

Effect of wheat bran derived prebiotic supplementation on gastrointestinal transit, gut microbiota, and metabolic health: a randomized controlled trial in healthy adults with a slow gut transit

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ABSTRACT

Acute intake of the wheat bran extract Arabinoxylan-Oligosaccharide (AXOS) modulates the gut microbiota, improves stool characteristics and postprandial glycemia in healthy humans. Yet, little is known on how long-term AXOS intake influences gastrointestinal (GI) functioning, gut microbiota, and metabolic health. In this randomized, placebo-controlled, double-blind study, we evaluated the effects of AXOS intake on GI function and metabolic health in adults with slow GI transit without constipation. Forty-eight normoglycemic adults were included with whole-gut transit time (WGTT) of >35 h receiving either 15 g/day AXOS or placebo (maltodextrin) for 12-wks. The primary outcome was WGTT, and secondary outcomes included stool parameters, gut permeability, short-chain fatty acids (SCFA), microbiota composition, energy expenditure, substrate oxidation, glucose, insulin, lipids, gut hormones, and adipose tissue (AT) function. WGTT was unchanged, but stool consistency softened after AXOS. 12-wks of AXOS intake significantly changed the microbiota by increasing *Bifidobacterium* and decreasing microbial alpha-diversity. With a good classification accuracy, overall microbiota composition classified responders with decreased WGTT after AXOS. The incretin hormone Glucagon-like protein 1 was reduced after AXOS compared to placebo. Energy expenditure, plasma metabolites, AT parameters, SCFA, and gut permeability were unchanged. In conclusion, intake of wheat bran extract increases fecal *Bifidobacterium* and softens stool consistency without major effects on energy metabolism in healthy humans with a slow GI transit. We show that overall gut microbiota classified responders with decreased WGTT after AXOS highlighting that GI transit and change thereof were associated with gut microbiota independent of *Bifidobacterium*. NCT02491125

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Introduction

Dietary fibers are well known for their beneficial health effects, but the exact underlying mechanisms are not fully understood. Specific dietary fibers termed prebiotics are fermented by the gut microbiota thereby altering the microbial composition and activity thus conferring a beneficial physiologic effect on the host.¹ Prebiotic intake can reduce postprandial glycemic and insulinemic excursions, increase satiety, improve the gut

barrier function and decrease inflammatory markers.² Short-chain fatty acids (SCFA) produced upon microbial prebiotic fermentation are suggested to mediate metabolic effects by modulating gut peptide release (peptide YY (PYY) and glucagon-like peptide-1 (GLP-1)), gut permeability, skeletal muscle, liver, and adipose tissue (AT) function.^{3,4} Combined data in systematic reviews regarding prebiotic interventions to improve functional gastrointestinal disorders⁵ or metabolic

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markers in type 2 diabetes⁶ and obesity⁷ are promising. Yet mostly due to study heterogeneity, regarding, for example, prebiotic fiber types, participant heterogeneity, duration of intervention as well as clinical endpoints, these reviews generally report a low to moderate level of evidence for the efficacy of prebiotics in the given disease context.

The gastrointestinal (GI) transit is an important for healthy gut functioning as well as in human energy metabolism. Upper intestinal transit is tightly involved in satiety signaling, the incretin response and postprandial glycemia, while lower intestinal transit determines nutrient availability, wash-out rate, and water availability, thereby shaping the ecologic niche of the gut microbiota.⁸⁻¹⁴ Conventionally, insoluble fibers are known to accelerate GI transit time possibly by increased stool bulk potentially caused by mechanical irritation and stimulation of mucus and water secretion in the large intestine.¹⁵ However, prebiotic modulation of gut microbiota and microbial metabolite production (e.g. SCFA, H₂) may also affect GI transit.⁸ Some prebiotics (namely fructans and galacto-oligosaccharides) may increase stool frequency and soften stool consistency more effectively in constipated compared to non-constipated participants supporting evidence that dietary fiber intake more effectively accelerates gastrointestinal transit in humans with initially longer transit.¹⁶⁻¹⁸

Short-term human studies with the novel prebiotic arabinoxylan-oligosaccharides (AXOS) derived from wheat bran have been reported to increased *Bifidobacterium* abundance and improve postprandial glycemia.¹⁹⁻²³ Further, AXOS intervention softened stool consistency and increased stool frequency in some,²³⁻²⁵ but not all short-term human interventions.^{20,22} Yet it is unknown how AXOS affects gastrointestinal transit and metabolic health in the long-term. We hypothesized that 12-week AXOS intake improves whole-gut transit time (WGTT, primary outcome) in healthy participants with a slow GI transit but without constipation. This selection was based on the assumption that participants who are non-constipated, yet not satisfied with their bowel habits (i.e. with slow GI transit) would be the likely population to use dietary fiber supplements such as AXOS to alleviate GI transit. Secondary outcomes were changes in gut microbiota composition, gastric emptying, oro-cecal transit, gut permeability,

fecal and plasma SCFA, energy and substrate metabolism, glycemic and insulinemic responses, gut hormones, systemic and local inflammatory markers as well as AT functioning after 12-week of AXOS intake compared to placebo.

Participants and methods

Study participants

Forty-eight healthy Caucasian men and women aged 20–55 years, with a body mass index of 20–30 kg/m² were recruited between August 2015 and December 2016 from the general population in the vicinity of Maastricht, the Netherlands. Initial recruitment was based on a defecation frequency of ≤4 per week. Participants with WGTT >35 h determined by radio-opaque marker method were included. Normoglycemia was determined by an oral glucose tolerance test according to the diagnostic criteria of the American Diabetes Association, 2010.²⁶ During a medical screening, eligibility was assessed including anthropometrics and medical history. Exclusion criteria were: Two or more symptoms of constipation according to ROME III criteria²⁷ to rule out any confounding effects of underlying pathologies (e.g. defecation disorders, outlet obstruction), other unknown pathologies or inevitable medication use. Further exclusion criteria were the diagnosis of diabetes, prior abdominal surgery, cardiovascular or gastrointestinal diseases, life expectancy shorter than 5 years, the use of antibiotics, laxatives, prebiotics or probiotics 3 months prior to the study or during study participation. Participants did not use β-blockers, lipid- or glucose-lowering medication or corticosteroids.

Study design

This study was a double-blind, randomized placebo-controlled parallel trial approved by the Medical Ethical Committee of Maastricht University Medical Center+ (METC 15-3-005) and was conducted according to the ethical standards of the Helsinki Declaration and in accordance with the Medical Research Involving Human Subjects Act (WMO). All patients provided verbal and written informed consent. Using block randomization, an independent researcher randomly allocated participants to AXOS or placebo group with stratification for age and sex.

After inclusion, two clinical investigation days (CID) before and directly after the 12-week intervention were performed to assess primary and secondary outcomes (Supplemental Figure 1). Participants were asked to ingest 5 g of AXOS powder (Cargill, Minnetonka, Minnesota, U.S.) or 5 g maltodextrin (C-PharmDry™, Cargill) three times per day with their regular meals for 12 weeks. AXOS preparations consisted of 71% of dry matter (DM) AXOS (degree of polymerization of the main AXOS fraction varies between 2 and 15 (average degree of polymerization: 3–8)), 10–14% DM β -glucan and 1–3% DM ferulic acid. AXOS and maltodextrin were provided as off-white powder, prepackaged in identical opaque sachets to be dissolved in tap water insuring the double-blind study design. Compliance was checked by the number of returned empty sachets. Participants were instructed to maintain habitual food intake and physical activity pattern throughout the study monitored by the short questionnaire to assess health-enhancing physical activity²⁸ and a weighed 3-day food record before and during the last week of the intervention (see supplementary methods). To assess gastrointestinal complaints, participants documented a 7-day gastrointestinal symptoms questionnaire including stool frequency and consistency using the Bristol stool scale (BSS)²⁹ the week before and during the last week of intervention. Quality of life was assessed via the 36-Item short form healthy survey of quality of life³⁰ before and after the intervention (see supplementary methods). Two days prior to the CIDs, participants were asked to refrain from intense physical activity, alcohol and natural ¹³C-enriched products (e.g. pineapple, corn, and cane sugar).

Clinical investigation day 1

Whole-gut transit time

Prior to clinical investigation day (CID) 1, on 6 consecutive days participants ingested 10 radio-opaque (Colonic Transit, P. & A. Mauch, Münchenstein, Switzerland) markers in the morning. On CID1, participants came fasted to the university and a single abdominal X-ray was taken 24 h after the last marker ingestion. Based on the number of markers visible on the abdominal X-ray, WGTT was calculated according to Metcalf et al.³¹

Gut permeability

Thereafter, participants ingested a multi-saccharide mix dissolved in 150 ml tap water containing 1 g sucrose (Van Gilse, Dinteloord, The Netherlands), 1 g lactulose (Centrafarm, Etten-Leur, The Netherlands), 1 g sucralose (Brenntag, Sittard, The Netherlands), 1 g erythritol (Nowfoods, Bloomingdale, U.S.), and 0.5 g of l-rhamnose (Danisco Sweeteners, Copenhagen, Denmark). Urine was collected in fractions to determine sucrose concentrations after 0–5 h for gastro-duodenal permeability, lactulose/rhamnose ratio after 0–5 h for small intestinal permeability, and the sucralose/erythritol ratio after 5–24 h for colonic permeability as described previously.³² Isocratic ion-exchange HPLC (Model PU-1980 pump) with mass spectrometry (Model LTQ XL, Thermo Fisher Scientific, Landsmeer, The Netherlands) was used to determine urinary sugar concentrations.

Clinical investigation day 2

Adipose tissue biopsy

After an overnight fast, abdominal subcutaneous adipose tissue (AT) needle biopsies were collected under local anesthesia lateral from the umbilicus in a subgroup of participants (AXOS n = 15, placebo n = 15). Biopsy specimens were fixed overnight in 4% paraformaldehyde and embedded in paraffin. Histological sections were cut to 8 μ m slices, mounted on microscope glass slides and dried overnight at 37°C. After hematoxylin eosin staining, digital imaging was performed using Leica DFC320 digital camera (Leica, Rijswijk, Netherlands) at \times 20 magnification (Leica DM3000 microscope; Leica). Computerized morphometric measurements of individual adipocytes were performed as previously described.³³ For AT mRNA expression, total RNA was extracted using TRIzol (Invitrogen, Breda, The Netherlands) for cDNA synthesis and real-time qPCR (iCycler, Bio-Rad, Veenendaal, NL, U.S.; primer sequences Supplemental Table 6). Results were normalized to 18S rRNA and expressed relative to baseline expression according to $\Delta\Delta$ Ct method.

Breakfast meal test and indirect calorimetry

After the AT biopsy, venous blood samples were taken at t = 0, 30, 60, 90, 120, 180, 240 and 300 min

after ingestion of a solid test meal consisting of two slices of white bread, a fried egg and 250 ml of chocolate milk (27 E% fat, 52 E% carbohydrate, 19 E% protein, 412 kcal). During fasting and postprandial, visual analogue scales of feelings of hunger, satiety, fullness, and desire to eat were assessed. Substrate oxidation and energy expenditure was measured by determining CO₂ production and O₂ consumption via an open-circuit-ventilated hood system (Omnicall, Maastricht University, The Netherlands) for 30 min during fasted conditions and in 15 min intervals during the 5 h postprandial state. Calculations of energy expenditure and the rate of fat and carbohydrate oxidation were calculated assuming 15% protein oxidation using the equations of Weir³⁴ and Frayn.³⁵

Upper gastrointestinal transit

Gastric emptying (GE) was measured using ¹³C-octanoic acid (100 mg, Campro Scientific bv, Veenendaal, The Netherlands) mixed into the standardized breakfast meal consumed at t = 0. Breath samples were collected at fasting and during the 5 h postprandial state, and analyzed using non-dispersive infrared spectrometry (IRIS, Wagner, Bremen, Germany).³⁶ Two fasting breath samples were obtained to measure baseline ¹³C-concentration to calculate delta over baseline and percentage of the dose ¹³C recovered (%/h) from the subsequent time points. Gastric emptying describing the time point at which 50% of the gastric content is excreted (T_{1/2}), and the time point of maximal excretion rate (T_{lag}) were calculated according to Ghoo et al.³⁷ Oro-cecal transit time (OCTT) was determined using the inulin hydrogen (H₂) breath test.³⁸ Participants ingested 5 g of Inulin (Cargill) dissolved in 150 ml tap water with the breakfast meal at t = 0. A hand-held device (Gastrolyzer, Bedford Scientific, Kent, UK) was used to measure breath H₂ (in ppm) as an indicator of at fasting and every 15 min during the 5 h postprandial state. OCTT was estimated from three consecutive measurements at least 10 ppm above baseline at fasting.

Biochemical blood analyses

Blood was collected in pre-chilled tubes and centrifuged at 3 000 x g at 4°C and plasma was snap-

frozen in liquid nitrogen and stored at -80°C until analyses. EDTA tubes (Becton Dickinson, Eysins, Switzerland) were used for SCFA, insulin, glucose, free fatty acids (FFA), triacylglycerol (TAG), free glycerol, lipopolysaccharide-binding protein (LBP), Tumor necrosis factor (TNF) α, Interleukin (IL) 6, IL-1β and IL-8 analyses. For GLP-1 and PYY, 20 μL of dipeptidyl peptidase-IV inhibitor (Merck, Darmstadt, Germany) was added into an EDTA or Aprotinin tube (Becton Dickinson), respectively. Glucose, FFA, and TAG were determined with enzymatic assays (Cobas Fara auto-analyzer, Roche, Basel, Switzerland). Free glycerol was measured after precipitation using an enzymatic assay (Enzytee™ Glycerol, Roche) and analyzed with the Cobas Fara auto-analyzer. Insulin and PYY concentrations were determined using radio-immunoassay (RIA) kits (Human PYY (3–36) Specific RIA, Merck). Total GLP-1 was measured with RIA as previously described.³⁹ LBP was measured using a noncommercial enzyme-linked immunosorbent assay (ELISA) as previously described.⁴⁰ IL-1β, IL-6, IL-8, and TNF-α were determined with an ELISA kit (Human ProInflammatory IL 4-Plex Ultra-Sensitive Kit, Meso Scale Diagnostics, Rockville, U.S.). Plasma SCFA were determined using liquid chromatography-mass spectrometry.⁴¹

Fecal sample collection and analyses

Feces wet weight was recorded by the participants for four consecutive days at home before CID1. From the first weighted stool, fractions of feces were collected in tubes for further analysis. Feces tubes were stored at -20°C in the participants' freezers and transported using ice packs and immediately stored at -80°C on arrival at the University for CID 1. Fecal calprotectin was determined using an ELISA kit (BÜHLMANN fCAL®, Basel, Switzerland). Fecal SCFA, branched-chain fatty acids (BCFA), succinate and lactate were measured using ion exchange chromatography with conductivity detection and normalized to dry weight (Brightlabs, Venlo, The Netherlands). Stool moisture, i.e., water percentage of a stool sample (approx. 3 grams) was calculated based on the ratio of the weight loss of a fecal sample

after 24 h of dry lyophilization to the weight of the fecal sample before.

Gut microbiota composition

Microbial DNA was extracted from fecal samples to perform 16S rRNA gene amplicon sequencing with Illumina HiSeq2500. DNA was isolated from 0.25 g feces with repeated bead beating followed by automated isolation and purification using a Maxwell 16 Tissue LEV Total RNA Purification Kit (Promega, Madison, U.S.). DNA was eluted in 50 µl of nuclease-free water and quantified by Nanodrop (ThermoScientific). The V4 region of the 16S rRNA gene was amplified with the double barcoded primer pair 515F (5'-GTGCCAGCMGCCGCGGTAA) – 806R (5'-GGACTACHVGGGTWTCTAAT) as previously described.^{42,43} Each sample was amplified in triplicate using Phusion hot start II high fidelity polymerase (Thermo Scientific). Forty µl PCR reactions contained 28.4 µL nucleotide-free water (Promega, Madison, USA), 0.4 µL of 2 U/µl polymerase, 8 µL of 5× Phusion Green HF Buffer, 0.8 µl of 10 µM stock solutions of each of the barcoded forward (515F) and reverse (806R) primers, 0.8 µL 10mM dNTPs (Promega) and 0.8 µL template DNA. Cycling conditions were as follows; Reactions were held at 98°C for 30 s and amplification proceeding for 25 cycles at 98°C for 10 s, 50°C for 10 s, 72°C for 10 s and a final extension of 7 min at 72°C. PCR products were checked for correct size on a 1% agarose gel and subsequently combined and purified with the CleanPCR kit (CleanNA, Alphen aan den Rijn, The Netherlands). Purified PCR products were quantified with Qubit using the dsDNA BR Assay Kit (Invitrogen, California, USA) and a composite sample for sequencing was created by combining equimolar amounts of amplicons (200 ng) from the individual samples. The resulting libraries were sent to GATC Biotech AG for 2X150nt sequencing on an Illumina HiSeq2000 instrument. Sequencing yielded 387 497 ± SD 164 372 reads per sample and sequence analysis was performed in NG-Tax using default settings.⁴² Paired-end libraries were demultiplexed using read pairs with perfectly matching barcodes. Amplicon sequence variants (ASV) were picked as follows: sequences were ordered by abundance per sample and reads were considered valid when their cumulative abundance was ≤0.1%. Taxonomy was assigned

using the SILVA reference database version128.⁴⁴ ASVs are defined as individual sequence variants rather than a cluster of sequence variants with a shared similarity above a specified threshold.

Statistics

To measure clinically relevant differences in WGTT of 30%, a power of 80% (β) and assuming an alpha of 0.05 (α), 22 participants per group were required. Estimating a 20% dropout rate, total recruitment included $n = 48$ participants. Data were tested for normality using a Shapiro Wilk test and were log-transformed in case of a non-parametric distribution and in case of ordinal data, a Wilcoxon signed rank tests was performed. Baseline differences were analyzed using unpaired Student's t-tests. Primary and secondary outcomes were compared using a 2-factor repeated measures analysis of variance with time (pre, post) and intervention (AXOS, placebo). In case of a significant time*intervention effect, a post hoc analysis was conducted with Bonferroni correction within both groups. *P*-values <0.05 were considered significant. Statistical analyses were performed using SPSS 22.0 (IBM, Armonk, U.S.). For the gut microbiota, analyses were performed in R version 3.4 (R Core Team, 2017). Distance-based redundancy analysis with weighted Unifrac was performed to calculate the multivariate intervention effect.^{45,46} Principal response curve (PRC) analysis was used to summarize differences in the microbiota composition between interventions over time.⁴⁷ Alpha diversity metrics were calculated using *vegan* package.⁴⁸ To classify responder status based on gut microbiota, we used random forests (RF) based on ASVs. Three outliers outside an overall 95% confidence interval on a weighted Unifrac based PCoA analysis were discarded. We evaluated the model using cross-validation as implemented in *caret*.⁴⁹ We randomly selected 75% of the samples to serve as the training set and consequently evaluated classification accuracy using the hold out set with 500 repetitions. The AUC was calculated with the cross-validation error using the *pROC*

package with 10 000 bootstraps for confidence intervals.⁵⁰

Results

Participant characteristics

Forty-eight participants were included and completed the study (Table 1, Supplemental Figure 2). No serious adverse events or gastrointestinal complaints (Supplemental Table 1) were reported, and compliance was checked by returned emptied sachets (236 ± 12 of 252 empty sachets were returned [93.7%]). There were no differences in BMI, body composition, dietary intake, physical activity and quality of life between groups before or after the intervention (Supplemental Table 2–4).

Softer stool consistency but no changes in whole gut transit after AXOS

No significant changes in WGTT, gastric emptying or OCTT were observed after AXOS intervention compared to placebo (Table 2). Independent of intervention or time, gastric emptying of the solid meal was uniformly overestimated, a known limitation of indirect breath test fitting.⁵¹ Yet, the unfitted percentage of ¹³C recovery was unchanged after AXOS intervention compared to placebo (Supplemental Figure 3). Stool frequency, stool weight, stool moisture, and fasting H₂ remained unchanged, however, BSS scores increased from harder stool type 1–2 toward softer stool types 3–4 after AXOS intervention (Table 2, Supplemental

Figure 4). WGTT and BSS were not correlated, neither at baseline nor when comparing changes after the intervention (Supplemental Figure 5). There were no changes in BSS scores after placebo, yet we observed that there was a non-significant increase of stool type 6 scoring (watery stool) after placebo intake. In general, we observed very irregular patterns of BSC types within 1 week, for example, having stool forms of type 2 and type 6 within 1 week. Thus, in the placebo group, this irregular pattern was maintained or even exacerbated, while we observed a tendency toward “normal” stool types (i.e., 3 and 4) after the AXOS intervention. Participants did not report the use of laxatives during the study as monitored via GI questionnaires.

AXOS intervention modulates gut microbiota

AXOS intervention significantly reduced alpha diversity ($P < .001$) and changed the microbiota composition ($P = .05$) compared to placebo (Figure 1(a)) after adjustment for age and sex. Furthermore, 15.1% of the variation in microbiota composition was explained by time ($P < 1 \times 10^{-5}$), and 4.4% ($P = .05$) by the time*intervention effect. Gut microbiota changes after AXOS intervention were mainly related to increased *Bifidobacterium* ASVs and, to a lesser extent, increased *Akkermansia*, *Prevotellaceae* NK3831 group, *Lactobacillus* and decrease of *Blautia*, *Eubacterium Hallii* group, *Coriobacteriaceae* UCG-003, and *Dorea* ASVs (Figure 1(b)). Nonetheless, the overall microbial responses to AXOS showed distinct individual patterns as exemplified by the *Blautia* and *Dorea* ASVs (Figure 1(c)).

Table 1. Baseline characteristics of the study participants.

Variables	AXOS (n = 24)	Placebo (n = 24)	P
Sex, male/female	6/18	6/18	
Age, y	36.1 ± 12.9	35.7 ± 11.0	.905
Weight, kg	72.7 ± 12	72.5 ± 11.7	.912
Body mass index, kg/m ²	24.7 ± 3.1	24.2 ± 2.3	.532
Body fat, %	25.4 ± 7.2	25.5 ± 8.4	.861
Fat mass, kg	18.4 ± 5.7	19.2 ± 7.6	.952
Systolic blood pressure, mmHg	116 ± 9	115 ± 11	.532
Diastolic blood pressure, mmHg	79 ± 5	76 ± 8	.125
Fasting glucose, mmol/L	4.8 ± 0.3	5.0 ± 0.3	.067
OGTT 2h plasma glucose, mmol/L	4.7 ± 1.2	4.9 ± 1.1	.503
Whole-gut transit time, h	70.8 ± 26	80.2 ± 21	.084

Values are given as means ± SD. Group differences were assessed using independent Student's t-test. OGTT oral glucose tolerance test, AXOS Arabinoxylan-Oligosaccharides

Fecal and plasma SCFA

Fecal SCFA, BCFA, succinate or lactate were unchanged after AXOS intervention compared to placebo (Figure 2(a–c), Supplemental Figure 6). Plasma acetate and butyrate decreased over time both after AXOS and placebo intervention with no differences between groups (Figure 2(d–f)).

Gut permeability and inflammation

Gut permeability, plasma LBP, fecal calprotectin, plasma IL-6, IL-8, TNF-α, and IL-1β were unchanged

Table 2. Gastrointestinal transit and stool parameters before and after AXOS and Placebo intervention.

Variable		AXOS (n = 24)	Placebo (n = 24)	P
Whole-gut transit, <i>h</i>	Pre	70.8 ± 26	80.2 ± 21	.266
	Post	71.8 ± 31	66.1 ± 30	
Oro-cecal transit, <i>min</i>	Pre	212 ± 95	235 ± 86	.725
	Post	235 ± 22	229 ± 18	
GE half time (T_{half}), <i>min</i>	Pre	191 ± 55	182 ± 49	.801
	Post	207 ± 38	195 ± 32	
GE lag time (T_{lag}), <i>min</i>	Pre	130 ± 43	128 ± 38	.235
	Post	150 ± 29	144 ± 25	
Fasting H ₂ , <i>ppm</i>	Pre	10.2 ± 6	9.9 ± 7	.992
	Post	12.2 ± 2.1	9.9 ± 8	
Stool frequency, <i>stools/week</i>	Pre	4.1 ± 2.8	4.0 ± 1.2	.880
	Post	5.0 ± 3.4	5.0 ± 1.7	
Stool wet weight, <i>g/day</i>	Pre	180 ± 22	196 ± 20	.735
	Post	145 ± 15	165 ± 15	
Stool moisture, %	Pre	69.6 ± 7.7	65.3 ± 8.7	.766
	Post	66.9 ± 11.2	61.1 ± 10.8	
BSS, <i>score/day</i>	Pre	3.0 [2.1–3.4]	3.3 [2.8–4.0]	AXOS * <i>P</i> = .018
	Post	3.5 [2.8–4.3]	3.3 [2.7–4.0]	Placebo * <i>P</i> = .676

Data are given in mean ± SD and for BSS in median ± IQR [25–75th]. Data (except BSS) were analyzed using 2-way repeated measure ANOVA with time (pre/post) and intervention as covariates. *P*-value represents time*intervention interaction. BSS scores were compared pre/post within groups with Wilcoxon signed-rank test represented by **P*-value. BSS scores significantly increased after AXOS but not after placebo intervention compared to baseline. GE gastric emptying, AXOS Arabinosyloxylan-Oligosaccharides, BSS Bristol stool scale

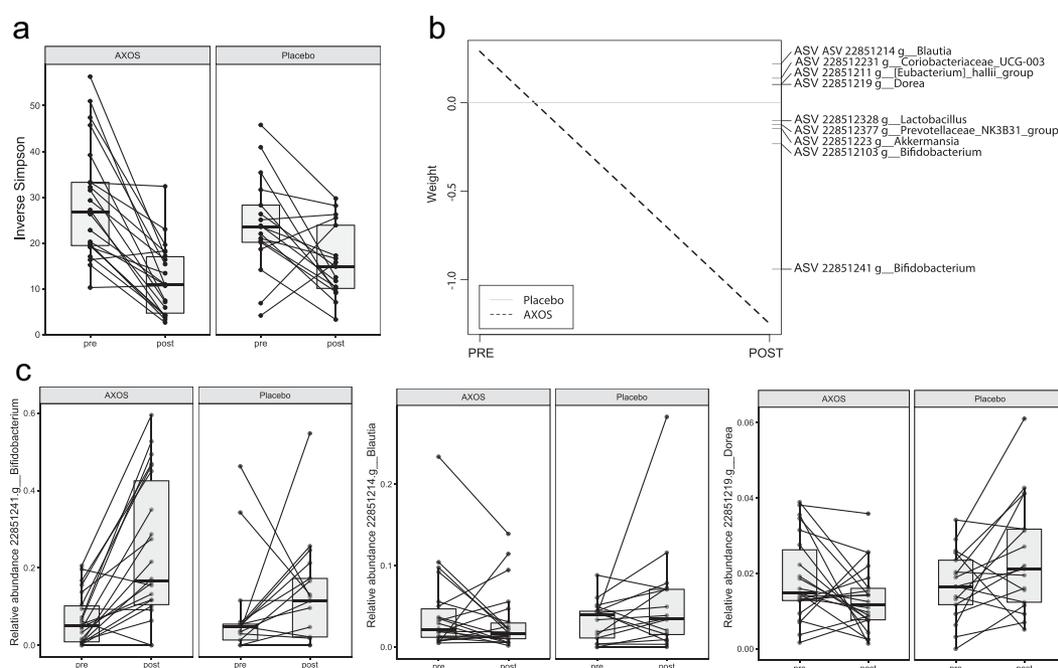


Figure 1. AXOS induces changes in gut microbiota. (a) Inverse Simpson index for alpha-diversity is reduced after AXOS intake. (b) Principal Response Curve summarizing the multivariate response of AXOS intervention versus Placebo over time ($P = .05$). ASVs with large deviations between AXOS and Placebo have high weights while taxa equally present in AXOS and Placebo have zero weight. ASVs with the highest weight plotted vertically on the right axis are the main drivers of the differences between interventions. ASVs that have a negative weight on the response curve follow the observed AXOS curve, whereas those with positive weights follow the opposite pattern. (c) Individual intervention responses of important ASVs. Individual changes depict the AXOS group ($n = 21$) and placebo ($n = 17$) before and after the intervention. ASV Amplicon sequence variant, AXOS Arabinosyloxylan-Oligosaccharides.

after AXOS intervention compared to placebo (Figure 2(g-i), Supplemental Table 5).

Energy metabolism, plasma metabolites and adipose tissue function

Postprandial fat oxidation tended to increase after AXOS intervention (iAUC_{0-5h}, ANOVA $P = .008$, post-hoc AXOS $P = .073$, placebo $P = .089$, Figure 3(a,b)). AXOS intervention did not affect energy expenditure, respiratory quotient and carbohydrate

oxidation (Figure 3(c-f)), yet AXOS intervention decreased early postprandial GLP-1 AUC 0-90min/min concentrations after a standardized breakfast meal as compared to placebo (ANOVA $P = .043$, post-hoc AXOS $P = .033$, placebo $P = .972$, Figure 3(g,h)) while PYY was unchanged after intervention (Figure 3(i)). Glucose, insulin, FFA, TAG, glycerol and appetite, hunger, satiety, and fullness ratings were not affected by AXOS intervention compared to placebo (Table 3, Supplemental Figure 7). AXOS intervention had no effect on adipocyte size and AT

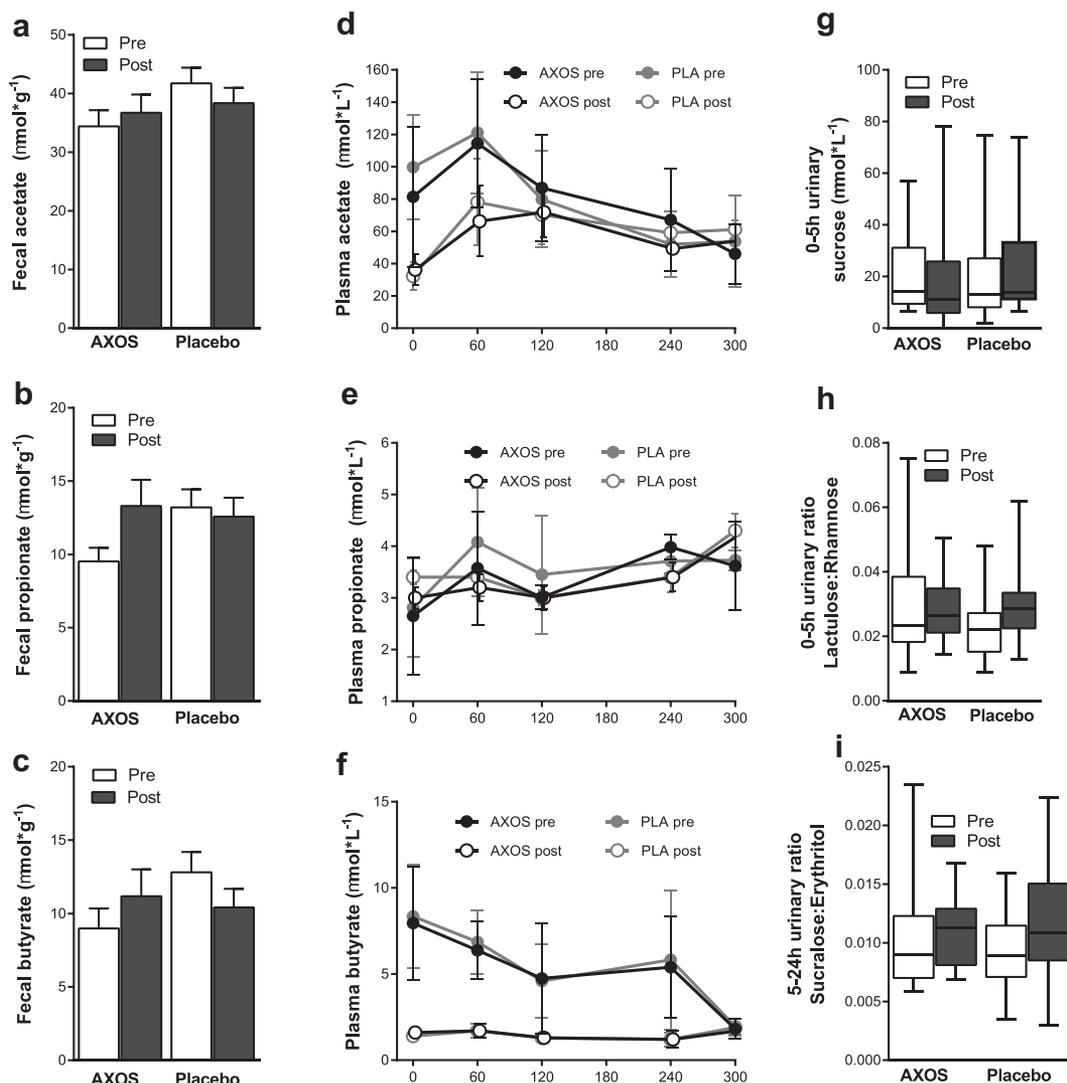


Figure 2. Short-chain fatty acid concentrations and gut permeability before and after AXOS ($n = 24$) and placebo intervention ($n = 24$). Data are given as means \pm SEM. Data were analyzed using 2-way repeated measure ANOVA with time (pre/post) and intervention as covariates. There are no differences between fecal (a) acetate (b) propionate, (c) butyrate or fasting and postprandial plasma (d) acetate, (e) propionate, (f) butyrate between groups as a result of intervention (time*intervention, ns). Urinary sugar excretions and ratios were compared with Wilcoxon signed-rank test pre vs. post within groups. There were no differences in urinary excretion rates of (g) 0-5 h sucrose (gastro-duodenal permeability) (h) 0-5 h Lactulose:Rhamnose ratio (small intestinal permeability) (i) 5-24 h Sucralose:Erythritol ratio (colonic permeability). AXOS Arabinoxylan-Oligosaccharides, PLA placebo.

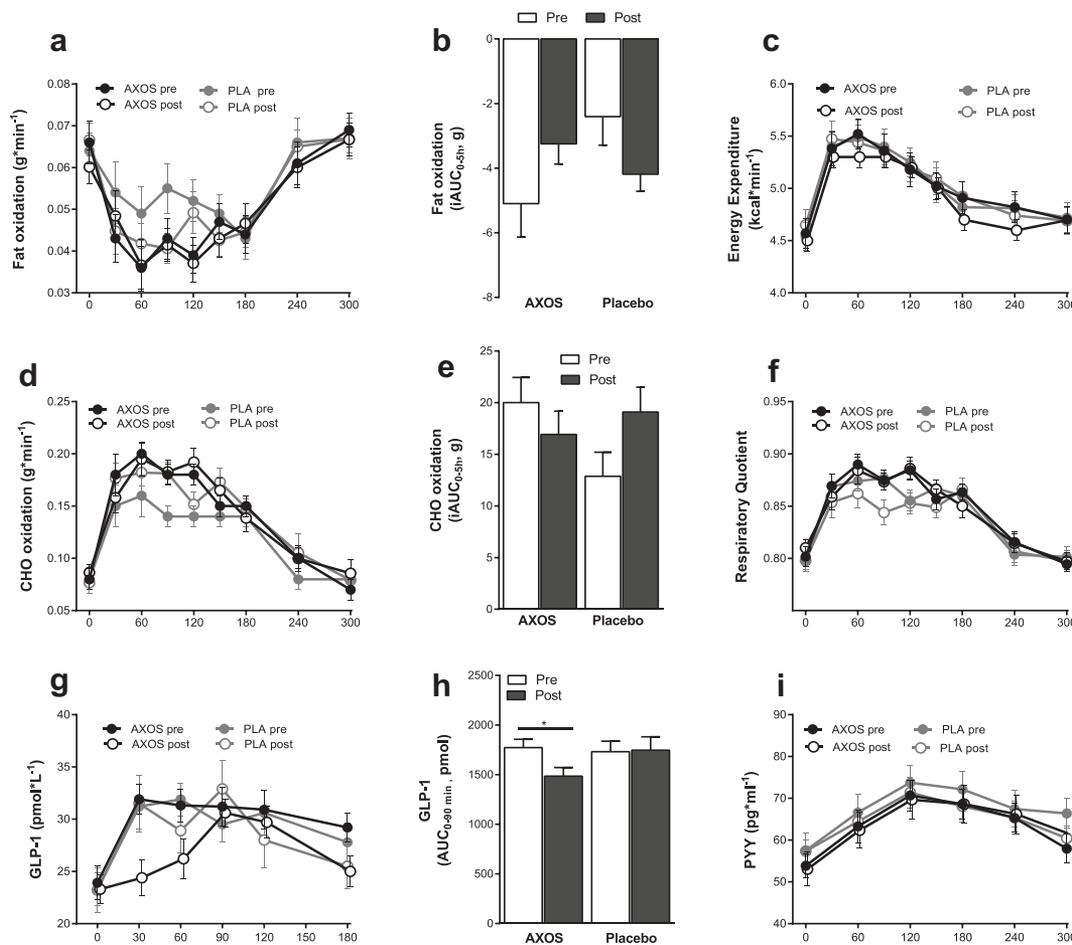


Figure 3. Energy expenditure, substrate metabolism, GLP-1 and PYY before and after AXOS ($n = 24$) and Placebo ($n = 24$) intervention. Data are mean \pm SEM and analyzed using 2-way repeated measure ANOVA with time (pre/post) and intervention as covariates. (a,b) Postprandial fat oxidation $iAUC_{0-5h}$ tended to increase after AXOS intervention (time*intervention $P < .01$, post-hoc AXOS $P = .073$, placebo $P = .089$). (c) Energy expenditure, (d) CHO oxidation, (e) CHO oxidation $iAUC$ and (f) RQ were unchanged between groups after intervention. (g,h) Postprandial GLP-1 $AUC_{0-90min}$ decreased after AXOS intervention compared to placebo (time*intervention $P = .043$, post hoc AXOS $P = .33$, placebo $P = .972$). (i) PYY was not affected by the intervention. AXOS Arabinoxylan-Oligosaccharides, CHO carbohydrate, GLP-1 glucagon-like peptide-1, PYY peptide YY, $iAUC$ incremental area under the curve, EE energy expenditure, RQ respiratory quotient, * $P < .05$.

mRNA expression of lipolytic enzymes (adipose tissue triglyceride lipase (ATGL), hormone-sensitive lipase (HSL), comparative gene identification 58 (CGI-58), lipid-droplet-associated protein (PLIN-1, Supplemental Figure 8)).

Microbiota composition relates to decreased WGTT after AXOS

To identify responders and non-responders, we defined responders as a $\geq 10\%$ decrease in WGTT and non-responders as unchanged/increase in WGTT after AXOS intervention. We used Random Forests analysis to determine gut microbiota patterns

characteristic for responders or non-responders after AXOS intervention. With a good classification accuracy (Area Under the Receiver Operating Characteristics curve, AUROC of 0.80, Figure 4(a)), we observed that overall microbiota composition classified decreased WGTT (responders) compared to unchanged/increased WGTT (non-responders) after AXOS intervention. ASVs important for this classification were mainly ASV 22851231 from the genus *Senegalimassilia*, family *Coriobacteriaceae* and an unclassified ASV 22851212 from the family *Lachnospiraceae* (Figure 4(b)), which showed higher abundances in responders compared to non-responders (Figure 4(c)).

Table 3. Circulating metabolites and insulin before and after AXOS and Placebo intervention.

Variables		AXOS (n = 24)	Placebo (n = 24)	P
Fasting				
Glucose, mmol/L	Pre	4.93 ± 0.06	4.95 ± 0.05	.586
	Post	4.94 ± 0.05	4.98 ± 0.05	
TAG, µmol/L	Pre	813 ± 56	896 ± 103	.534
	Post	706 ± 59	860 ± 119	
FFA, µmol/L	Pre	547 ± 37	536 ± 41	.961
	Post	567 ± 50	553 ± 38	
Glycerol, µmol/L	Pre	81.5 ± 8	84.4 ± 7	.410
	Post	91.0 ± 11	83.7 ± 7	
Insulin, mU/L	Pre	8.4 ± 0.6	8.8 ± 0.9	.821
	Post	7.8 ± 0.6	7.9 ± 0.5	
Postprandial (0–5 h) plasma metabolites				
Glucose, iAUC/min	Pre	0.11 ± 0.09	0.32 ± 0.01	.576
	Post	0.14 ± 0.09	0.27 ± 0.09	
TAG, iAUC/min	Pre	117 ± 24	179 ± 22	.183
	Post	175 ± 39	177 ± 21	
FFA, iAUC/min	Pre	−268 ± 31	−243 ± 33	.620
	Post	−302 ± 42	−253 ± 24	
Glycerol, iAUC/min	Pre	−15 ± 5	−22 ± 6	.171
	Post	−25 ± 9	−17 ± 5	
Insulin, iAUC 0-5 h/min	Pre	23.1 ± 2.5	20.4 ± 1.9	.316
	Post	22.5 ± 2.6	22.1 ± 2.1	

Data are mean ± SEM and analyzed using 2-way repeated measure ANOVA with time (pre/post) and intervention as covariates. *P*-value represent time*intervention interaction. There were no differences between groups as a result of intervention. TAG Triacylglycerol, FFA free fatty acids, iAUC incremental area under the curve, AXOS Arabinoxylan-Oligosaccharides

Discussion

This is the first study investigating the effects of 12-week AXOS intake on combined measures of gastrointestinal functioning and metabolic health in healthy participants with a slow GI transit. AXOS intervention did not affect whole-gut transit (WGTT) but significantly increased fecal *Bifidobacterium*, decreased

microbial diversity and softened stool consistency compared to placebo. Further, responder analysis showed that overall microbiota composition accurately classified responders with decreased WGTT as well as non-responders with unchanged/increased WGTT after AXOS intervention. Postprandial fat oxidation tended to increase and early postprandial GLP-1 significantly decreased after AXOS while upper intestinal transit, stool frequency, SCFA, gut permeability, inflammatory markers as well as glucose, insulin and lipid metabolism and adipose tissue functioning were not affected by AXOS.

Bifidobacterium spp. has been shown to accelerate gastrointestinal transit in healthy humans when administered as probiotics.^{52,53} In this study, the AXOS-mediated bifidogenic effect did not translate into changes in quantitative measures of gastrointestinal transit but was accompanied by softer stool consistency consistent with previous studies.^{23–25} WGTT and BSS were not correlated in this study which may explain the different outcomes between the rather subjective BSS and the quantitative radio-opaque marker method. After the AXOS intervention, we observed an average BSS change of 0.5, which may be debatable with regards to its clinical relevance. According to FDA guidelines, change in a BSS of more than 1 is considered clinically relevant in inflammatory bowel disease diarrhea and constipation subtypes.⁵⁴ However, to date, there is no specific suggestion for clinically relevant changes in BSS in the healthy population. Thus, an increase of 0.5 in the Bristol stool score may be regarded as

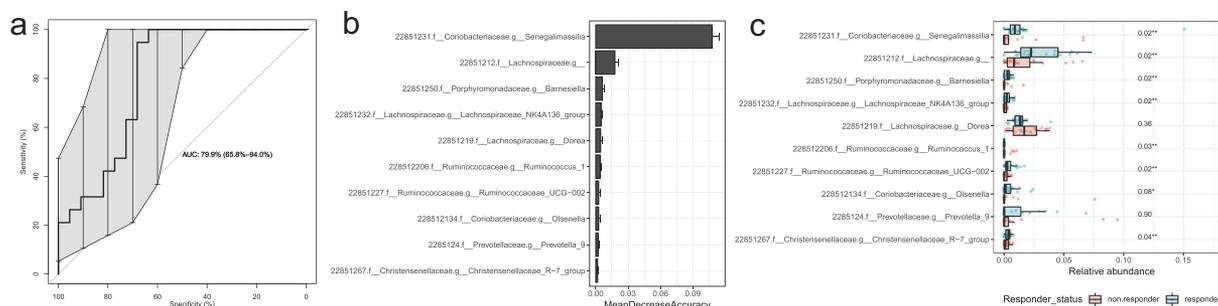


Figure 4. Identification of microbial ASVs as markers for responders with decreased WGTT after AXOS intervention by random forests models with 500-fold cross-validation. (a) The cross-validation error-based AUC was 79.9% with 10 000 bootstraps for a 95% confidence interval (65.8–94.0%). (b) The top 10 microbial ASVs that were important for the classification of responders with decreased WGTT. On the x-axis, MeanDecreaseAccuracy displays the decrease of model accuracy if the predictor ASV would be removed. (c) The abundance of the top 10 microbial groups in responders and non-responders after AXOS intervention. FDR adjusted *P*-values are given of a Wilcoxon signed-rank test between responders and non-responders. WGTT whole-gut transit time. AUC, area under the curve, AXOS, Arabinoxylan-Oligosaccharides, FDR false discovery rate.

normalization of the stool consistency while the clinical relevance in a healthy population needs yet to be validated.

Despite being of lower avDP than other Arabinoxylans or other well-known prebiotics, AXOS with avDP <10 have been shown to induce a bifidogenic effect in animals and humans.^{20,22,55,56} This indicates that AXOS indeed reaches the colon inducing changes in the gut microbiota composition, which most likely occurs mainly in the proximal colon. Possible AXOS-mediated mechanisms contributing to softer stools may be an increased fecal bacterial biomass, increased water binding capacity or stimulation of host mucus production.⁵⁷ Further, AXOS intake decreased microbial alpha diversity, while high microbial diversity is commonly regarded as a biomarker for a stable, healthy gut ecosystem. However, hard stool has been associated with increased alpha diversity reflecting a diversification as an adaptation to a changing ecosystem during long colonic passage (i.e., depletion of nutrients, switch from microbial saccharolytic to proteolytic fermentation, microbial competition, decreased water availability).¹⁰ Thus, microbial diversity should be interpreted within the physiological context: in participants with slow GI transit, softer stool after AXOS intervention may have contributed to the observed reduced alpha diversity which may reflect the AXOS induced shifts in the microbial and colonic ecosystem. Further, reduced alpha diversity may have been a result of the selective stimulation of *Bifidobacterium* growth, and not necessarily represent a reduced microbiota stability. Despite observing no changes in WGTT after AXOS intake compared to placebo, the within-participants variability was remarkable in response to the AXOS intervention. Thus, we further explored the association between responders with a decreased WGTT and non-responders with an unchanged/increased WGTT after AXOS intervention and the gut microbiota composition. We observed that the overall gut microbiota composition could accurately classify responder status indicating that the relationship between gut microbiota and GI transit is potentially bidirectional. Responsible for this classification were mainly *Senegalimassilia* ASV (family *Coriobacteriaceae*, phylum *Actinobacteria*), and an unclassified ASV from the family *Lachnospiraceae* (phylum *Firmicutes*) which were significantly more abundant in the

responders compared to non-responders. Unfortunately, not much is known about *Senegalimassilia* and the ASV from the family of *Lachnospiraceae*, which is among the most abundant in the human gut, making it difficult to speculate on their mode of action. Other predictors were common members of the gut microbiota from different families and phyla. Hence, changes in GI transit may be mediated by a microbial network comprising species of these families rather than the selective stimulation of *Bifidobacterium*.

Colonic SCFA may affect gastrointestinal motility via stimulation of enterochromaffin cells amongst other mechanisms.⁵⁸ Despite the increase of acetate-producing *Bifidobacterium*, plasma and fecal SCFA concentrations were not affected by AXOS intervention. Of note, we observed a time-dependent decrease in plasma acetate and butyrate in both groups reflecting a high temporal variability in SCFA metabolism. Circulating acetate and butyrate have several sources: 1) direct exogenous ingestion via food sources, 2) indirect exogenous production by colonic bacterial fermentation of non-digested food components, and 3) endogenous production from protein, lipid, and carbohydrate catabolism in mainly the liver.⁴ Plasma SCFA concentrations are thus the net result of SCFA production, absorption and splanchnic extraction/hepatic metabolism, and are therefore difficult to interpret. Since rate of appearance from endogenous or exogenous sources can only be acquired by using stable isotope tracer techniques, we cannot provide this information for the present study.

Early postprandial GLP-1 was reduced after AXOS intervention, whilst PYY, insulin, glucose concentrations as well as ratings of hunger and satiety were unchanged. Acute AXOS studies reported inconsistent effects on GLP-1,^{19,59} however, arabinose derived from arabinoxylan fermentation has been proposed to be rapidly fermented in the small intestine as observed by an early postprandial increase of plasma arabinose and xylan after ingestion of wheat-bran rich pasta accompanied by a reduced rate of endogenous glucose appearance rate compared to control wheat bread.⁶⁰ The authors of the latter study propose that the increased availability of arabinoxylans may have increased luminal viscosity and thereby may interfere with small intestinal glucose

breakdown and absorption. Further, arabinose itself has been shown to partly inhibit sucrase and maltase activity in *in vitro* and in rodent models,⁶¹⁻⁶³ which potentially may attenuate breakdown of glucose and thus early glucose-dependent GLP-1 stimulation as observed in our study. While AXOS intervention tended to increase postprandial fat oxidation, there was no effect of AXOS on other metabolic outcomes including energy expenditure, glucose, and insulin, or lipids or adipocyte functioning nor on gut permeability, plasma LBP, fecal calprotectin or other systemic inflammatory markers.

A strength of this study was the thorough phenotypical characterization of participants regarding the subjective and quantitative assessment of gastrointestinal parameters and metabolic profile, as well as the controlled randomized design. A limitation of this study is that the inclusion of healthy participants may have left less room for improvement in metabolic health parameters compared to metabolically compromised individuals with obesity or type 2 diabetes. To summarize, 12-week of AXOS intervention strongly increases *Bifidobacterium*, reduces microbiota diversity and softens stool consistency without changes in WGTT and metabolic health. Overall microbiota accurately classifies responders with decreased WGTT indicating that changes in transit were associated with gut microbiota composition independent of *Bifidobacterium*. Hereby, we substantiate the current notion that there is a complex bidirectional interaction between the gut microbiota and GI transit and that prebiotic stimulation of *Bifidobacterium* was not effective in improving GI transit in this study population. Further studies are warranted to disentangle the connections between the gut microbiota and GI functioning and whether these play a role in metabolic health.

Abbreviations

ASV	amplicon sequence variant
AT	adipose tissue
AUC	area under the curve
AXOS	arabinoxylan-Oligosaccharides
CID	clinical investigation day
FFA	free fatty acids
GE	gastric emptying
GLP-1	glucagon-like peptide-1

OCTT	oro-cecal transit time
PYY	peptide YY
GPR	g-protein coupled receptor
LBP	lipopolysaccharide-binding protein
OGTT	oral glucose tolerance test
SCFA	short-chain fatty acid
T2DM	type 2 diabetes mellitus
TAG	triacylglycerol
TNF- α	tumor necrosis factor- α
IL	interleukin
WGTT	whole-gut transit time

Disclosure of potential conflict of interest

No potential conflicts of interest were disclosed.

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Data sharing

Sequence data have been deposited in the European Nucleotide Archive, accession number [ENA: PRJEB32919].

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