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Modulating the Gut Microbiota with Alginate Oligosaccharides In Vitro

Grégoire Bouillon [1,2,†], Olav Gåserød [2,‡], Łukasz Krych [1], Josué L. Castro-Mejía [1], Witold Kot [3], Markku T. Saarinen [4], Arthur C. Ouwehand [4,*], Dennis S. Nielsen [1] and Fergal P. Rattray [1,§]

1 Department of Food Science, University of Copenhagen, Rolighedsvej 26, Frederiksberg C, DK-1958 Copenhagen, Denmark
2 International Flavors and Fragrances, Industriveien 33, N-1337 Sandvika, Norway
3 Department of Plant and Environmental Sciences, University of Copenhagen, Thorvaldsensvej 40, Frederiksberg C, DK-1871 Copenhagen, Denmark
4 International Flavors and Fragrances, Sokeritehtaantie 20, 02460 Kantvik, Finland
* Correspondence: arthur.ouwehand@iff.com; Tel.: +358-40-5956-353
† Current address: Chr. Hansen A/S, Bøge Alle 10, 2970 Hørsholm, Denmark.
‡ Current address: Gnosis by Lesaffre, Mustads vei 1, 0283 Oslo, Norway.
§ Current address: Novozymes A/S, Biologensvej, 2, DK-2800 Lyngby, Denmark.

Abstract: Alginate oligosaccharides (AOS) are non-digestible carbohydrates from brown kelp. As such, they are dietary fibers and may have prebiotic potential. Therefore, we investigated the capacity of gut bacteria to utilize AOS with single-strain cultures and as a complex bacterial community. *Bifidobacterium adolescentis*, *Lacticaseibacillus casei* and *Lacticaseibacillus paracasei* showed weak growth (relative to unsupplemented medium; $p < 0.05$) in the presence of AOS and alginate, while strong growth ($p < 0.01$) was observed for *Bacteroides ovatus* when grown with alginate as carbohydrate source. *Enterococcus faecium* and *Enterococcus hirae* were for the first time reported to be able to grow on AOS. Further, AOS as substrate was investigated in a complex bacterial community with colonic fermentations in an in vitro gut model. The in vitro gut model indicated that AOS increased short-chain fatty acid (SCFA) levels in donors with a low endogenous SCFA production, but not to the same level as inulin. *Bacteroides* was found to dominate the bacteria community after in vitro gut simulation with alginate as substrate. Further, stimulation of *Bacteroides* was observed with AOS in the gut model for two out of three donors with the third donor being more resistant to change. Our results allowed the identification of AOS utilizers among common gut species. The results also demonstrated the capacity of AOS to elevate SCFA levels and positively modulate the gut microbiota during in vitro simulated colon fermentations, although some subjects appear to be resilient to perturbation via substrate changes.

Keywords: prebiotic; alginate; alginate oligosaccharides; intestinal microbiota; microbial metabolites; short-chain fatty acids; colon simulation

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1. Introduction

In recent decades, development of functional foods has rapidly become a major focus of research [1]. The capacity of a food ingredient to selectively stimulate the growth of beneficial microbes is a key criterion for a prebiotic effect [2]. Numerous studies have been conducted to better understand the role and composition of the gut microbiota (GM) and its modulation by nutrition [3]. The composition of the GM has been reported to be highly variable even among people of the same region of the world [4]. Moreover, although a large number of oligosaccharides have been studied for prebiotic effects and are commonly incorporated into certain food preparations [5] their utilization by beneficial and nonbeneficial microbes is still open to different interpretations [6]. This underlines the need for a deeper understanding of the modulatory effect of a prebiotic food ingredient on the GM prior to its incorporation in a food product.

Alginate is an abundant polymer and represents up to 40% of the dry matter of brown seaweed, with about 30,000 metric tons produced industrially every year [7]. Alginate is a linear and naturally occurring copolymer of two main sugars: β-D-mannuronate (M) and α-L-guluronate (G) linked in 1,4-glycosidic bonds and organized in consecutive G residues (GGGGGG), M residues (MMMMMM), or alternating M and G residues (GMGMGM) [8]. Alginate is used in the food industry as a thickening agent with its viscosity directly correlated to its molecular weight and concentration. Moreover, alginate has the ability to form heat-stable gels through G-block-Ca^{2+} interactions described as the "egg-box model", where the calcium ions sit in the structural void of the polymer and form hydrogels by trapping water molecules [9]. Consequently, the gelling and thickening properties of alginate make it one of the most extensively used additives in the food industry [10,11].

The depolymerized form of alginate known as alginate oligosaccharide (AOS) has been investigated for diverse applications in the food and agriculture industries but also for human health applications [12]. Alginate is poorly digested in vivo, and the porcine microbiota has been reported to require a 39-day adaptation period prior to being able to degrade the oligomer [13]. After adaptation, the porcine microbiota was able to successfully utilize a broad range of AOS [14]. In rats, daily intake of AOS (2.5% of diet) over a two-week period increased fecal bifidobacteria and lactobacilli by 13-fold and 5-fold, respectively, while the abundance of *Enterobacteriaceae* and enterococci decreased [15]. However, in vitro fermentation of AOS using pig fecal samples or human fecal samples increased the abundance of *Bacteroides* and the production of short-chain fatty acids (SCFA), with no effect on the bifidobacteria or lactobacilli community [16,17]. *Bacteroides ovatus* was also established as the main gut bacterium able to degrade the 1,4-glycosidic bond of alginate [18]. While current literature indicates different effects of AOS on the gut microbiota, it also clearly demonstrates its potential as a gut microbiota modulatory agent.

In this study, a screening of several main representative bacteria of the GM was carried out in a single-strain approach to investigate their capacity to use AOS for growth. The modulation of the GM by the fermentation of AOS was further investigated using an in vitro colon model, CoMiniGut [19]. The dynamics of the simulated GM community was studied using Illumina-based NxtSeq-based 16S rRNA gene amplicon high throughput sequencing, and SCFA production was estimated using gas-chromatography analysis. To the best of our knowledge this study is the first to combine the single-strain approach of a broad range of gut bacteria with the use of an in vitro gut model, and will help to reach a better understanding of the prebiotic potential of AOS.

2. Materials and Methods

2.1. Substrates and Strains

Sodium alginate was extracted from the stem of the brown seaweed *Laminaria hyperborea* (supplied by IFF, Sandvika, Norway). The alginate was subsequently subjected to high-temperature acid hydrolysis, followed by neutralization with Na_2CO_3 before spray drying in order to produce the alginate oligosaccharide (AOS) with a purity of at least 90% [20]. Inulin was purchased from Sigma Aldrich (Søborg, Denmark). All bacteria used in the Bioscreen C study (Table 1) were collected from frozen stocks kept at both IFF facilities and the University of Copenhagen, or supplied from public collections. These strains were selected according to the following criteria: relevance for human gut microbiota and with published work with AOS, to investigate potential pre- and probiotic synergy, and availability in the culture collections.

Table 1. Growth after 24 h under anaerobic conditions of gut bacteria in basal medium supplemented with either glucose, AOS, alginate or inulin (n = 6).

Species	Source *	Strain Name	Glucose [a]	AOS [a]	Alginate [b]	Inulin [a]
Bifidobacterium breve	BCCM/LMG	LMG 13208	+++	-	-	-
Bifidobacterium longum	IFF	Bl-05	+++	-	-	-
Bifidobacterium lactis	IFF	Bl-04	+++	-	-	-
Bifidobacterium bifidum	IFF	Bb-06	+++	-	-	+++
Bifidobacterium adolescentis	DSMZ	DSM 20083	+++	+	+	+++
Bacteroides vulgatus	BCCM/LMG	LMG 17767	+++	-	-	-
Bacteroides acidifaciens	DSMZ	DSM 15896	+++	-	-	-
Bacteroides thetaiotaomicron	DSMZ	DSM 2079	+++	-	-	-
Bacteroides fragilis	DSMZ	DSM 2151	+++	-	-	-
Bacteroides ovatus	DSMZ	DSM 1896	+++	-	++	-
Escherichia coli	ATCC	ATCC 43888	+++	-	-	+++
Enterobacter cloacae	UCPH	NTCT 11572	+++	-	-	-
Klebsiella pneumoniae	UCPH	c132-98 WT	+++	-	-	-
Salmonella enterica Typhimurium	UCPH	SML 27C	+++	-	-	-
Cronobacter sakazakii	DSMZ	DSM 4485	+++	-	+	-
Lactobacillus acidophilus	BCCM/LMG	LMG 9433	+++	-	-	-
Lacticaseibacillus rhamnosus	DSMZ	DSM 20021	+++	-	-	-
Lacticaseibacillus casei	DSMZ	DSM 20011	+++	+	+	-
Lacticaseibacillus paracasei	NCIMB	NCFB 151	+++	+	+	+++
Enterococcus faecium	DSMZ	DSM 2146	+++	+	-	++
Enterococcus hirae	DSMZ	DSM 3320	+++	+	+	++
Clostridium clostridioforme	DSMZ	DSM 933	+++	-	-	-
Anaerostipes hardus	DSMZ	DSM 3319	+++	-	-	-

[a] added at 10 g L^{-1}; [b] added at 4 g L^{-1} (-) no growth; (+) weak growth; (++) moderate growth; (+++) strong growth. * Source of bacteria strain: Belgium Coordinated Collection of Microorganism (BCCM/LMG); German Collection of Microorganism and Cell Culture (DSMZ); International Flavors & Fragrances (IFF); National Collection of Industrial, Food and Marine Bacteria (NCIMB); American Type Culture Collection (ATCC); University of Copenhagen (UCPH).

2.2. Basal Colon Media

Basal medium was prepared as described by Wiese et al. [19]. Briefly, the following reagents (all purchased from Merck, Darmstadt, Germany) were dissolved in one liter of ultrapure water (Milli-Q®, Merck, Darmstadt, Germany): 0.5 g bile salts, 2 g peptone, 2 g yeast extract, 0.1 g NaCl, 0.04 g K_2HPO_4, 0.04 g KH_2PO_4, 0.01 g $MgSO_4 \cdot 7H_2O$, 0.01 g $CaCl_2 \cdot 6H_2O$, 2 g $NaHCO_3$ and 2 mL Tween-80. After full dissolution, the pH of the medium was adjusted to 5.6 using 1M HCl and anaerobic conditions were generated using the Hungate boiling system prior to autoclaving at 121 °C for 20 min. Finally, 0.002 g hemin, 10 µL vitamin K1 and 0.5 g L-cysteine HCl were aseptically added under anaerobic conditions. The medium was further supplemented with 10 g L^{-1} of glucose, AOS or inulin, used as a reference prebiotic. A concentration of 4 g L^{-1} of alginate was used to avoid too high a level of viscosity. Once all ingredients were fully dissolved, the medium was filter-sterilized and kept anaerobically at 4 °C in the dark, prior to use in both in vitro screening method using Bioscreen C (Growth Curves Ltd., Helsinki, Finland) and in vitro fermentation within the CoMiniGut.

2.3. Single-Strain Growth Experiment

The single-strain growth experiment was performed as described by Mäkeläinen et al. [21]. Prior to each run, pre-cultures were prepared in fresh glucose containing medium to maximize the growth over 24 h. The cultures were centrifuged at 4000× g for 10 min at 4 °C. The pellet was resuspended in saline solution (0.9% NaCl) and diluted accordingly to obtain a 10^6 CFU mL^{-1} inoculum. Twenty µL of inoculum was anaerobically added to 180 µL of each medium into a 10 × 10-well honeycomb plate. All treatments and appropriate controls were tested in triplicate, and every experiment was performed in duplicate (n = 6). Incubation was carried out using a Bioscreen C. Anaerobic conditions (80% N_2, 10% H_2, and 10% CO_2) were maintained using a Concept 400 anaerobic chamber (Ruskin Technologies, Leeds, UK). The optical density (OD 600 nm) was measured every 30 min for 24 h. The area

under the curve for each growth kinetic was computed after subtracting the blank values. Significant differences between data sets compared to the basal medium were determined by two-way ANOVA, and $p < 0.05$ was considered significant.

2.4. Faecal Sample Preparation

Fresh stool samples were collected from 3 healthy adults (18–65 years of age), healthy as self-reported and without antibiotics or probiotics intake 3 months prior to donation. The samples were donated anonymously and no further details were collected in accordance with the ethical approval (Ethical Committee of the Capital Region of Denmark, registration number H-20028549) were collected and homogenized in a 1:1 ratio with phosphate saline solution (per liter: 8 g NaCl, 0.2 g KCl, 1.44 g Na_2HPO_4 and 0.24 g K_2HPO_4) with 30% (*v/v*) glycerol in a stomacher bag for 120 s using a stomacher (Stomacher 400; Seward, Worthing, UK) at medium speed. Due to limitation in the collection process, fecal slurries with a final glycerol content of 15% (*v/v*) were aliquoted into cryotubes and stored at −60 °C prior to further use to minimize variation between fermentation. Faecal sample preparations were used separately and not pooled.

2.5. In Vitro Gut Model CoMiniGut

The in vitro gut fermentations were carried out using the CoMiniGut model as described by Wiese et al. [19]. In brief, the model consisted of a climate box with five parallel anaerobic reactor units. A circulating water bath connected to a heat-exchange plate and coupled with a ventilation system ensured a stable and evenly distributed heat throughout the experiment within the climate box. Each unit contained a 5 mL reaction vessel with a magnetic stirrer, an anaerogen bag (AN0020D; ThermoScientific, Waltham, MA, USA) and a resazurin indicator (Anaerobe Indicator Test; Sigma-Aldrich, St. Louis, MO, USA). The units were placed on a magnetic stirrer bench and pH probes were inserted into each reactor. The pH was monitored using a 6-channel pH meter and a data logger (Consort multi-parameter analyzer C3040) and connected to a laptop running Matlab scripts for pH control (ver. R2015a; The MathWorks, Inc., Natick, MA, USA). The pH was maintained at the desired level during the fermentation by addition of 0.5 M NaOH through a multichannel syringe pump connected to the reactors using injection needles (Frisenette, Knebel, Østjylland, Denmark) and controlled by the Matlab script. The pH was set to incrementally increase from 5.7 to 6.1 over the first 8 h of fermentation to mimic the proximal colon, from 6.1 to 6.5 over the following 8 h to mimic the transverse colon and from 6.5 to 6.8 over the last 8 h to mimic the distal colon.

2.6. Fermentation Conditions

The five reactor units were assembled the day prior to the experiment to generate anaerobic conditions. Fecal glycerol stocks were thawed for 30 min at refrigerated temperatures prior to the experiment. One millilitre of fecal stock (1:1 ratio of fecal matter with PBS 15% glycerol) was diluted with 4 mL of 0.1 M PBS pH 5.6. CoMiniGut reaction vessels were aseptically filled with 4.5 mL of basal medium and further inoculated with 0.5 mL of fecal slurry to achieve a final inoculation at 1% original fecal matter. Using the control media or media supplemented with AOS, alginate or inulin, the fermentations were performed for each fecal sample in quadruplicate. After 24 h, the products of fermentation were aliquoted in cryotubes and kept at −60 °C until further analysis.

2.7. SCFA Analysis

The level of acetic, butyric, propionic, valeric, isobutyric, 2-methylbutyric, isovaleric and lactic acids were measured as previously described by Ouwehand et al. [22]. One hundred µL of internal standard (20 mM pivalic acid) was mixed with 300 µL of water, 250 µL of saturated oxalic acid solution and added to 100 µL of the simulator sample. After thorough mixing, the sample was kept at 4 °C for 60 min and subsequently centrifuged at 13,000× *g* for 5 min. Supernatant (1 mL) was analyzed by GC using a glass

column packed with 80/120 Carbopack B-DA/4% on Carbowax 20M stationary phase (Supelco, Bellefonte, PA, USA) at 175 °C and with helium as the carrier gas at a flow rate of 24 mL min^{-1}. The temperature of the injector and the flame ionization detector were 200 and 250 °C, respectively. The concentration of acetic acid, propionic acid and butyric acid were determined using previously established standard curves. One-way ANOVA was performed (significance level $p < 0.05$) using the package "ggpubr" of the Rstudio platform.

2.8. DNA Extraction

One hundred µL from each gut reactor was used for genomic DNA extraction using the Bead-beat Micro AX Gravity kit (A&A Biotechnology, Gdynia, Poland) following the protocol of the manufacturer. The bead-beating step was performed in 3 cycles of 15 s each at a speed of 6.5 ms^{-1} in a FastPrep-24TM Homogenizer (MP) at room temperature. DNA quantity was measured using Qubit® (dsDNA HS assay; Invitrogen, Carlsbad, CA, USA).

2.9. Amplicon Sequencing

The bacterial community composition was determined using high throughput tag-encoded 16S rRNA gene amplicon NxtSeq-based sequencing (Illumina, San Diego, CA, USA). The V3 region of the 16S rRNA gene was amplified using primers compatible with the Nextera Index Kit (Illumina) NXt_338_F:50- 5′-TCGTCGGCAGCGTCAGATGT GTATAAGAGACAGACWCCTACGGGWGGCAGCAG-3′ and NXt_518_R: 5′-GTCTCGTG GGCTCGGAGATGTGTATAAGAGACAGATTACCGC GGCTGCTGG-3′ (Integrated DNA Technologies; Leuven, Belgium). The PCR reactions and library preparation were performed as described previously [23].

2.10. High Throughput Sequencing and Data Treatment

The raw data set containing pair-ended reads with corresponding quality scores were merged and trimmed using fastq_mergepairs and fastq_filter scripts implemented in the UPARSE pipeline. The minimum overlap length was set to 10 base pairs (bp). The minimum length of merged reads was 150 bp, the maximum expected error E was 2.0, and the first truncating position with quality score was $n \leq 4$. Purging the dataset from chimeric reads and constructing zero radius Operational Taxonomic Units (zOTU) were conducted using the UNOISE3 pipeline [24]. The Green Genes (13.8) 16S rRNA gene collection was used as a reference database [25]. Quantitative Insight Into Microbial Ecology 2 (QIIME2) open source software [26] (version 2019.4.0) was used for the subsequent analysis steps. Principal coordinate analysis (PCoA) plots were generated with the diversity core-metrics-phylogenetic workflow based on 10 UniFrac distance metrics calculated using 10 subsampled OTU tables. The number of sequences taken for each jack-knifed subset was set to 90% of the sequence number within the most indigent sample, hence 14,000 reads per sample. Analysis of similarities (ANOSIM) was used to evaluate group differences using weighted and unweighted [27] UniFrac distance metrics that were generated based on rarefied zOTU tables. The relative distribution of the genera registered was calculated for unified and summarized in genus level zOTU tables. Alpha diversity measures expressed as observed species values (sequence similarity 97%) were computed for rarefied zOTU tables. Differences in alpha diversity were determined using the alpha-group-significance workflow.

3. Results

3.1. Single-Strain Growth Analysis

A single-strain screening of selected bacteria was carried out in vitro (Table 1) to assess their potential for the utilization of AOS. Very little or no growth was observed for all the species inoculated into the basal medium (without carbohydrate), while the addition of glucose was sufficient to give satisfactory growth for all species tested after 24 h incubation. These initial results confirmed that the conditions were suitable for the growth of all tested bacteria in the presence of a carbon source. *Bif. adolescentis*, *L. casei*, *L. paracasei* and *E. hirae*

showed a moderate yet significant growth ($p < 0.05$) in presence of both AOS and alginate relative to baseline. Strong growth ($p < 0.01$) was observed for *B. ovatus* using alginate as substrate, while *Bif. adolescentis*, *L. casei*, *L. paracasei* and *E. hirae* showed no growth in the presence of AOS. In comparison, significant growth ($p < 0.01$) was observed for *Bif. bifidum* Bb-06, *Bif. adolescentis*, *E. coli*, *L. paracasei*, *E. faecium*, and *E. hirae* in the presence of inulin.

3.2. SCFA Production during In Vitro Simulated Colon Passage

The production of SCFA was measured as an indication of the fermentation capacity by the simulated GM (Table 2). Although valeric, isobutyric, 2-methylbutyric, isovaleric and lactic acids were also measured, these acids were only present in trace amounts and under the certainty thresholds (results not shown). Interestingly, AOS and alginate mainly stimulated the production of acetic acid compared to the control from 14.4 ± 0.4 to 26.4 ± 0.5 and 25.9 ± 3.2 μmol mL^{-1}, respectively, for donor 1, and from 13.3 ± 3.9 to 29.2 ± 5.7 and 21.9 ± 4.2 μmol mL^{-1} for donor 3. Additionally, the level of propionic acid was increased for donor 3 in presence of both AOS and alginate from 2.0 ± 0.3 to 5.0 ± 1.7 and 2.8 ± 0.2 μmol mL^{-1}, respectively. These results indicate the capacity of AOS to stimulate acetic acid production in a donor-dependent manner. Inulin strongly increased the level of acetic acid for each donor, while butyric and propionic acid production was increased by inulin in a donor-dependent manner for donor 1 and donor 3, and no significant difference was observed for donor 2. Additionally, no significant increase in the level of any analyzed SCFAs was observed for donor 2 in the presence of both AOS and alginate. Inulin fermentation resulted in the highest activity and led to the largest amount of total SCFA with every donor.

Table 2. Concentration of SCFA after 24 h fermentation in the gut model *CoMiniGut* for each substrate ($n = 4$).

Donor	Substrate	Concentration of Fatty Acids (μmol mL^{-1})			
		Acetic Acid	**Butyric Acid**	**Propionic Acid**	**Total**
1	Control	14.4 ± 0.4	0.9 ± 0.0	1.6 ± 0.0	16.90
	AOS	26.4 ± 0.5 ***	0.6 ± 0.1 ***	1.6 ± 0.1	28.60 *
	Alginate	25.9 ± 3.2 **	1.0 ± 0.2	2.6 ± 0.6 *	29.50 *
	Inulin	60.5 ± 5.6 **	5.1 ± 1.8 *	17.9 ± 5.4 **	83.50 **
2	Control	23.4 ± 0.8	2.7 ± 0.3	2.0 ± 0.1	28.10
	AOS	28.3 ± 5.7	2.3 ± 0.2	2.6 ± 0.4	33.20
	Alginate	23.3 ± 6.2	1.0 ± 0.6 **	2.0 ± 0.5	26.30
	Inulin	44.5 ± 6.9 **	4.1 ± 7.7	1.8 ± 0.7	50.40 **
3	Control	13.3 ± 3.9	1.9 ± 0.5	2.0 ± 0.3	17.20
	AOS	29.2 ± 5.7 **	2.3 ± 0.6	5.0 ± 1.7 *	36.50 *
	Alginate	21.9 ± 4.2 *	1.0 ± 0.2 *	2.8 ± 0.2 **	25.70 *
	Inulin	30.7 ± 8.0 *	22.6 ± 4.2 **	4.7 ± 1.1 *	58.00 **

Significative differences compared to control are expressed as * (p-value ≤ 0.05); ** (≤0.01) and *** (≤0.001).

3.3. Changes in the Simulated GM Composition during Gut Model CoMiniGut

Alpha diversity analysis using Richness (observed species; Figure 1) and Shannon indices (Figure 2) revealed significant differences in bacterial diversity with all three tested carbohydrates. As expected, the highest diversity measured with both indexes was found in the control fermentation for all donors. The fermentation of AOS, alginate and inulin resulted in a similar index value for the overall analysis; however, some donor-dependent differences were also observed. A higher diversity of the bacteria community was found with AOS for donor 1 and donor 2 compared to inulin, while the opposite result was observed in donor 3.

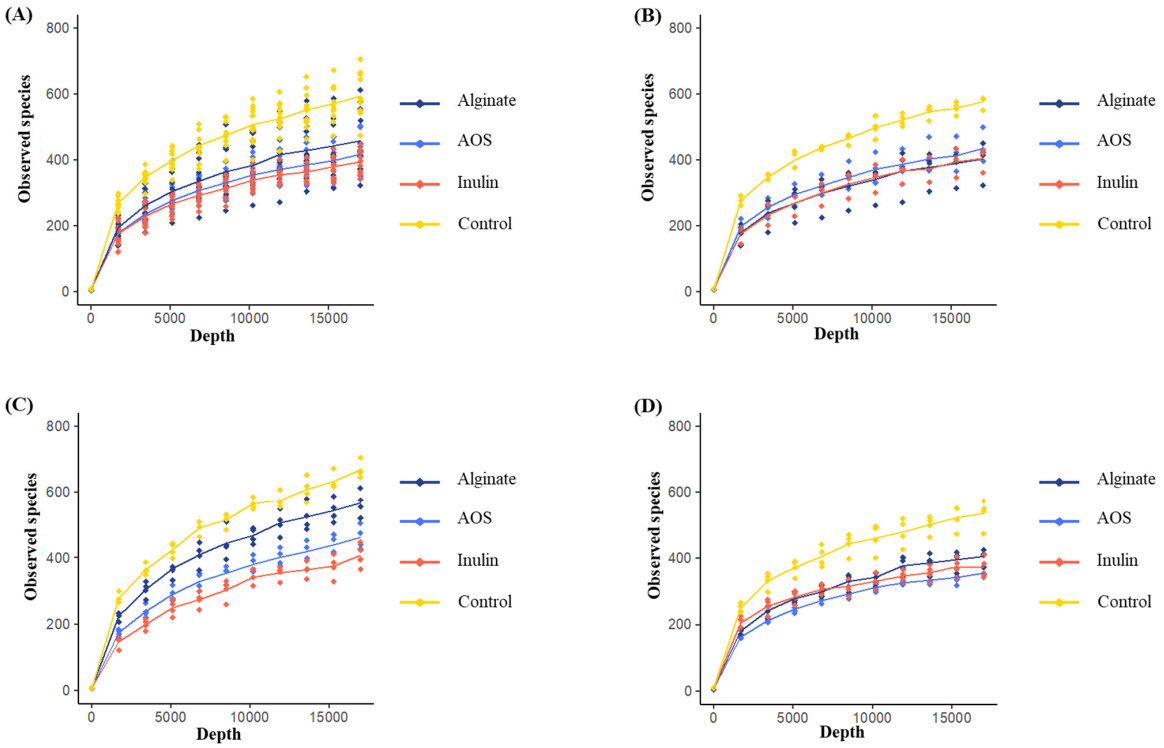

Figure 1. Richness rarefaction analysis. The Richness index (observed species per fermentation) was calculated for each treatment using the samples of combined donors (**A**), or for each individual donor: donor 1 (**B**), donor 2 (**C**) and donor 3 (**D**). The highest number of species observed were obtained in the control fermentation.

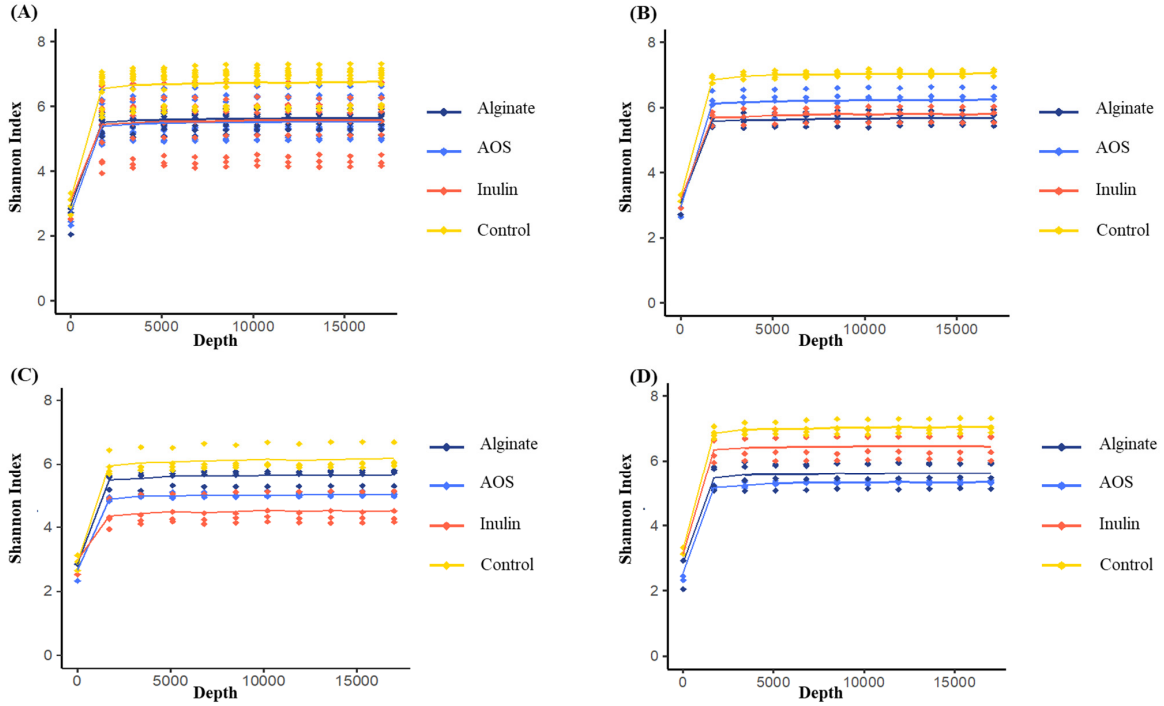

Figure 2. Shannon rarefaction analysis. The Shannon index was calculated for each treatment using the samples of combined donors (**A**), or for each individual donor: donor 1 (**B**), donor 2 (**C**) and donor 3 (**D**). Highest index value was observed in the control fermentation for each donor, while the effect of each substrate appeared to be donor dependent.

An analysis of composition of microbes (ANCOM) [28] test confirmed significant changes within microbial relative abundance and/or presence–absence due to substrate (Figure 3). Bacteria belonging to the genus *Bacteroides* overall had higher relative abundance in the fermentations with AOS and alginate. *B. ovatus* was found to be significantly represented among these fermentations compared to the control fermentation for each of the three donors. Furthermore, inulin strongly stimulated the *Bifidobacterium* community across all donors. Although similar features were noted among fermentations, the relative frequency of bacteria observed for each community was found to be strictly donor-dependent. Clear effects on the gut microbial composition within the AOS were also more specific for each donor. The relative abundance of *Bif. adolescentis* was slightly stimulated after fermentation with AOS compared to the control only for donor 1. The relative abundance of *Clostridiaceae* and *Enterobacteriaceae* markedly decreased after fermentation with AOS compared to the control for donor 2 and 3. All tested substrates caused a donor-dependent modulation of the simulated GM. However, the relative abundance of *Bacteroides* was significantly increased for all substrates.

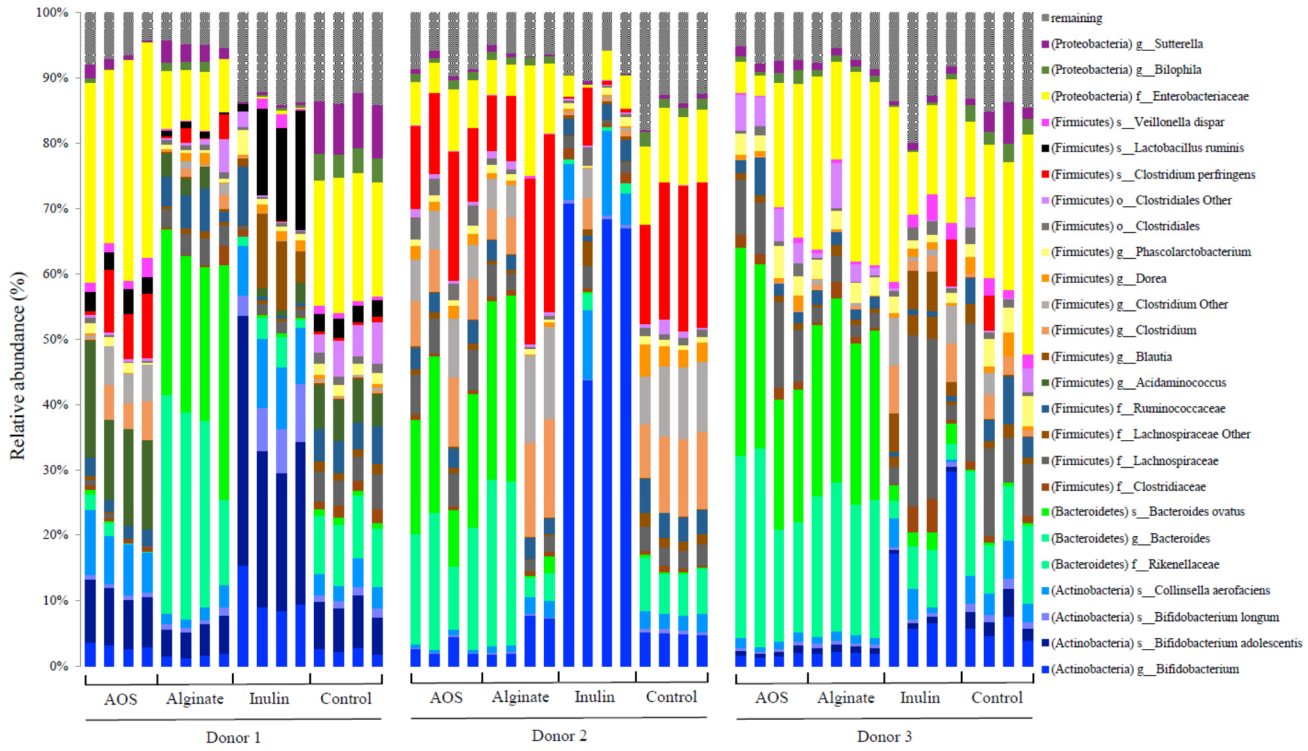

Figure 3. Relative abundance of bacterial community of the three donors in in vitro colon simulations performed in quadruplicate. The bar chart presents the bacterial relative distribution of 25 of the most abundant taxa determined by 16S rRNA gene amplicon sequencing (NxtSeq, Illumina). A clear increase in the bifidobacteria community was observed with inulin for each donor, while alginate oligosaccharide (AOS) and alginate stimulated *Bacteroides* abundance.

Beta-diversity analysis based on unweighted (qualitative) and weighted (quantitative) UniFrac distance matrices showed significant effects on gut microbial composition due to substrate (Figure 4A,B), and donor (Figure 4E,F). Although the donor effect was the strongest using unweighted UniFrac-based analysis, indicating the presence of donor-specific taxa, the proportions of the most abundant groups of bacteria were affected by the carbohydrate, resulting in the somewhat stronger effect of the carbohydrates (Figure 4B) than the donors (Figure 4F), observed with the weighted UniFrac-based analysis. Moreover, clear changes in the simulated GM were observed after 24 h for every donor in both abundance and diversity (Figure 4C,D).

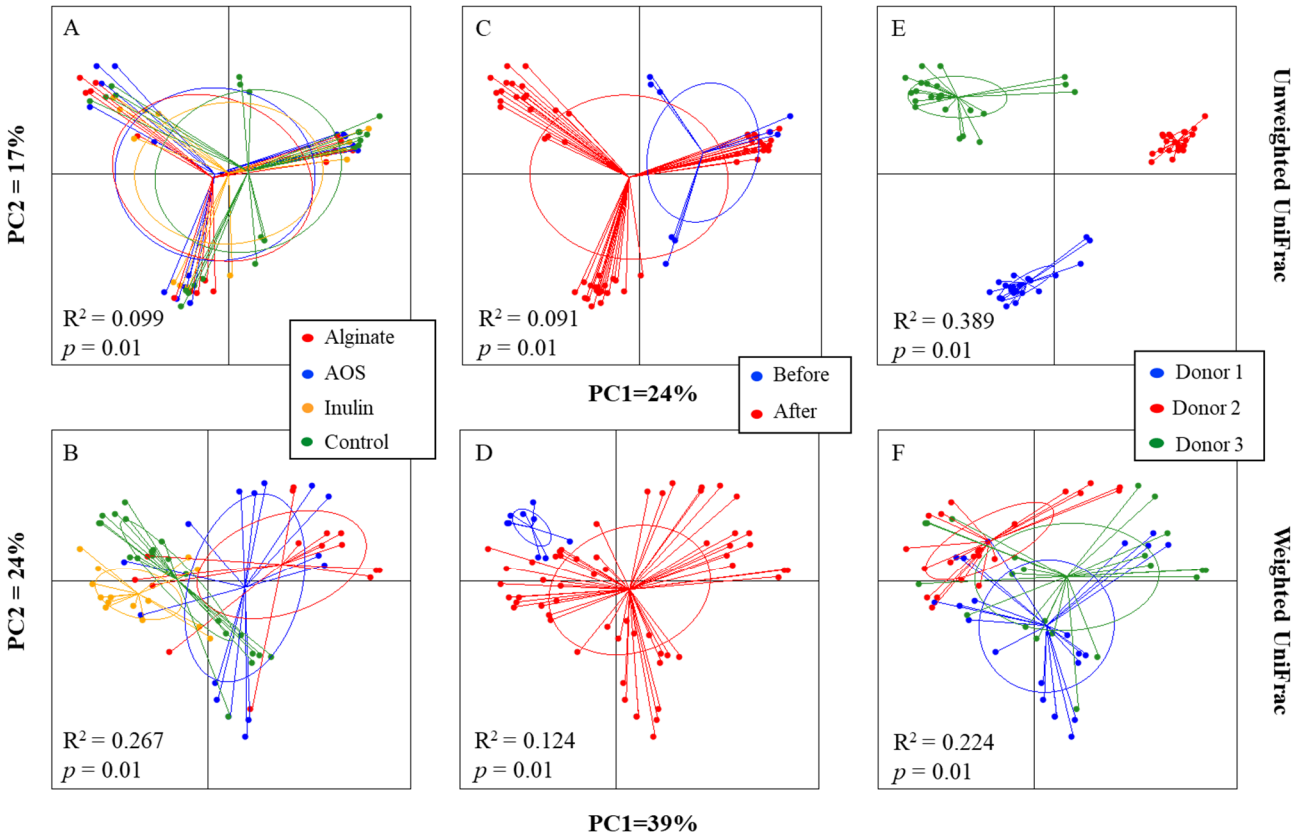

Figure 4. Beta-diversity analysis demonstrating differences in microbial composition of in vitro simulated colon fermentations. The Principal Coordinates Analysis (PCoA) plots are based on unweighted (**A**,**C**,**E**) and weighted (**B**,**D**,**F**) UniFrac distance matrices projecting similarities in microbial community between categories. AOS, alginate and inulin had little effect on the bacterial qualitative composition (**A**) and a pronounced effect on the bacterial relative abundance (**B**). The effect of fermentation on qualitative and quantitative characteristics of microbial community was weak yet significant (**C**,**D**). The donor effect was the strongest and clear on both unweighted (**E**) and weighted (**F**) UniFrac distance based on PCoA plots. The PERMANOVA results are given in each plot.

4. Discussion

An initial screening was carried out to gain insight into the capacity of AOS to influence bacterial growth. The growth of *Bif. adolescentis*, *L. casei* and *L. paracasei* was slightly stimulated in the presence of AOS, while no growth of the other tested bifidobacteria or lactobacilli was observed. Interestingly, previous studies reported that *Bif. longum*, *Bif. breve*, *Bif. bifidum*, *Bif. adolescentis*, *Bif. infantis* and *L. delbrueckii* subsp. *bulgaricus* were stimulated in the presence of AOS [15,29]. *Bacteroides ovatus* and *Bacteroides thetaiotaomicron* were also previously reported to be involved in the degradation of alginate and its derivatives [18]. *Bacteroides* spp. have been described as the main carbohydrate utilizers of the human microbiota, accounting for a large number of glycoside hydrolases (GH) and polysaccharide lyases (PL) genes per genome [30]. A significant amount of GH and PL genes were also found in the genome of certain bifidobacteria [30]. The capacity of these bacteria to grow in alginate and AOS-supplemented medium may be linked to their capacity to produce enzymes capable of degrading these substrates into smaller sugars. However, some of these studies evaluated the addition of enzymatically depolymerized alginate into skim-milk media or carbohydrate-free MRS [15,29]. In the present study, the use of colon basal medium aimed to recreate conditions similar to the colon model while enabling a better appreciation of the effect of AOS, as the basal medium is a less favorable growth environment compared to commonly used nutrient-rich media. Moreover, the AOS used here were obtained through acid hydrolysis of alginate. Thus, although similarities were

found within the previous studies, differences indicated the capacity of certain bacteria to use AOS as substrate may be strain-dependent. It is also thought that this capacity is influenced by the chemical structure of AOS which is linked to the method used to produce these. Recent studies reported a relationship between the structure of the AOS and their biological properties [31,32]. Therefore, it is believed that further investigation of such structure–activity relationship for the prebiotic capacity of AOS should be considered.

The biological properties of SCFA, such as the acidification of the colon, reduced risk of pathogen colonization, modulation of water and salt retention, energy supply for colonocytes and peripheral tissues, as well as the stimulation of mucin production through modulation of the gene expression of epithelial cells, have driven the search for food ingredients that can stimulate the production of these metabolites [33]. In the present study, it was demonstrated that the level of acetic acid increased after 24 h fermentation in the presence of AOS compared to control conditions for donor 1 and donor 3, by 80% and 119%, respectively. For donor 3, the level of acetic acid was similar to that detected in the fermentation with inulin. A tendency to increase acetic acid was also noted in donor 2, although it was not statistically significant. These results indicated the capacity of AOS to stimulate SCFA production in a donor-dependent manner, although SCFA levels were often lower than those found in the fermentation with inulin. These results were in agreement with previous studies carried out in vitro using human GM inoculum [18], as well as some in vivo studies performed in weaned pigs and broiler chickens [34,35], which indicate that AOS may have prebiotic potential.

A UniFrac analysis confirmed the donor-specific aspect of each simulated GM profile, which was consistent with the current literature on the variability of GM composition among individuals. An elevated proportion of *Bacteroides*, especially *B. ovatus* was found for all donors after fermentation with AOS. Although these results are in agreement with earlier in vitro fermentation studies [17,18], they do not correlate entirely with the single-strain growth experiments, where we failed to observed growth of this species on AOS (Table 1). Along with an increased proportion of *Bacteroides*, a donor-dependent effect was observed for other bacterial taxa. A reduction in the relative abundance of *Clostridiaceae* and *Enterobacteriaceae* families was observed for donor 2 and 3 after the fermentation with AOS. This correlates with the single-strain growth experiments where *E. coli*, *E. cloacae*, *K. pneumoniae* and *S. enterica* Typhimurium failed to grow on AOS and thus were out-competed by species that were able to utilise this substrate. *Bif. adolescentis* was mainly observed in donor 1 and to a lesser extent in donor 3. *Bif. adolescentis* showed growth with AOS and inulin in the single-strain culture tests. In the in vitro colon simulations, AOS only modestly increased the relative proportion of *Bif. adolescentis* in donor 1 while no effect was observed on *Bif. adolescentis* in donor 3. Moreover, inulin substantially increased the relative abundance of *Bif. aolescentis* only in donor 1, which was in line with the single-strain growth experiments. However, inulin showed stimulation of the growth of genus *Bifidobacterium* in all three donors, in agreement with the single-strain screening. Although several lactobacilli and enterococci species were observed to grow on AOS in the single-strain testing, their numbers were too low to be observed in the community analyses.

Although only three donors were included in the present study, it is clear that there is a donor-dependent ability to utilise AOS and alginate. The influence of the host microbiota composition on its ability to utilise alginates has recently been reported [36] and is also influenced by cross-feeding between members of the intestinal microbiota [37]. The absence of cross-feeding in the single-strain growth experiments may be one of the explanations why the results do not fully correlate with the in vitro colon simulations.

Therefore, it can be suggested that although the single-strain approach led to a better understanding of the capacity of certain species of bacteria to use AOS for their growth, the presence of a complex bacteria community such as in the colon is necessary to further investigate the impact of AOS onto the simulated GM. Although AOS led to limited growth improvement in single-strain culture, important changes in the gut microbiota profile were noted after fermentation in the colon-model approach. As previously mentioned, AOS

and alginate stimulated the growth of only a few specific bacterial taxa. Accordingly, the lower value obtained by the Richness and Shannon indices of the gut model carried out with the substrates was explained by the stimulation of these specific bacteria and therefore their preferential growth among the simulated GM community compared to the control. Overall, AOS possesses the capacity to shape the GM in vitro, although this effect differs by its selectiveness and its intensity from the effect of inulin. The use of the combined approach led to a better understanding of the results of each experiment.

5. Conclusions

This study aimed to provide a better insight into the prebiotic capacity of AOS. For the first time using an in vitro approach, the effect of AOS on both bifidobacteria and *Bacteroides* communities was observed and explained by donor-dependent variations, while the capacity of certain gut bacteria to utilize AOS was validated through the single-strain method. Although of a lesser intensity compared to inulin, the production of acetic acid was increased by AOS in a gut-model fermentation. AOS effectively stimulated the growth of *Bacteroides* in all fecal samples tested, while they also increased the abundance of *Bif. adolescentis* and decreased the abundance of *Clostridiaceae* and *Enterobacteriaceae* families in a donor-dependent manner. These results were in agreement with previous findings and confirmed the potential of AOS to be used as a prebiotic ingredient, although more studies are needed to further evaluate the prebiotic capacity and the link with the chemical structure of AOS.

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