μ M), 2 μ L PCR Grade Nucleotide Mix (Roche Diagnostics), 2 U Phusion[®] Hot Start II High-Fidelity DNA polymerase and 1 $\times$ HF buffer (Finnzymes). Amplification consisted of an initial denaturation at 98°C for 30 s; five cycles of denaturation at 98°C for 10 s, annealing at 52°C for 20 s, and elongation at 72°C for 20 s; and a final extension at 72°C for 10 min. PCR products were purified using the HighPrep[™] PCR kit (MagBio Genomics, Gaithersburg, MD, USA).

Amplicons were quantified using the Qubit[®] dsDNA BR Assay Kit (Life Technologies, Carlsbad, CA, USA), and 40 samples, each labeled with a unique barcode, were equimolarly pooled. Pooled libraries were sequenced at GATC Biotech AG (Constance, Germany) by Illumina Miseq sequencing.

2.5.3 Sequence analysis

Sequencing reads were demultiplexed and analyzed using QIIME 1.8.0 as described before [20], while using the Silva 111 reference database Operational taxonomic units (OTUs) clustering was performed at 97% sequence identity.

2.5.4 Statistical analysis

The results were analyzed using Graphpad Prism (La Jolla, CA, USA). The parametric distribution of data was confirmed using Kolmogorov–Smirnov test. Results are expressed as mean $\pm$ SEM. Statistical comparisons were performed using paired *t*-test for parametrically distributed data. Where non-parametric distribution could be demonstrated, we applied Mann–Whitney test. *p* < 0.05 was considered as statistically significant.

Univariate testing of differences for individual microbial groups was processed by using a Mann–Whitney test. The Benjamini–Hochberg method was used for adjustment after multiple testing [22]. To relate changes in bacterial community composition to environmental variables, redundancy

Table 1. Constituent monosaccharide composition (mol%) and carbohydrate content (w/w%) of pectins^{a)}

Pectin	Monosaccharides (mol%)								Total carbohydrate (w/w%)	DM (%)	DA(%)
	Rha	Fuc	Ara	Xyl	Man	Gal	Glc	UA			
Low methyl esterified pectin	0	0	1	0	0	2	0	96	80	29	1
High methyl esterified pectin	0	0	1	0	0	3	0	95	79	53	1
Sugar beet pectin	3	0	13	1	0	18	1	63	78	58	25
Soy pectin	2	3	24	6	1	42	4	19	71	4	17

a) Rha: rhamnose; Fuc: fucose; Ara: arabinose; Xyl: xylose; Man: mannose; Gal: galactose; Glc: glucose; UA: uronic acid. DM: degree of methyl esterification, moles methanol per 100 moles of UA residues. DA: degree of acetylation, moles acetic acid per 100 moles of UA residues.

analysis (RDA) was used as implemented in CANOCO 5 [23]. Explanatory variables were considered to significantly affect microbial composition at $p < 0.05$.

3 Results

3.1 Characteristics of pectin substrates

The constituent monosaccharide compositions of the four pectins are shown in Table 1. Both low-methyl esterified pectin and high-methyl esterified pectin were mainly composed of uronic acid (UA, ≥ 95 mol%). The degrees of methyl esterification (DM) of low- and high- methyl esterified pectins were 29 and 53%, respectively. The degrees of acetylation (DA) of these two pectins were around 1%. Sugar beet pectin was mainly composed of UA (63 mol%), next to galactose (18 mol%), and arabinose (13 mol%). The DM and DA of sugar beet pectin were 58 and 25%, respectively. Less UA (19 mol%) was present in soy pectin compared to sugar beet pectin. The main constituent monosaccharide in soy pectin was galactose (42 mol%), followed by arabinose (24 mol%), xylose (6 mol%), glucose (4 mol%), fucose (3 mol%), and rhamnose (2 mol%). The DM and DA of soy pectin were 4 and 17%, respectively. The molecular weight distributions of the four pectins were quite similar (Supporting Information Fig. 1), ranging between 100 and 1000 kDa.

3.2 Characterization of diet nonstarch polysaccharides (NSP) and digesta

To study the fermentation of pectin and the consequent effects of pectin on the fermentation of other dietary fibers, the diet and digesta were characterized with respect to constituent monosaccharide composition of NSP (Table 2). The main constituent monosaccharides in the NSP fraction of CONT were xylose (40 mol%), glucose (27 mol%), arabinose (19 mol%) and UA (8 mol%). As expected, higher proportions of UA were present in the NSP of pectin-supplemented diets. The molar percentage of galactose was higher for SBP (6 mol%) and SSPS (11 mol%) than for CONT (3 mol%). Sup-

plementation of citrus pectin did not change the arabinose to xylose (A/X) ratio compared to controls, while supplementation of soy pectin and sugar beet pectin did. With respect to cecal and colonic digesta, slightly higher molar proportions of UA were found in LMP- and HMP-fed rats than in CONT-fed rats. A/X ratios for cecal digesta from CONT- and HMP-fed rats were higher than those of related diets. In contrast, A/X ratios for cecal digesta from SBP- and SSPS-fed rats were lower than those of related diets. The ratio of the sum of arabinose and xylose to UA [(Ara+Xyl)/UA] for cecal digesta from CONT-fed rats was lower compared to that of the CONT diet. The (Ara+Xyl)/UA ratio was much higher for cecal digesta from pectin supplemented groups than for their corresponding diets. A/X ratios of colonic digesta from all groups were slightly higher than those of the corresponding cecal digesta. Overall, the molar compositions of monosaccharides in colonic digesta were rather similar to those in cecal digesta (Table 2).

3.3 Digestibility of carbohydrate and protein in cecum and colon

Due to the low amount of digesta in rats, cecal and colonic digesta from the animals in the same treatment group were pooled to attenuate the individual variation for the TiO₂ analysis. Consequently, the digestibility values are indicative rather than statistically justified (Table 3).

In the cecum, similar digestibilities of NSP ($\approx 56\%$) were observed for LMP-, SBP- and SSPS-fed rats, which levels were higher than those in CONT- (36%) and HMP-fed rats (39%). Slightly lower digestibilities of protein (63%) and starch (98%) were found in HMP-fed rats than for CONT-fed rats. In the colon, apparent digestibility of NSP in pectin supplemented diet-fed rats was lower than in CONT-fed rats (69%).

Next to the digestibility of total NSP, the fermentation of individual constituent monosaccharides of NSP was analyzed (Table 3). Cecal digestibilities of arabinose and xylose were lower in HMP-fed rats, and higher in LMP-, SBP- and SSPS-fed rats than in CONT-fed rats. Colonic digestibilities of arabinose, xylose and glucose were lower in rats fed with pectin supplemented diets than in CONT-fed rats. Higher

Table 2. Constituent monosaccharide composition (mol%) and carbohydrate content (w/w%) of diet, cecal digesta, and colonic digesta^{a)}

Group	Sample	Monosaccharides (mol%)							Total carbohydrate (w/w %)	Ara/Xyl	(Ara+Xyl)/UA
		Rha	Ara	Xyl	Man	Gal	Glc	UA			
CONT	Diet	0	19	40	2	3	27	8	34	0.48	7.4
	Cae	1	17	31	2	3	36	9	29	0.54	5.2
	Col	1	19	33	2	4	32	9	26	0.59	5.7
LMP	Diet	0	15	32	2	3	19	28	40	0.47	1.6
	Cae	2	15	30	3	3	31	16	30	0.49	2.8
	Col	2	17	29	3	4	33	13	27	0.58	3.7
HMP	Diet	0	14	30	2	3	19	31	36	0.47	1.4
	Cae	2	16	29	2	3	33	14	27	0.55	3.1
	Col	2	17	28	3	4	32	14	27	0.61	3.2
SBP	Diet	1	17	33	2	6	23	18	38	0.51	2.9
	Cae	1	15	31	3	3	39	8	27	0.48	5.7
	Col	2	17	31	3	4	34	9	27	0.55	5.4
SSPS	Diet	1	19	34	2	11	23	12	39	0.56	4.4
	Cae	1	14	30	3	3	40	8	25	0.48	5.5
	Col	1	15	30	3	4	38	8	27	0.50	5.6

a) Diet: total nonstarch polysaccharides segment of diet; Cae: cecal digesta; Col: colonic digesta. Ara/Xyl: mole ratio of arabinose to xylose; (Ara+Xyl)/UA: mole ratio of the sum of arabinose and xylose to uronic acid.

cecal and colonic fermentabilities of UA were found in rats fed with pectin supplemented diets than in controls. Cecal digestibilities of added pectin were calculated to be 91, 82, 96, and 101% in LMP-, HMP-, SBP-, and SSPS-fed rats, respectively. Both citrus pectins showed approximately 92% digestion at the end of the colon. Colonic digestibility of cellulose was lowered after supplementing pectin to the diets. The digestibility of β -glucan in rats fed with

pectin supplements was comparable to that in CONT-fed rats ($\approx 98\%$, w/w).

3.4 Formation of organic acids in cecum and colon

The main fermentation products detected in the digesta were SCFAs including acetate, propionate, butyrate, and lower

Table 3. Apparent digestibility (%) of protein, starch, and carbohydrates in the cecum and colon of rats fed with diets basal diet chow and pectin added diets

	CONT		LMP		HMP		SBP		SSPS	
	Cae	Col	Cae	Col	Cae	Col	Cae	Col	Cae	Col
Protein	66.3	78.4	69.8	72.6	63.4	69.0	69.5	74.1	69.3	73.4
Starch	98.7	99.4	98.8	99.1	98.3	99.1	98.7	99.2	98.9	99.1
NSP ^{a)}	36.3	68.8	55.7	65.0	38.8	56.4	55.9	64.9	54.5	62.8
Glc	11.2	65.0	27.9	39.7	4.3	34.5	19.4	42.5	13.2	33.8
β -glucan ^{b)}	96.2	98.1	97.4	98.1	96.3	97.0	98.6	98.1	96.6	98.0
Cellulose	−4.4	58.9	13.3	27.4	−15.0	21.3	4.7	32.2	−1.9	22.2
Ara	48.1	69.8	55.8	60.6	26.3	44.7	62.8	66.4	68.2	71.9
Xyl	56.0	76.3	58.6	68.5	37.6	57.5	62.5	70.4	63.1	68.6
Ara+Xyl ^{c)}	53.5	74.3	57.7	66.0	34.0	53.4	62.6	69.1	64.9	69.8
UA	29.3	60.2	73.2	82.0	66.5	81.9	79.9	82.1	69.0	74.6
Pectin ^{d)}	–	–	90.8	92.7	82.3	91.5	96.1	111.6	100.5	98.4

a) NSP: nonstarch polysaccharides;

b) Mixed lineage (1-3),(1-4)- β -D-glucan;

c) Sum of arabinose and xylose; in CONT, LMP, and HMP, it stands for the amount of arabinoxylans;

d) The digestibility of galacturonic acid present in the backbone of pectins; it is calculated from the digestibility of uronic acid with the assumption that supplementation of pectin did not affect the digestibility of glucuronic acid.

Figure 1. Lactate (A), Total SCFA (B), acetate (C), propionate (D), butyrate (E), and branched SCFA (F) concentrations in cecal and colonic digesta of rats fed the different diets for 7 weeks. Values are mean $\pm$ SEM. Within samples from cecum, values without a common letter are significantly different ($p < 0.05$), values with a same letter are not significantly different ($p > 0.05$). Number of samples from cecum and colon differ within the same treatment, $p < 0.05$.

amounts of lactate, iso-butyrate and iso-valerate (Fig. 1). Lactate concentrations were low in both cecum and colon and not different between groups ($p > 0.05$). Total SCFA concentrations in cecum were higher in LMP- and SSPS-fed rats than in CONT-fed rats. The increase of total SCFA concentration was significant only in LMP- and SSPS-fed rats. The concentrations of acetate, propionate, butyrate, and branched SCFAs (iso-butyrate and iso-valerate) were significantly higher ($p < 0.05$) in LMP-fed rats than in CONT-fed rats. Also significant increases ($p < 0.05$) were found for the analyzed individual SCFA (except acetate) in SSPS-fed rats compared to CONT-fed rats. In contrast, fermentation of HMP and SBP resulted in lower concentrations (Fig. 1) and proportion (Supporting Information Table 2) of butyrate relative to CONT, LMP, and SSPS, although differences were not significant. In the colon samples, the concentrations of acetate, propionate, butyrate and branched SCFAs in any of the pectin-fed rats did not significantly differ from those in CONT-fed rats (Fig. 1).

3.5 Microbiota composition in cecum and colon

The most prevalent phyla in all samples were Firmicutes and Bacteroidetes, with Verrucomicrobia, Actinobacteria, and Candidate_division_TM7 contributing smaller proportions of the sequencing reads (Supporting Information Fig. 2). In general, the levels of Firmicutes in both cecal and colonic digesta were higher in rats fed with pectin supplemented diets than in CONT-fed rats. In contrast, Bacteroidetes generally decreased in relative abundance in rats fed with the pectin-added diets.

At genus level (Fig. 2), most genera in the cecum and colon were influenced by pectin supplementation. Generally, effects were more pronounced in cecum than in colon. Mem-

bers of the genus *Lactobacillus* were stimulated by pectin in SBP ($p < 0.01$) and LMP ($p = 0.01$, without correction) during cecal fermentation in rats (Supporting Information Table 3). Unidentified microbes in the family Lachnospiraceae were significantly ($p < 0.01$) increased in the cecum of SBP-fed rats. *Incertae_Sedis* in the family Lachnospiraceae were higher in cecal digesta from SBP- ($p < 0.05$) and SSPS-fed rats ($p < 0.01$) than in CONT-fed rats. Unclassified microbes in the family S24-7 (order Bacteroidales) were decreased in cecum of HMP- ($p < 0.05$) and SBP-fed rats ($p < 0.01$). In the cecum, the proportion of an unclassified genus in the family Peptostreptococcaceae was lower in SBP- ($p = 0.01$) and SSPS-fed rats ($p = 0.037$, without correction) than in CONT-fed rats. Decreases ($p < 0.01$) of *Akkermansia* relative abundance were found in SBP- and SSPS-fed rats, but not in the citrus pectins-fed rats. Shifts of colonic microbiota composition induced by pectins showed a similar trend, although the differences were mostly not significant (Supporting Information Table 3).

To relate changes in microbiota composition to the different diets and different SCFAs as main metabolites of microbial fermentation, relative abundance of 35 genus-level phylogenetic groups were subjected to redundancy analysis (RDA). The RDA included proportions of lactate, acetate, propionate, butyrate, branched SCFAs, and diets as explanatory variables. Both cecal and colonic samples in LMP-, HMP-, SBP-, and SSPS-fed rats clustered separately from those in CONT-fed rats (Fig. 3), although individual SCFA as explanatory variables did not have a significant contribution (Monte Carlo Permutation test, $p > 0.05$). Cecal samples from SBP-fed rats clustered separately from those from SSPS-fed rats. Samples in the two groups were clearly separated from those in LMP- and HMP-fed rats. Colonic samples from LMP-, HMP-, and SSPS-fed rats clustered together.

Figure 2. Bacterial community at the genus level in the caecum and the colon of rats fed with control diet chow (CONT), low methyl esterified pectin enriched diet (LMP), high methyl esterified pectin enriched diet (HMP), sugar beet pectin enriched diet (SBP), or soy pectin enriched diet (SSPS). Un: unclassified; IS: Incertae_Sedis.

4 Discussion

4.1 Fermentation pattern of NSP present in the basic diet

The NSP of the diets that mainly originates from ingredients wheat and oats in CONT were not completely fermented after passing the rat intestine. Fecal digestibility of total NSP in CONT diet were comparable to those reported for diets based on wheat and oats [24]. Fermentation of cereal NSP present in CONT led to a high proportion of butyrate, which could be attributed to the stimulating effect of AXs on the production of butyrate, as reported before [25]. Regarding microbiota composition, we found that members of the genus *Lactobacillus*, an unclassified genus from family S24-7 (order Bacteroidales) and members of the family Peptostreptococcaceae were predominant in caecum and colon of rats fed with CONT diet. Previous findings on AXs and β -glucan from barley also demonstrated their selectivity of *Lactobacillus* for these polysaccharides [26].

4.2 The fermentation pattern of citrus pectin-enriched diets is DM dependent

Supplemented pectins, which are structurally different from cereal DFs, resulted in changes in fermentation characteristics. Both citrus pectins showed excellent fermentability in the large intestine of rats. Nonetheless, the high-methyl esterified pectin was fermented less efficiently than low-methyl esterified pectin in the caecum, in line with a previous study that suggested that pectin degradation was due to pectate lyase

and pectin methyl esterase [6]. We found that low-methyl esterified pectin more strongly stimulated the growth of lactobacilli than high-methyl esterified pectin. Lactobacilli have displayed higher growth rates on low-methyl esterified pectin than on high-methyl esterified pectin as a carbon source [27].

In the present study, the location of fermentation of the NSP and its polymeric constituents, AXs and cellulose, was shifted from the caecum to the colon in HMP- but not in LMP-fed rats. The variance of cecal digestibility of the two citrus pectins (10%, Table 3) does not seem to fit the significant ($p < 0.05$) difference of cecal SCFA levels (Fig. 1). The shift of fermentation site of DFs in the large intestine is interesting and explainable. The fermentation of pectin might have started in the ileum of rats due to presence of pectin degrading microbiota (e.g. lactobacilli) [28]. Because the low-methyl esterified pectin was more efficiently or earlier degraded than the high-methyl esterified pectin, lower amounts of low-methyl esterified pectin were available in the caecum of LMP-fed rats. Although the supplemented pectin accounted for 20% of the DFs present in the pectin-enriched diets, still 80% of the DFs was similar to those in CONT. The cecal microbiota already adapted to the AXs in LMP-fed rats as indicated by the comparable fermentability (Table 3) and SCFA proportion (Supporting Information Table 2) to controls, where AXs were the main substrate for microbiota. In contrast, a higher amount of high-methyl esterified pectin might have been present for microbiota in caecum of HMP-fed rats. Consequently, the fermentation of AXs present in HMP was shifted from the caecum to the colon. The cecal fermentability of AXs (Table 3) and production of cecal SCFAs (Fig. 1) were much lower in HMP- than in LMP-fed rats. Because citrus pectins have been suggested to be a good

Figure 3. Differential modulation of cecal (A) and colonic (B) microbiota composition by pectins. Correlation triplots based on a redundancy analysis (RDA) depicting the relationship between cecal and colonic microbiota composition and the differences induced by pectins. Proportions of lactate, acetate, propionate, butyrate, branched SCFA, and the different diets were included as explanatory variables. These variables explain 29.4% of total variation, and 59.1% of that variation was explained by the first two canonical axes (A). These variables explain 35.4% of total variation, and 49.9% of that variation was explained by the first two canonical axes (B).

source for acetate production [18], the increased ratio of fermented pectin to cereal NSP in the cecum of HMP-fed rats resulted in higher proportion of acetate and lower proportion of butyrate. The carbohydrate availability in the large intestine also shaped the microbiota by impacting fibrolytic microorganisms, as reported before [29]. The increase of Firmicutes upon dietary supplementation of pectins in the present study might associate with the diet high in DF as reported before [30]. Although a lower Bacteroidetes:Firmicutes ratio was reported in obese people [31] and in *ob/ob* mice [32], more recent

studies showed no evidence for such association [33,34]. More subtle changes occurred in pectin-enriched diet fed individuals at the species level. A significant increase of *Bacteroides* by citrus pectins as reported before [6,7] was not observed here. Our results showed the increased relative abundance of *Lactobacillus* as previously reported [6]. This could be due to the changes in proportions of different fermented DFs as a result of the shifted fermentation site in the large intestine. The increase of *Lactobacillus* for LMP and HMP in the present study was not accompanied by a higher concentration of lactate in the digesta, possibly due to lactate utilization by butyrate-producing Lachnospiraceae, Ruminococcaceae, and Bacteroidaceae [35].

4.3 Influence of branched pectins on fermentation pattern

We also included sugar beet pectin and soy pectin in our investigation. The HG in soy pectin has been reported to be 4–10 residues in length [36], which is quite short compared to HG from sugar beet pectin (± 100 residues) [37]. Despite higher levels of side chains and acetylation than citrus pectin, the HG length of sugar beet pectin is closer to those of both citrus pectins (114–138 residues [37]) than of soy pectin.

In the present study, the total cecal SCFA concentration in SSPS-fed rats was significantly higher than that in CONT-fed rats, which is due to the high fermentability of soy pectin and constant cecal fermentability of other DFs in SSPS-fed rats. In contrast, total cecal SCFA concentration in SBP-fed rats was not different from that in controls, possibly due to the sugar beet pectin-induced shift in fermentation of other DFs. This is similar to high-methyl esterified pectin as discussed above, which could be due to the similarity in HG length and degree of methyl esterification between both pectins. Rapid fermentation of the two branched pectins tended to differentiate from each other with respect to the proportion of individual SCFA in the cecum of rats. Our results are in accordance with previous data that sugar beet pectin fermentation preferably stimulated acetate production, whereas fermentation of soy pectin enhanced propionate production [38].

Sugar beet pectin and soy pectin branched with arabinan and galactan showed different effects on the microbiota than citrus pectins. A strong bifidogenic effect was obtained with arabinan and galactan previously [7], but bifidobacteria poorly utilize polygalacturonic acid, methyl esterified citrus pectin and rhamnogalacturonan oligosaccharides [7]. We did not detect any *Bifidobacterium* in digesta from SBP- and SSPS-fed rats, in line with previous reports [39]. Supplementation of sugar beet pectin and soy pectin resulted in a significant increase of Firmicutes, which was also reported before for citrus pectins [38]. The relative abundance of *Akkermansia* was significantly lower in SBP- and SSPS-fed rats than in CONT, indicating lower levels of mucin degradation. Compared to the two branched pectins, the citrus pectins showed less

inhibition on *Akkermansia*. The difference most probably was due to slight differences in fermentability of the fibers, which is structure dependent. Consistent with our results, AXs and inulin also resulted in lower cecal abundances of the mucin-degrading *Akkermansia* [40]. Although pectins supplemented to the diets have been reported to increase *Akkermansia*, it is only in the high-fat setting [41]. Increased level of *Akkermansia* has been associated with alleviation of obesity and type 2 diabetes [42]. In contrast, in the present study, pectin-a lower relative amount of *Akkermansia* correlated with a lower weight gain of the pectin fed rats (data not shown). Sugar beet pectin and soy pectin supplementation increased the abundance of Lachnospiraceae, Ruminococcaceae, and Bacteroidaceae, several members of which are known for their abilities to produce butyrate for health promotion [8].

Compared to LMP- and HMP-fed rats, a broader variety of microbial enzymes including arabinanases, galactanase, etc., could be present in the cecum of SBP- and SSPS-fed rats, as suggested by the high cecal fermentability of the two complex heteroglycans. Although sugar beet pectin and soy pectin were fermented as quick as the citrus pectins, SCFAs and microbiota were clearly different between the groups. The fermentation of less easily fermentable AXs was also impaired in the large intestine of rats by more easily fermentable pectins. Preferred utilisation of one DF over the other by microbiota in the large intestine of pigs was previously described for resistant starch over AXs [12]. The competitive fermentation could take place within microorganisms able to ferment multiple substrates, or between different species that each have specific substrate preferences in a complex microbial food-web [43]. In the present study, the fermentation patterns of DFs in the cecum of rats were significantly influenced by the supplementation of different pectins (Fig. 3A). In contrast, the differences in microbiota and SCFAs between the rats fed different diets were small in the colon of rats (Fig. 3B). This could be explained by the extensive cecal pectin fermentation, resulting in the situation that DFs available for the colonic microbiota were mainly cereal AXs and cellulose as for controls.

In conclusion, pectin supplementation shapes location of fermentation of DFs by microbiota in the large intestine, which is attributed to the rapid fermentation of pectins and consequently shifted fermentation of other consumed DFs (e.g. cereal AXs). Our results suggest that pectins can confer beneficial health effects through gut microbiota modulation, although the modulating effects were only significant for some of the main genera present. More in-depth studies are necessary. Such modulation by pectins may potentially associate with eating behaviour and fat deposition mediated by SCFAs. It remains to be elucidated whether these effects are valid in other mammals (e.g. humans and pigs) for health-promoting properties.

All authors participated in the design of the study. L.T. analyzed samples, performed data analysis and interpretation, and wrote the manuscript. H.Sc. and H.G. contributed to data in-

terpretation and the manuscript. J.S. and A.S. carried out the rat experiment, contributed to the manuscript. K.B., T.B., and H.Sm. analyzed the microbiota composition, contributed to data interpretation and the manuscript.

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