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Separation and Identification of Individual Alginate Oligosaccharides in the Feces of Alginate-Fed Pigs

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Supporting Information

ABSTRACT: This research aimed to develop a method for analyzing specific alginate oligosaccharides (AOS) in a complex matrix such as pig feces. The data obtained were used to study alginate degradation by the microbiota in the large intestine during adaptation, including the individual variation between pigs. A method using an UHPLC system with an ethylene bridged hybrid (BEH) amide column coupled with MS^n detection was able to distinguish saturated and unsaturated AOS with DP 2−10. Isomers of unsaturated trimer and tetramer could be separated and annotated. In the feces, saturated and unsaturated AOS were present. The presence of unsaturated AOS indicates that the microbiota produced alginate lyase. The microbiota utilized unsaturated AOS more than saturated AOS. The results also suggested that guluronic acid at the reducing end of AOS inhibits the utilization by microbiota during the first weeks of adaptation. After adaptation, the microbiota was able to utilize a broader range of AOS.

KEYWORDS: *alginate, mannuronic acid, guluronic acid, UHPLC, MS, BEH amide*

INTRODUCTION

Alginate is a linear polysaccharide that is composed of guluronic acid (G) and mannuronic acid (M).[1] As a dietary fiber, alginate is not absorbed in the small intestine and becomes a substrate for the microbiota, mainly in the large intestine.[2] It has been shown that the digestibility of alginate by the microbiota in the large intestine of pigs[3] was limited compared to the digestibility of pectin,[4] which is also mainly composed of uronic acids. Even after 39 days of adaptation to the alginate-containing diet, <60% of alginate was utilized by the microbiota in the large intestine.[3] The remaining alginate was excreted in the feces. Less than 10% of the alginate in the feces was soluble in hot water. The water-soluble fraction of alginate in the feces may consist of alginate oligosaccharides (AOS).

In vitro, AOS may have various physiological effects toward different cells, such as human keratinocyte,[5] human endothelial cells,[6] and neuron-like cells.[7] It has also been shown that AOS were able to interact with the immune system of mice in vitro[8] and in vivo after oral administration.[9] Moreover, AOS were demonstrated to have prebiotic activity in vitro[10] as well as in vivo in rats.[11] Additionally, AOS were able to inhibit colonization of the large intestine of chickens by the pathogen *Salmonella enteritidis*.[12]

In most experiments, AOS used in the study were a mixture of unsaturated AOS produced from enzymatic degradation of alginate by alginate lyase. Such mixtures contain AOS with various degrees of polymerization (DP) as well as isomers of AOS with the same DP having different ratios and sequences of G and M residues. It was found that some effects were exerted by AOS with specific chemical structures.[5,6,13] Therefore, it is of interest to develop analytical methodology for the quantification of specific AOS in in vivo samples, such as intestinal digesta or feces.

AOS resulting from alginate degradation in the large intestine are present in a complex matrix. Hence, to be able to investigate which specific AOS are present in digesta or fecal samples, a method that can analyze specific AOS in complex matrices is necessary. Ideally, the method should be able to separate AOS on the basis of the DP as well as separate isomers of the same DP.

Current methods for analyzing specific AOS often involve tedious purification of the specific isomers before analysis of those using MS and NMR.[14,15] Although this method provides the highest accuracy, it may not be applicable for biological samples because the amount of sample available for analysis is often limited and the concentration of AOS in the sample might not allow purification to single isomers.

Separation techniques, such as high-performance anion exchange chromatography with pulsed amperometric detection (HPAEC-PAD) and capillary electrophoresis (CE), were able to separate AOS with different DP and isomers.[16−20] Nevertheless, purified standards are necessary to be able to identify the compounds, and some compounds coeluted.[17] To overcome the need of purified standards and the interference by coelution of different compounds, the separation methods can be coupled with mass spectrometry detection (MS^n). MS^n detection allows identification of the compounds on the basis of their molecular masses and fragmentation patterns. Hence, the use of MS enables the detection of compounds that coelute, if they have different molecular masses. Unfortunately, the HPAEC methods used, which could separate AOS based on DP and isomers,[17] are not compatible with the MS^n detector due to the high salt concentration used during analysis. Other LC-MS^n methods

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that have been applied for AOS include MS^n-compatible AEC[21] and reversed-phase liquid chromatography (RP-LC),[22] but the isomers were not separated[21] or were not identified.[22]

Recently, LC-MS^n with a porous graphitized carbon column[23] or with an ultrahigh-performance liquid chromatography (UHPLC) ethylene bridged hybrid (BEH) amide column[24,25] has been used to separate and identify various neutral and acidic oligosaccharides. For acidic oligosaccharides, such as pectins, the UHPLC-BEH amide column, which separates compounds on the basis of the principle of hydrophilic interaction chromatography (HILIC), was able to separate unsaturated and saturated pectin oligosaccharides, as well as pectin oligosaccharides with different degrees of methylation or acetylation.[24,25] The method used for pectins is assumed to be applicable for alginate, because both pectins and alginate have a backbone of uronic acids.

Hence, the present research aimed to develop an LC-MS^n method using an UHPLC-BEH amide column to separate and identify specific AOS in a complex matrix. It was also aimed to analyze AOS present in the feces of alginate-fed pigs, to understand how alginate is degraded by the microbiota in the large intestine of the pigs during adaptation.

MATERIALS AND METHODS

Unless otherwise noted, the chemicals and solvents used were of analytical grade. Water used for analysis and extractions was Milli-Q water (Millipore, Bedford, MA, USA). The chemicals, solvents, and water used for UHPLC-MS^n eluents were of ULC-MS grade.

Preparation of Alginate Standards. A G-rich alginate and an M-rich alginate were obtained from SKW Biosystems (Rubi, Spain). The alginate (Pectacon M-5761) that was used for the in vivo study[3] was from Acatris (Bunschoten, The Netherlands).

The alginates were fractionated to obtain M-rich and G-rich alginate fractions using the method described by Haug et al.[26] In short, the alginates were partially hydrolyzed in 1 M oxalic acid at 100 °C for 10 h. The fraction insoluble in the acid was solubilized at neutral pH, followed by pH adjustment to 2.85 ± 0.05. At this pH, the insoluble fraction (G-rich fraction) was subsequently separated from the soluble fraction (M-rich fraction) by centrifugation (1500*g*, 10 min, 25 °C). After neutralization to pH 7.0, the alginate fractions were precipitated in 80% (v/v) ethanol and dried overnight in a vacuum oven at 50 °C.

Unsaturated AOS were prepared by dissolving alginate or alginate fractions in water (5 mg/mL) and adjusting the pH to 7.0 ± 0.2. Alginate lyase from *Sphingomonas* sp. (Megazyme, Bray, Ireland) was diluted to 500 μg protein/mL using 100 mM Tris buffer, pH 7.2, containing 1 mg/mL bovine serum albumin (BSA) as was recommended by the supplier. An aliquot of 10 μL of enzyme was added to 1 mL of substrate solution. After incubation in a shaking incubator (40 °C, 24 h), the enzyme was inactivated by boiling for 10 min.

Saturated AOS were prepared by dissolving alginate or alginate fractions in water at 5 mg/mL. After the pH of the solution had been adjusted to 4.0 ± 0.2 using HCl solution, the alginate was partially hydrolyzed at 120 °C for 3 h.[27]

For analyses using UHPLC-MS^n, 100 μL of the 5 mg/mL solutions of AOS was diluted to 1 mg/mL by adding 25 μL of ammonium formate buffer (eluent C given in Analysis of AOS), 125 μL of water, and 250 μL of acetonitrile. After mixing, the mixture was centrifuged (14000*g*, 5 min, 20 °C), and the supernatant was used for analysis.

Extraction of AOS from Pig Feces. Freeze-dried feces were obtained from pigs fed a diet containing retrograded starch and alginate. The experimental setup has been described elsewhere.[3] The experimental protocols were reviewed and approved by the Ethical Committee of Wageningen University (Wageningen, The Netherlands; DEC no. 2011088.c).

AOS were extracted by suspending 500 mg of freeze-dried feces in 5 mL of water, followed by boiling for 15 min with frequent mixing. After centrifugation (14000*g*, 5 min, 20 °C), the supernatant was collected, and the pellet was washed with 3 mL of water. The supernatant from the washing was combined with the first supernatant, as crude extract of soluble materials.

The hydrophobic compounds in the extract were removed using Sep-Pak C18 cartridges (Waters Corp., Milford, MA, USA), which had been activated with methanol and washed with water. The flow-through of the sample was collected, and the cartridge was washed once with water. The washing was combined with the flow-through of the sample. After drying under a stream of air, the dried soluble compounds were resolubilized in 4 mL of water.

An aliquot (225 μL) of this solution was mixed with 250 μL of acetonitrile and 25 μL of ammonium formate buffer (eluent C given in Analysis of AOS). After centrifugation (14000*g*, 5 min, 20 °C), the supernatant was used for analysis using UHPLC-MS^n.

Analysis of AOS. The samples were analyzed using an Accela UHPLC system (Thermo Fisher Scientific, San Jose, CA, USA) coupled with a Velos Pro ESI-Ion Trap-MS^n (Thermo Fisher Scientific). The separation was performed using an Acquity UPLC BEH amide column (1.7 μm, 2.1 mm × 150 mm; Waters Corp.) preceded by a VanGuard BEH amide precolumn (1.7 μm, 2.1 mm × 50 mm), which was kept at 35 °C. An aliquot of 5 μL of sample was injected onto the column using an autosampler while the sample tray was kept at 20 °C. Before injection of the sample, the needle was washed with 20% (v/v) and 75% (v/v) acetonitrile solution, as strong and weak wash, respectively.[24]

The eluents used were water containing 1% (v/v) acetonitrile (A), acetonitrile (B), and a buffer solution containing 200 mM ammonium formate, 2% (v/v) formic acid, and 2% (v/v) acetonitrile (C). A gradient elution was used for analyzing the AOS. The buffer (eluent C) was constantly used at 5% (v/v) of total eluent. The elution started with isocratic elution of 20% A for 1 min, followed by a 50 min gradient from 20 to 45% A to elute the AOS. The column was then cleaned of the remaining compounds using a gradient to 55% A within 4 min and isocratic elution of 55% A for 1 min. Re-equilibration of the column with 20% A was performed for 14 min before injection of the next sample. The flow rate was constant at 500 μL/min, and a 1:9 ASI flow splitter (Analytical Scientific Instruments, El Sobrante, CA, USA) was used so that only 50 μL/min of eluent went into the MS^n system for detection.

The MS detection was in negative mode, with source heater temperature at 225 °C and capillary temperature at 350 °C. The ion source voltage was −4.5 kV. The sheath gas, auxiliary gas, and sweep gas flow were 33, 10 and 2, respectively (arbitrary units). The MS^n collection parameters was set according to the parameters described elsewhere,[24] with the scan range of the MS set at m/z 300−2000. The data were processed using Xcalibur version 2.1.0 SP1 (Thermo Fisher Scientific).

Molecular Mass Distribution. Molecular mass distribution of alginate or alginate fractions were analyzed using high-performance size exclusion chromatography (HPSEC) as described elsewhere.[3]

Alginate Content, Uronic Acid Composition, and Apparent Digestibility of Alginate in Pigs. The analyses of uronic acid composition of alginate, alginate content in pig feces, and the calculation of the apparent digestibility were performed according to the methods described elsewhere.[3]

RESULTS AND DISCUSSION

Uronic Acid Composition of Reference Alginate Materials. The uronic acid compositions of alginate fractions obtained after partial hydrolysis in 1 M oxalic acid and precipitation at pH 2.85 of G-rich alginate, M-rich alginate, and Pectacon alginate are presented in Table 1. The G-rich fraction with the highest G/M ratio (18.6) was obtained from G-rich alginate, and the M-rich fraction with the lowest G/M ratio (0.2) was obtained from M-rich alginate. These fractions were used further as sources of AOS, to assist in the identification of AOS isomers produced from Pectacon alginate, which was the alginate used in the in vivo study (see Building an Alginate Oligosaccharide UHPLC-MSn Library).

Separation of Specific Alginate Oligosaccharides. To obtain unsaturated AOS, the G- and M-rich alginate fractions and Pectacon alginate were degraded using alginate lyase. Saturated

Table 1. Uronic Acid Composition of Alginate and Alginate Fractions Obtained from Partial Acid Hydrolysis and Separation by Precipitation at pH 2.85

sample	mol % G[a]	mol % M[b]	G/M
M-rich alginate	49	51	1.0
insoluble at pH 2.85	92	8	12.0
soluble at pH 2.85	**13**	**87**	**0.2**[c]
G-rich alginate	78	22	3.5
insoluble at pH 2.85	**95**	**5**	**18.6**[d]
soluble at pH 2.85	22	78	0.3
Pectacon M-5761	70	30	2.3
insoluble at pH 2.85	95	5	17.9
soluble at pH 2.85	18	82	0.2

[a]G, guluronic acid. [b]M, mannuronic acid. [c]M-rich fraction. [d]G-rich fraction.

AOS were obtained by partial hydrolysis of alginates in HCl solution at pH 4.0. The extent of alginate degradation by the two depolymerization methods is illustrated by the change in molecular mass as analyzed by HPSEC (Figure 1). The alginate and alginate fractions were degraded by acid, as shown by the lower molecular masses of the molecules in the acid hydrolysates compared to the molecular mass of the starting material. It was also shown in Figure 1 that the extents of depolymerization by acid were similar for the Pectacon alginate and the selected alginate fractions.

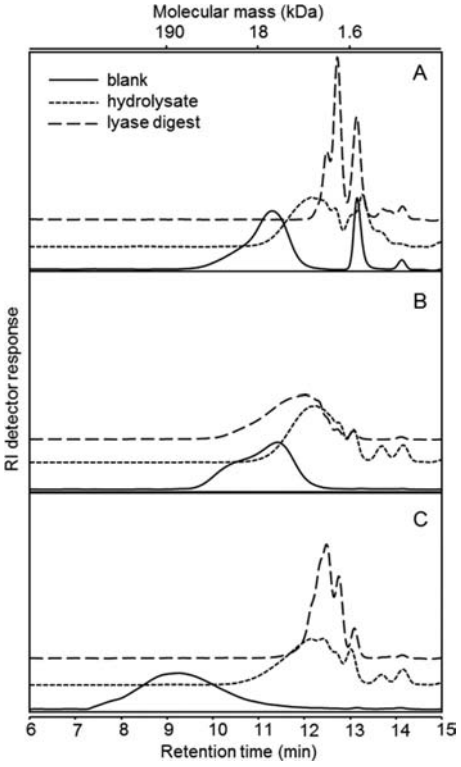

Figure 1. HPSEC elution patterns of M-rich alginate fraction (A), G-rich alginate fraction (B), and Pectacon alginate (C) before (blank) and after partial hydrolysis in acid at pH 4.0 (hydrolysate) or after digestion with alginate lyase (lyase digest). The upper *x*-axis shows the molecular mass based on calibration using pullulan standards.

Figure 1 also demonstrates that the alginate and alginate fractions were considerably degraded by alginate lyase. Pectacon alginate and M-rich alginate fraction were more degraded by alginate lyase than the G-rich alginate fraction (Figure 1B). This can be explained by the specificity of the commercial alginate lyase toward poly-β-D-mannuronate, as was described by the supplier. Nevertheless, the enzyme was not strictly specific, and part of the lyase digest of the G-rich alginate fraction had a molecular mass similar to that of the hydrolysate. Therefore, AOS are expected to be present in lyase digests and hydrolysates of the alginate and alginate fractions.

The unsaturated AOS in the lyase digests and saturated AOS in the hydrolysates were then analyzed by UHPLC-MS^n. The elution gradient that was previously developed for galacturonic acid oligomers[24] was modified to obtain a better separation between AOS isomers. Unsaturated and saturated AOS with DP 2−10 were identified on the basis of the m/z values of the parent ions (Figure 2). Clusters of peaks with similar m/z values are isomers of AOS with the same DP. Figure 2 showed that unsaturated AOS eluted before saturated AOS with the same DP. This has also been found for unsaturated and saturated pectin oligomers analyzed using the BEH amide column and was assumed to be due to the lack of the hydroxyl group at the C4 position of the uronic acid residue at the nonreducing end of unsaturated pectin oligomers.[24]

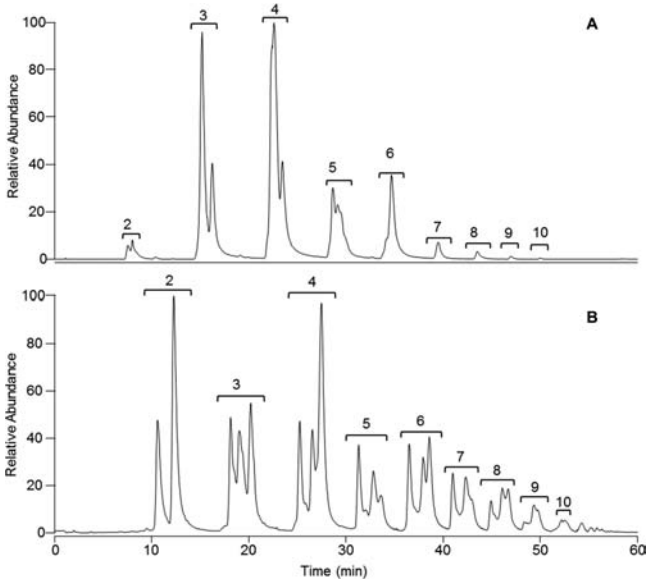

Figure 2. Elution patterns of unsaturated (A) and saturated (B) alginate oligosaccharide (AOS) separated by the UPLC-BEH amide column coupled with MS detection. The AOS were obtained from Pectacon alginate after digestion by alginate lyase and after partial acid hydrolysis, respectively. The numbers denote the degree of polymerization of the AOS.

Building an Alginate Oligosaccharide UHPLC-MS^n Library. The elution gradient used for AOS was able to partially separate isomers, especially those with DP 2−4, as indicated by the clusters of peaks with similar m/z values (Figure 2). Due to the unavailability of purified standards for each isomer, the isomers were identified on the basis of the paper of Zhang et al.,[27] in which MS^2 spectra of different purified AOS were described. The MS^2 fragments were annotated according to the nomenclature described by Domon and Costello.[28]

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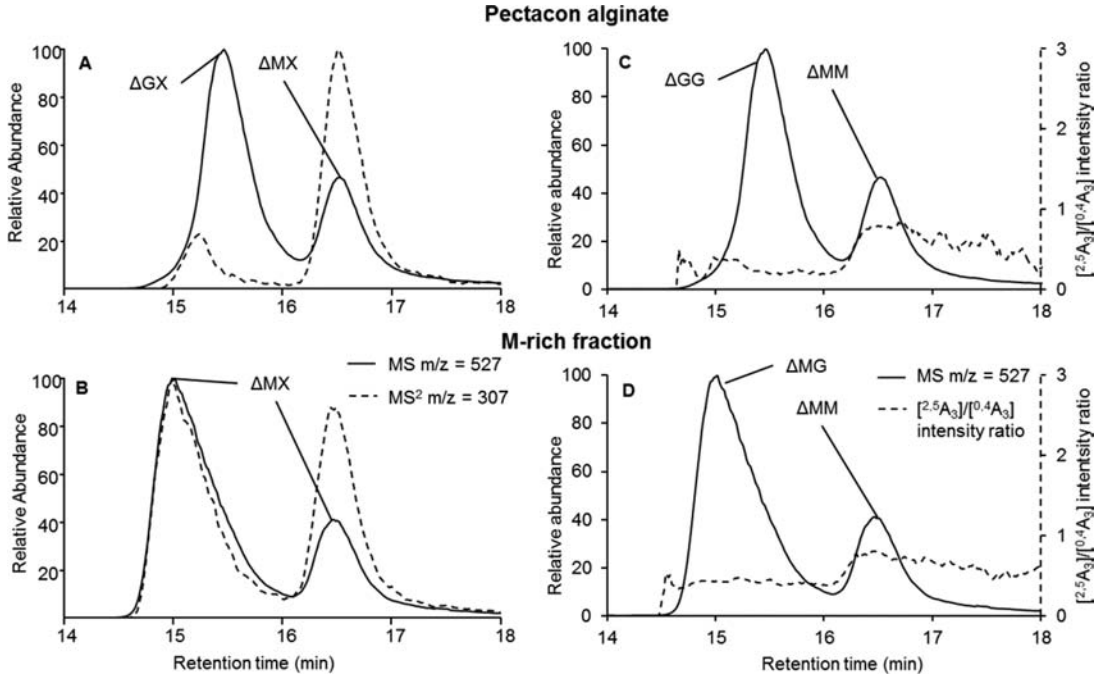

Figure 3. Zoomed-in UHPLC-MS^n patterns of unsaturated trimer isomers from Pectacon alginate and alginate fraction rich in mannuronic acid (M-rich fraction). The internal residues were identified on the basis of the patterns of d-Z_2 (m/z 307; A, B). The reducing end was annotated on the basis of the $[^{2,5}A_3]/[^{0,4}A_3]$ intensity ratio (C, D). Δ, 4-deoxy-L-erythro-hex-4-enopyranosyluronate; G, guluronic acid residue; M, mannuronic acid residue; X, unknown uronic acid residue.

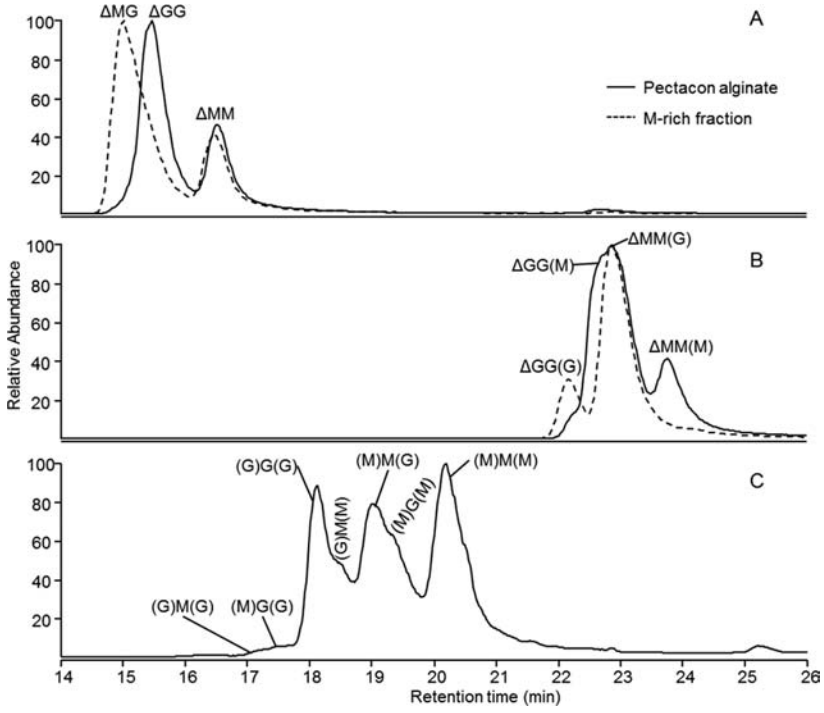

Figure 4. Summarized overview of the UHPLC-MS^n elution patterns of isomers of unsaturated alginate trimers, m/z 527 (A), unsaturated alginate tetramers, m/z 703 (B), and saturated alginate trimers, m/z 545 (C), separated on the UPLC BEH amide column with online MS^n detection. Δ, 4-deoxy-L-erythro-hex-4-enopyranosyluronate; G, guluronic acid; M, mannuronic acid. The parentheses indicate putative annotation of the sugar residue.

Identification of isomers was first attempted for the unsaturated trimers (Figure 3). Unsaturated AOS have uniform nonreducing ends because 4-deoxy-L-erythro-hex-4-enopyranosyluronate (Δ; "unsaturated uronic acid residue") is formed at the nonreducing end regardless of the type of uronic acid.[29] Hence, there are four possible isomers of unsaturated trimer: ΔGG, ΔGM, ΔMG, and ΔMM. In the Pectacon alginate lyase digest, there seemed to be two isomers of unsaturated trimer, as shown by the two peaks in Figure 3A.

The identification of the internal residues was based on the presence of decarboxylated Z_n ions (d-Z_n) in the MS^2 spectra. It has been reported that AOS with an M internal residue produced

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a fragment of decarboxylated Z_n ions (d-Z_n), whereas an isomer with a G internal residue did not produce this fragment.[27] For unsaturated trimer, the respective ion is d-Z_2 (m/z 307). Figure 3A shows that the trimer eluting at 16.53 min had the d-Z_2 fragment in its MS^2 spectra. Hence, it could be concluded that this trimer contained an internal M residue. In the MS^2 spectra of the other trimer, which was eluting at 15.47 min, the d-Z_2 fragment in its MS^2 spectra was also present, although the intensity was low. Nevertheless, the highest intensity was reached at a slightly lower retention time (15.24 min), indicating that there was coelution of different isomers. By comparing the results with those of the lyase digest of the M-rich alginate fraction (Figure 3B), it became clear that there is another trimer with an M internal residue eluting at around 15.02 min. As the retention times of the same compounds from the same sample can shift up to 0.3 min due to analytical variations, it is possible that the isomer in the Pectacon digest that eluted at 15.24 min is the same as the isomer in the digest of M-rich alginate fraction that eluted at 15.02 min. Therefore, it could be concluded that the Pectacon alginate lyase digest contained three isomers of unsaturated trimer: two isomers with an M internal residue, one of which was present in low amount and partly coeluted with the isomer containing a G-internal residue. The results illustrate that an overlay of the full MS base peak chromatogram for m/z 527 and the MS^2 base peak chromatogram for d-Z_2 (m/z 307) can assist the identification of coeluting isomers when they have different internal residues.

After the internal residues were recognized, the reducing ends of the unsaturated trimers were annotated by comparing the intensities of internal fragmentation products $[^{2,5}A_3]$ and $[^{0,4}A_3]$, which have m/z values of 449 and 453, respectively. Zhang et al.[27] reported that the ratio would be lower when the reducing end was G than when the reducing end was M. As the ratio is also influenced by the internal uronic acid residues, the method is best applicable for annotating isomers that differ only at the reducing end. By overlaying the ratio obtained at every time point with the full MS base peak chromatogram for DP 3 (m/z 527), the reducing ends of the unsaturated trimer with M internal residue could be identified (Figure 3C,D). For the isomer with G internal residue (retention time 15.47 min), the $[^{2,5}A_3]/[^{0,4}A_3]$ ratio was low. Hence, the reducing end was annotated as G, representing an isomer of ΔGG (Figure 3C). Using this method, the three isomers of unsaturated trimer could be identified. ΔMG eluted before ΔMM, suggesting that isomers with G at the reducing end elute earlier than similar isomers with M at the reducing end, as is summarized in Figure 4A.

A similar approach was used to annotate the isomers of unsaturated tetramer (Supporting Information, Figure S-1), but the annotation of the reducing end was more complicated because of the large overlap between the peaks (Supporting Information, Figure S-1C,D). In this case, the information obtained from the trimers, that isomers with G at the reducing end may elute earlier, was used to annotate the reducing ends. As a result, four isomers of unsaturated tetramer were putatively identified (Figure 4B).

For isomers of saturated AOS trimer, the annotation was more challenging, because the nonreducing end can be either a G or an M residue. As a consequence, theoretically there can be eight isomers for a trimer: GGG, GGM, GMG, MGG, MGM, MMG, GMM, and MMM. Following the described approach, the internal residues were identified from the d-Z_2 fragment, and the reducing end as well as the nonreducing end was annotated putatively by comparing the $[^{2,5}A_3]/[^{0,4}A_3]$ ratio, combined with the assumption that the presence of G decreases the retention time. In addition, the abundance of the isomers in G-rich alginate fraction or M-rich alginate fraction (Supporting Information, Figure S-2) was also taken into account. For example, an isomer of saturated AOS trimer that is abundant in G-rich fraction (G/M ratio 18.6) was expected to be rich in G. As a result, seven isomers in the acid hydrolysate of Pectacon alginate were putatively annotated. The elution behavior as well as the annotation of isomers of saturated trimer is summarized in Figure 4C.

Figure 4 shows that part of the peak clusters illustrated in Figure 2 was formed by isomers of AOS. Despite the inability to absolutely annotate each isomer peak, the analysis method described above was proven to be useful for analyzing specific AOS in complex mixtures. The information obtained and the AOS UHPLC-MS^n library were then applied for analyzing AOS in pig feces to understand the mechanism of alginate degradation in the large intestine of pigs.

Alginate Oligosaccharides in Pig Feces. In previous research, it has been shown that after feeding with a diet containing 5%(w/w) alginate, the feces of the pigs contained 15−20% alginate based on dry matter, even after 74 days of adaptation, with <10% (w/w) of the alginate being water-soluble.[3] Despite the low amount of soluble alginate, the presence of AOS could be detected. An example of the elution patterns obtained from the water-soluble fecal extract is presented in Figure 5. In the base peak chromatogram (Figure 5A), a number of AOS could already be recognized. By filtering the base peak chromatogram for m/z values specific for AOS parent masses, it was shown that both unsaturated AOS (Figure 5B) and saturated AOS (Figure 5C) were present in the feces.

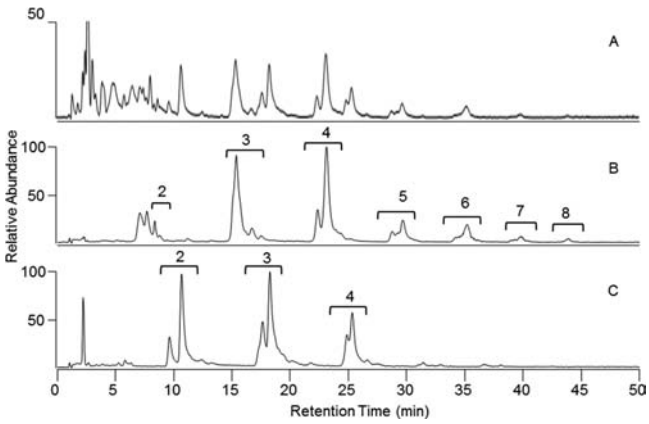

Figure 5. Elution patterns of alginate oligosaccharides (AOS) extracted from the feces of an alginate-fed pig (pig E, day 7), after separation by the UPLC-BEH amide column coupled with MS detection. The base peak chromatogram (m/z 300−2000) (A) was filtered for the m/z values of unsaturated AOS (B) and saturated AOS (C) as necessary. The numbers denote the degree of polymerization of the AOS.

Assuming that the MS signal intensities are similar for DP 2−8, the total amount of AOS is reflected by the peak area of the AOS. For comparison between samples from the same pig, the AOS peak area for a fecal sample from a given pig was compared relatively to the highest AOS peak area achieved for the same pig (Figure 6). For both pigs, the highest total AOS content in the feces was achieved on day 7. The average total transit time of feed in the gastrointestinal system of growing pigs (initial average body weight = 30 kg) was 45 and 37 h for solid and liquid phases, respectively.[30] Therefore, the increase of total AOS content in

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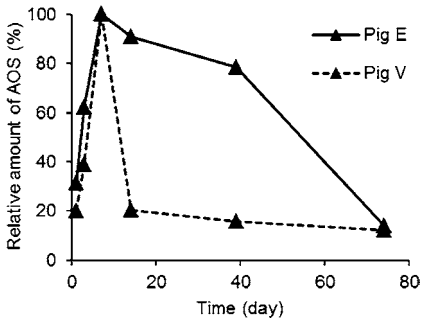

Figure 6. Relative amounts of AOS in the feces of pigs during 74 days of feeding with alginate-containing diet. The total AOS content in the feces of a given pig is expressed in comparison to the highest total AOS content reached for the same pig, based on total AOS peak area as analyzed by UHPLC-MS^n.

the feces between days 3 and 7 suggested that the microbiota was adapting to alginate, and alginate-degrading enzymes were produced. Nevertheless, after 74 days, the total AOS contents in the feces of both pigs were similarly low, suggesting, besides being able to degrade alginate to AOS, the microbiota was also able to utilize the AOS efficiently. The more efficient utilization of AOS was supported by the lower fecal alginate content on day 74 compared to alginate content on day 39 (Table 2). Between

Table 2. Total and Soluble Alginate Contents and Apparent Digestibility of Alginate in the Feces of Alginate-Fed Pigs during a Feeding Period up to 74 Days

	soluble alginate		total alginate		apparent digestibility	
time (days)	% (w/w)	G[a]/M[b] ratio	% (w/w)	G/M ratio	G	M
pig E						
14	1.8	2.2	21.8	4.5	0.10	0.54
39	1.7	1.9	23.6	4.8	0.21	0.61
74	0.5	2.7	17.0	9.4	na[c]	na
pig V						
14	0.9	2.0	21.2	4.6	0.16	0.57
39	0.9	1.6	23.9	4.5	0.24	0.61
74	0.4	1.9	19.1	6.1	na	na

[a]G, guluronic acid. [b]M, mannuronic acid. [c]na, apparent digestibility for day 74 could not be calculated because the sample did not contain any indigestible marker.

days 7 and 74, there was individual variation between the pigs in alginate degradation, which will be addressed in more detail under Individual Variation between Pigs in Alginate Degradation.

The same data obtained from the UHPLC-MS^n that were used to determine the total AOS content were also used to quantify unsaturated and saturated AOS of different DP. Early in the adaptation period (days 1−3), the relative amount of unsaturated AOS was lower than (pig E) or similar to (pig V) the amount of saturated AOS (Figure 7). As the pigs adapted to the diet, the proportion of the unsaturated AOS increased, with individual differences between pigs. The highest proportion of unsaturated AOS was reached on day 39 for pig E, whereas it was reached on day 7 for pig V. After reaching its maximum, the proportion of unsaturated AOS declined gradually during the feeding period.

The increasing amounts of unsaturated AOS during the early period of adaptation (Figure 7) showed that the inclusion of

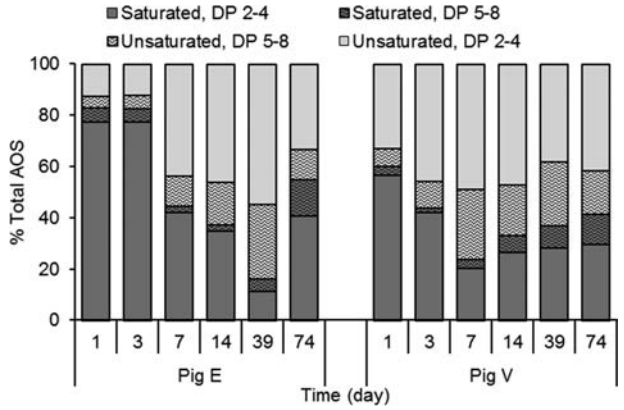

Figure 7. Proportions of unsaturated and saturated alginate oligosaccharides (AOS) with different degrees of polymerization (DP) in the feces of pigs during 74 days of feeding with alginate-containing diet. The proportions are based on the total AOS peak area based on UHPLC-MS^n elution patterns.

alginate in the diet stimulated the production of alginate lyase by the microbiota in the large intestine of pigs. It has been reported that alginate lyase, which comprises the majority of alginate-degrading enzymes,[29] is also produced by rumen microbiota[31] and human large intestinal microbiota.[32]

With the assumption that alginate lyases were the main alginate-degrading enzymes produced by the microbiota, the saturated AOS were formed only from the nonreducing end of every alginate molecule. Hence, saturated AOS were expected to be present in small amounts compared to unsaturated AOS. In contrast, on days 1−3 the amounts of saturated AOS were relatively high compared to unsaturated AOS (Figure 7). This indicates that before the microbiota was adapted to alginate, saturated AOS accumulated. With a longer adaptation period, the microbiota has more ability in utilizing saturated AOS, but the utilization of saturated AOS was still lower than the utilization of unsaturated AOS, as was shown by the increasing proportion of saturated AOS relative to that of unsaturated AOS between days 39 and 74 for both pigs.

By zooming in on the AOS elution patterns for the fecal samples (Figure 5) and comparing the retention times and the MS^n spectra of the AOS isomers that were present in the feces with the retention times of putatively identified AOS (see Building an Alginate Oligosaccharide UHPLC-MSn Library), the different isomers of saturated trimer, unsaturated trimer, and unsaturated tetramer of the AOS in the fecal samples could be annotated. Figure 8 depicts the identified AOS, which were present in the fecal samples on day 7. For both pigs, the dominant unsaturated trimer was ΔGG, followed by ΔMG (Figure 8A). The dominant unsaturated tetramers were ΔGG(G) and ΔMM(G) (Figure 8B). AOS composed of only M residues (ΔMM and ΔMMM) were present in very low amounts. From the results of the apparent digestibility (Table 2), it is shown that M was utilized more than G. Hence, it is concluded that the low amounts of ΔMM and ΔMMM were a result of extensive utilization and not because of a low digestibility of M by the microbiota.

The preferential utilization of ΔMM and ΔMM(M) over ΔMG and ΔMM(G) up to day 7 indicates that the presence of G at the reducing end inhibits the utilization of the AOS in a nonadapted situation. This was also supported by the remaining saturated trimers (Figure 8C). Nevertheless, after a longer adaptation period up to 74 days, there was no accumulation of

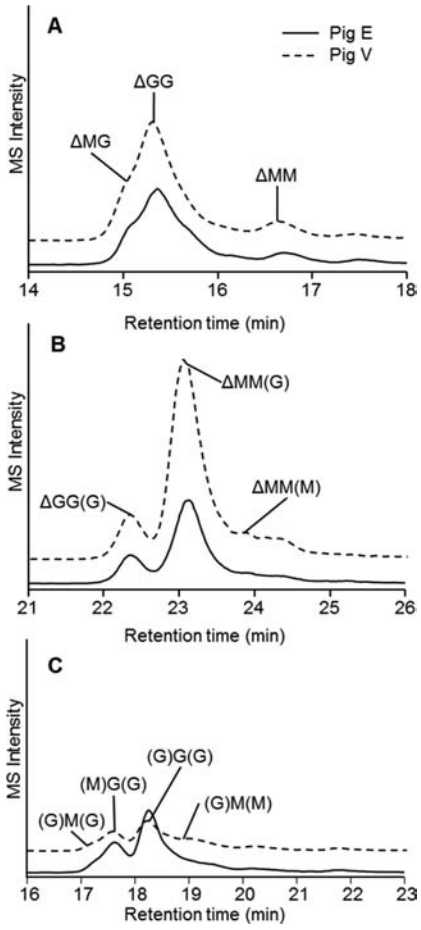

Figure 8. Zoomed-in UHPLC-MS^n elution patterns of unsaturated alginate oligosaccharides (AOS) trimer (A), unsaturated AOS tetramer (B), and saturated AOS trimer (C) in the feces of two alginate-fed pigs 7 days after the pigs were fed solely with experimental diets. Δ, 4-deoxy-L-erythro-hex-4-enopyranosyluronate; G, guluronic acid; M, mannuronic acid. The parentheses indicate putative annotation of the sugar residue. The complete elution pattern of the AOS in the feces of pig E can be viewed in Figure 5.

specific AOS (results not shown), which means that after adaptation the microbiota was able to utilize a broad variation of AOS.

Individual Variation between Pigs in Alginate Degradation. The AOS composition in the feces was also used for investigating individual variation between the two pigs used in this study, specifically on alginate degradation in the large intestine. Figure 7 shows that the highest proportion of unsaturated AOS was achieved more quickly for pig V than for pig E. This result seemed to indicate that pig V adapted to alginate more quickly than pig E. This hypothesis was supported by the comparison of the total AOS content of the samples (Figure 6). Although both pigs reached the highest total AOS content on day 7, for pig V it decreased sharply between days 7 and 14. On the contrary, the total AOS content for pig E was about constant up to day 39. The steep decline of total AOS content for pig V indicated rapid utilization of AOS, which signifies quick adaptation to alginate.

Individual variation between pigs is also evident from the apparent digestibility data (Table 2). On day 14, the digestibility of G for pig E (0.10) was lower than that for pig V (0.16). This again supports the assumption of rapid adaptation of pig V compared to pig E. After adaptation for 39 days, the digestibilities of G and M were similar for the two pigs, but the feces of pig E contained more soluble alginate compared to pig V. On day 74, despite having similar soluble alginate levels, pig E had a lower total alginate content with a higher G/M ratio than that of pig V. The soluble alginate content in the feces of pig E also had a higher G/M ratio than in the feces of pig V. With a high G/M ratio for soluble alginate, it was expected that there would be relatively more G-containing AOS in the feces of pig E on day 74. However, this difference was not observed in the AOS profile due to the very low amounts of AOS with DP 2−4 and the inability to identify isomers larger than DP 4, which were present in higher amounts than the smaller AOS (Figure 7). Nevertheless, the lower total alginate content and higher G/M ratio on day 74 suggest that the microbiota in pig E may have a higher ability to solubilize and utilize G than the microbiota in pig V. These results show that the microbiota composition in the large intestine was influenced not only by diet but also by other factors. Individual genetic variation may also have an important role, as has been reported before.[33]

In summary, the presence of AOS in the feces of alginate-fed pigs demonstrated that oligosaccharides are present in the large intestine as results of polysaccharide degradation. The ability to identify these oligosaccharides, such as demonstrated in this paper for AOS, is essential to the assessment of the presence of certain oligosaccharides once they are shown to be potentially physiologically active through metabolic processes in the large intestine.

■ ASSOCIATED CONTENT

Supporting Information

Figures for putative annotation of unsaturated tetramer and saturated trimers. This material is available free of charge via the Internet at http://pubs.acs.org.

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Notes

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