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Randomized Control Trials

Reinforcement of intestinal epithelial barrier by arabinoxylans in overweight and obese subjects: A randomized controlled trial Arabinoxylans in gut barrier



CLINICAL



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A R T I C L E I N F O

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SUMMARY

Background & aims: Obesity and metabolic diseases are associated with alterations in microbial composition and impaired gut barrier. Previous *in vitro* and animal studies have shown that arabinoxylans (AX) have the potential to modulate gut microbiota and gut barrier and therefore could have a protective role. Primary aim of the study was to investigate the effect of AX on intestinal permeability. Secondary aims included the effect of AX on gene transcription and protein expression of tight junctions (TJ), intestinal microbiota composition and activity, immune response and metabolic markers in overweight and obese individuals.

Methods: In this randomized, double-blind, placebo-controlled trial, 47 overweight subjects were randomly assigned to groups receiving 7.5 g/d AX (n = 16), 15 g/d AX (n = 17) or 15 g/d placebo (n = 14) for 6 wks. Intestinal permeability was investigated using a multi-sugar test. Sigmoid colon tissue was obtained from a subgroup (n = 26) for analyzing gene transcription and mucosal expression of TJ proteins. Fecal samples were collected to assess microbial composition and activity. Furthermore, the production of cytokines by stimulated peripheral blood mononuclear cells (PBMCs) was examined. Blood was also sampled for measuring metabolic markers.

Results: No significant changes in gastrointestinal permeability and TJ protein expression were observed after 6 wks AX supplementation compared to placebo. However, gene transcription of occludin was upregulated in the 7.5 g AX group, and transcription of claudin-3 and claudin-4 were upregulated in the 15 g AX group compared to placebo. Furthermore, fecal microbiota diversity was decreased after 6 wks 15 g AX treatment, but no change in relative abundance of dominant phyla was observed. AX intake significantly decreased fecal pH and increased fecal concentrations of total SCFAs, acetate, propionate and butyrate, compared to placebo. Additionally, a decreased TNF α production by stimulated PBMCs was observed after 15 g AX treatment. No changes in metabolic markers were detected.

Conclusions: Regular consumption of AX resulted in a more beneficial fermentation profile in overweight and obese individuals. Further studies are required to assess whether such fermentation profile will translate into improved gut barrier function and immune health.

The trial has been registered at ClinicalTrials.gov with study ID number NCT01877044.

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1. Introduction

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The gastrointestinal (GI) epithelial barrier is a complex system that includes an active local immune defense, a physical barrier comprised of a network of epithelial cells which are connected by

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| Abbrevia | ations |
|----------|--|
| ANOSIM | analysis of similarity |
| AX | arabinoxylans |
| BC | Bray–Curtis |
| CVD | cardiovascular disease |
| DM2 | diabetes mellitus type 2 |
| GI | gastrointestinal |
| GSRS | gastrointestinal symptom rating scale |
| IFNγ | interferon-gamma |
| L/R | Lactulose/L-rhamnose |
| MLCK | myosin light-chain kinase |
| MUMC+ | Maastricht University Medical Center+ |
| PBMC | peripheral blood mononuclear cells |
| PBS | phosphate-buffered saline |
| РСоА | principal coordinate analysis |
| PHA | Phytohemagglutinin-M |
| QUICKI | quantitative insulin sensitivity check index |
| SCFA | short chain fatty acids |
| S/E | sucralose/erythritol |
| TJ | tight junction |
| ZO | zonula occludens |
| | |

tight junction (TJ) proteins to control permeability, a mucus layer, secretion of antimicrobial peptides, intestinal microbiota and luminal microbial metabolism [1]. Recent research has focused on intestinal microbiota and their effects on intestinal physiology, nutrient digestion, luminal metabolism and immune function [2]. Short-chain fatty acids (SCFA) are the end products of fermentation of non-digestible carbohydrates by intestinal microbiota and have an important role in maintenance of intestinal homeostasis. Changes in gut microbiota composition may affect gut barrier function via changes in expression, localization and distribution of TJ proteins, thereby influencing gut permeability [3]. Changes in gut barrier function are regarded as early events or triggers in the development of various intestinal diseases, and also in the development of systemic metabolic diseases, such as diabetes mellitus type 2 (DM2) and cardiovascular diseases (CVD) [4].

Modulation of gut microbial communities by prebiotics appears to be an interesting concept to enhance the gut barrier, to treat or even prevent the onset or aggravation of chronic diseases. Prebiotics are non-digestible food ingredients selectively stimulating growth and/or activity in the GI microflora conferring potential health benefits to the host [5]. A limitation of most prebiotics is their rapid fermentation in the proximal colon. Arabinoxylans (AX), the most abundant non-digestible carbohydrates present in wheat, form a novel class of potential prebiotics [6]. Due to their structure, AX rely on a whole spectrum of (microbial) enzymes for degradation. They are more gradually fermented along the colon, resulting in more distal fermentation [6,7]. AX exist in different forms, ranging from long-chains to enzymatically modified short-chain fractions. In vitro research has shown that the AX form affects the fermentation pattern and immune function, high-molecular weight AX being most active [7,8]. Promising outcomes of high-molecular weight AX on the gut barrier [9,10], gut microbiota [9,11,12], immune system [9] and metabolic markers [9] have been shown both in in vitro and in animal studies. To date, no human data are available on the combined effect of such AX on intestinal environment, gut barrier and immune system.

Therefore, the purpose of this study was to investigate the effects of a high-molecular weight AX concentrate on the gut barrier, intestinal microbiota, immune system and metabolic control in humans in overweight and obese individuals. We hypothesized that 6 wks AX supplementation ameliorates the gut barrier, improves colonic microbiota composition and its metabolic activities, improves the immune system, enhances metabolic control and is well-tolerated in overweight and obese individuals. We specifically chose to study an overweight and obese population, as overweight and obesity are associated with an impaired gut barrier function. Primary aim of this study was to investigate the effect of AX on intestinal permeability, measured by a multi-sugar test. Secondary, we aimed to investigate the effect of AX intake on gene transcription and protein expression of TJ, on fecal microbiota composition and fecal pH, ammonium and SCFA concentrations, on cytokine production by stimulated peripheral blood mononuclear cells (PBMCs), on blood concentrations of glucose, insulin and lipids, and on GI tolerance.

2. Materials and methods

The Medical Ethics Committee of the Maastricht University Medical Center (MUMC+) approved the trial and the study was performed at the MUMC+ from March 2012 to December 2012 in full accordance with the principles of the Declaration of Helsinki of 1964 as amended in 2013 and with the Dutch Regulations on Medical Research involving Human Subjects (WMO, 1998). Before participation all participants provided written informed consent.

2.1. Subjects

Healthy overweight and obese volunteers aged 18-70 v with a BMI between 28 and 35 kg/m² were recruited by local advertisement. Key exclusion criteria were: any medical condition that might interfere with the study and might jeopardise the health status of the participant; smoking; abuse of alcohol (>20 consumptions/wk) and drugs; no consistently stable body weight for at least 6 months $(\pm 2 \text{ kg})$; plans to lose weight or following an energy restriction diet during study period; use of medication, vitamin- or mineral supplements, consumption of pro-, pre- or synbiotics during study period; use of antibiotics in the 90 days prior to start of the study; pregnancy and lactation; history of any side effects towards the intake of pro-, pre-, or synbiotic supplements of any kind. Throughout the trial, subjects consumed their habitual diet. Before start of the study, subjects were informed about the prohibited pre-, pro- and synbiotics and food products containing them.

To assess the difference in GI permeability between this study population and a group of age and sex matched healthy lean subjects, we used the GI permeability data obtained with exactly the same protocol in healthy controls. These data have recently been published by our group [13].

2.2. Design and intervention

This study was set up as a randomized, placebo-controlled, double-blind, parallel group study. Each subject was tested on three occasions, while a subset of these subjects additionally provided sigmoid mucosal tissue samples on two separate occasions. Participants were randomly assigned in a double-blind fashion to one of the three intervention arms: 7.5 g AX, 15 g AX or a placebo (15 g maltodextrin). An independent and blinded person generated the randomization list, using a computerized method. All participants and investigators remained blind to intervention until all analyses were finalized.

Participants were instructed to refrain from consumption of alcohol and strenuous physical exercise on the day before each test day. After an overnight fast, subjects handed in a fecal sample on the first test day. Fecal samples were collected one day before or on a test day, and stored at -20 °C until arrival at the study site. Subsequently, venous blood samples were collected from an antecubital vein in the fore-arm. Then, subjects ingested a multi-sugar drink, to assess gastrointestinal permeability, and collected full urine output for 24 h. A subset of the participants underwent a standard flexible sigmoidoscopy without bowel preparation on the second test day. Seven mucosal tissue samples of approximately 5 mg each were obtained from the sigmoid colon by using a standard forceps (diameter: 2.8 mm) and directly after collection frozen in liquid nitrogen. Two tissue samples were embedded in Tissuetek[®] (Sakura Finetek, Tokio, Japan) prior to freezing. After completion of the baseline measurements, participants received the study product for the first 3 wks. After 3 wks of daily supplementation, the third test day was organized. Again, a fecal sample was handed in, blood samples were taken and study products for another 3 wks were provided. After 6 wks of daily administration of the study product, the fourth and fifth test days took place. Measurements were identical to the baseline measurements performed during the first and second test days, respectively. The same subgroup of subjects underwent again a sigmoidoscopy. Also, all participants had to complete a questionnaire at weekly intervals, to assess the presence of GI symptoms, stool frequency and stool consistency. To assess compliance, participants were asked to collect the empty sachets and to return these at the last visit.

2.3. AX concentrate and placebo

BioActor BV (Maastricht, The Netherlands) supplied AX with a purity of 62%, degree of substitution of 0.7, and a varying degree of polymerization, with an average above 60; the composition of the batch used for the study was 67% non-starch polysaccharides (62% AX), 18% protein, 0.5% lipids, 3.8% ash. Maltodextrin (Glucidex IT 19; Roqette Frères, Lestrem Cedex, France) served as placebo. The study products were provided to subjects as a powder in sachets. The placebo group received per day two sachets, each containing 7.5 g Maltodextrin, the 7.5 g AX group received per day two sachets, each containing 3.75 g AX combined with 3.75 g Maltodextrin and the 15 g AX group received per day two sachets, each containing 7.5 g AX. Subjects were asked to stir the content of one sachet in 200 mL liquid (e.g. juice, water, milk) and ingest the solution twice daily (every morning and every evening) for 6 wks.

2.4. Gastrointestinal permeability

GI permeability was investigated using a multi-sugar test, quantifying 24-h urinary excretion of five different ingested sugars reflecting the permeability of 4 segments of the GI tract. Firstly, fasted volunteers emptied their bladder and drank the multi-sugar drink, consisting of 1 g lactulose (Centrafarm, Etten-Leur, the Netherlands), 0.5 g L-rhamnose (Danisco, Copenhangen, Denmark), 1 g erythritol (Now Foods, Bloomindale, IL, USA), 1 g sucralose (Brenntag, Sittard, the Netherlands) and 1 g sucrose (Van Gilse, Dinteloord, the Netherlands) dissolved in 150 mL tap water. Participants were instructed to collect their 24 h urine output in two different fractions; one fraction containing the 0-5 h urine output and a second fraction containing the 5–24 h urine output. It was not allowed to ingest any foods or drinks, except for water, throughout the first urine fraction (0-5 h) collection. Gastroduodenal and small intestinal permeability were reflected by the 0-5 h urinary sucrose excretion and the lactulose and L-rhamnose (L/R) ratio, respectively. In 5–24 h urine, the sucralose to erythritol (S/E) ratio represented colonic permeability, while in 0-24 h urine it indicated whole gut permeability. After collection of the 24 h urine output, total volume was quantified and urine aliquots were frozen at -80 °C. Urinary sugars were analyzed using HPLC-MS as reported earlier [14]. The intestinal permeability test and the analysis of urine samples in the study of Mujagic et al. [13] was performed in exactly the same way and done by the same research group as in our study.

2.5. Gene transcription of TJ and associated proteins

Gene transcription of TJ and associated proteins (claudin-3, claudin-4, occludin, myosin light-chain kinase (MLCK) and zonula occludens-1 (ZO-1)) in sigmoid colon tissue samples was determined by quantitative real-time polymerase chain reaction, as earlier presented by Pijls et al. [15]. Housekeeping genes included GAPDH and 18S RNA. Data are shown as normalized expression ratios.

2.6. Protein concentrations of claudin-3

Sigmoid mucosal tissue was used for the determination of claudin-3 protein concentrations. Biopsy specimens were kept in liquid nitrogen and ground in a nitrogen cold mortar. The powder was gently scraped with a spatula of the auger and dissolved in 150 µl phosphate-buffered saline (PBS) (Invitrogen 10010, pH 7.4) containing protease inhibitor cocktail (Sigma P8340) with a concentration from 10 µl PI/ml PBS and centrifuged for 20 min at 10.000 rpm at 4 °C. Supernatant was stored at -80C. The concentration of claudin-3 was determined with the sandwich ELISA kit for human claudin-3 (Cloud-Clone Corp., Houston USA Kit: SEF293Hu). The microtitre plate provided in this kit was precoated with an antibody specific to claudin-3. Standards of samples (samples were 1-5 diluted) were added to the suitable microtitre plate wells together with a claudin-3 specific biotin-conjugated antibody. Then, avidin conjugated to horseradish peroxidase was applied on each microplate well and incubated. Subsequently tetramethylbenzidine substrate solution was added, and only the wells containing claudin-3, biotin-conjugated antibody and enzyme-conjugated avidin exhibited a color change. Addition of sulphuric acid solution ended the enzyme-substrate reaction. The change in color was quantified using spectrophotometry (wavelength: 450 nm \pm 10 nm). The concentration of claudin-3 in the tissue was defined by comparison of the O.D. of the tissue sample to that of the standard curve. Total protein determination in the sigmoid tissue was done using Pierce® BCA Protein Assay Kit (ThermoFisher, Waltham, MA USA). Samples were 1-15 diluted. Data are presented as pg claudin-3 per ug total protein.

2.7. Immunofluorescence staining of ZO-1 and occludin

Immunofluorescent staining of ZO-1 and occludin was done as presented earlier [16]. Leica TCS SPE confocal microscopy (Leica Micro systems GmbH, Mannheim, Germany) was used to analyze images. Obtained images were quantified by calculating the AUC. Briefly, to assess the fluorescent staining of both TJ proteins in the TJ region, mucosal tissue was exposed to confocal analysis of Z regions perpendicular to the apical cell surface of the epithelium. Three randomly selected areas within 3 different Z regions were analyzed. The middle part of the villi was used for analyzing the staining. Image J software created the plot profiles reflecting staining intensity and AUC was calculated using Matlab R2013a software (Natick, MA, USA).

2.8. Microbiota composition and activity

2.8.1. DNA extraction

To extract DNA from fecal samples (100 mg) samples were resuspended in 1 mL of lysis buffer (Tris/HCl (100 mM pH 8.0),

100 mM EDTA, 100 mM NaCl, 1% (wt/vol) polyvinylpyrrolidone and 2% (wt/vol) SDS). Cells were lysed in a Fast Prep[®]-24 instrument (MP Biomedicals, Solon, OH, USA) for 30 s at 1800 rpm and extractions were performed using phenol-chloroform-isoamyl ethanol (25:24:1). DNA was precipitated with ice-cold isopropanol and resuspended in 100 μ l of TE buffer (10 mM Tris–HCl, 1 mM EDTA [pH 8.0]). The amount and quality of DNA was measured using an ND 1000 spectrophotometer (ThermoFisher, Waltham, MA, USA).

2.8.2. Gut microbiota composition

We used 16S targeted DNA-based Illumina with MiSeq platform to assess the gut microbiome structure in the fecal samples. Primers for the 16S rRNA gene amplified the hypervariable region V1–V2. After sequencing, quality filtering was performed as previously described and sequences were taxonomically annotated using RDP database with 80% threshold [17]. Sequencing depth was normalized to the minimum reads per sample, using the Phyloseq package from R. Rarefaction curves and biodiversity indices were calculated using the vegan package from R. Rarefaction curve analysis showed that the use of at least 10.000 reads was sufficient to sample the complete diversity of the library.

The following metrics of alpha diversity were determined: observed richness, Shannon diversity index and Simpson evenness. Beta diversity was assessed by Bray–Curtis (BC) dissimilarity. BC is an ecological diversity index representing the extent of change in microbiota composition. This index takes into consideration both the bacterial taxa detected in a specimen, and their relative presence.

2.8.3. Fecal pH, ammonium and SCFA determination

Fecal samples were collected by the participants and stored at -20 °C until handed in at the study site. For SCFA and ammonium analyses, aliquots of approximately 1 g of fecal samples were diluted and homogenized with 6 mL demineralized water. After removal of the particulate material by centrifugation (10 min, 500 \times g), the supernatants were stored at -20 °C prior to analysis. SCFA concentrations in the supernatants were determined using gas chromatography with flame ionization detector, based on the SCFA concentration analysis described by Possemiers et al. [18]. The sum of acetate, propionate, butyrate, isobutyrate, valerate, isovalerate, caproate and isocaproate represent total SCFA. Ammonia concentrations in the supernatants were measured using the method previously presented [19]. To determine fecal pH, an aliquot of approximately 1 g feces was homogenized by mixing into 10% (w/w) demineralized water. Directly after homogenization pH was assessed.

2.9. Production of cytokines by stimulated peripheral blood mononuclear cells

Blood was collected in sodium heparine tubes (Becton & Dickinson, Franklin Lakes, NJ, USA) and used within 1 h for the isolation of PBMCs. To examine *ex vivo* cytokine production by PBMCs, cells were isolated from whole blood and stimulated with Phytohemagglutinin-M (PHA). PHA-stimulation is used to bind antigen-presenting cells and T-cells and thereby PHA induces T-cell proliferation. This technique is used to enlarge the current activities of the T-cells present in the culture. Lymphoprep gradient centrifugation was performed according to the manufacturer's instructions (Takeda Nederland B.V., Hoofddorp, The Netherlands). Freshly isolated cells were diluted in RPMI 1640 culture medium containing HEPES and L-glutamine (ThermoFisher, Waltham, MA USA), with 1% added Penicillin/Streptomycin, Sodium Pyruvate, and heat inactivated human serum pool. Cells were seeded 1 * 10⁶ in 24well flat bottom culture plates (Greiner Bio-One B.V., Alphen a/ d Rijn, The Netherlands) and T-cell proliferation was stimulated with PHA (Roche Diagnostics Nederland B.V., Almere, The Netherlands). After 48 h, the culture medium was harvested and stored at -80 °C until further analysis. Interferon-gamma (IFN γ) was measured using a sandwich ELISA according to the manufacturer's instructions (eBioscience, Vienna, Austria). IL-2, IL-10, IL-12p40 and TNF α were measured with a multiplex chemoluminescence assay (Meso Scale Diagnostics, Rockville, Maryland USA).

2.10. Blood lipids, glucose and insulin

Blood samples were collected at baseline, after 3 wks of supplementation and at the end of the supplementation period, respectively. Serum concentrations of glucose, total cholesterol, LDL- and HDL-cholesterol, and triglycerides were measured using spectrophotometry. Plasma insulin was determined using a luminescence-enhanced immunoenzymatic assay. Insulin sensitivity was estimated by the quantitative insulin sensitivity check index (QUICKI) index as: $1/(\log insulin 0 h + \log glucose 0 h)$.

2.11. Gastrointestinal tolerance

The gastrointestinal symptom rating scale (GSRS) was used to assess the presence of GI symptoms. This questionnaire consists of 15 seven-point graded items that can be combined into 5 symptom clusters reflecting reflux, abdominal pain, indigestion, diarrhea, and constipation. The absence of troublesome symptoms is represented by a score of 1, while a score of 7 reflects very inconvenient GI symptoms [20]. Defecation frequency and stool consistency were assessed using the Bristol stool chart. Subjects completed these questionnaires at weekly intervals during the study period.

2.12. Statistical analyses

The primary outcome of the study was the effect of 6 wks 7.5 g AX and 15 g AX supplementation on GI permeability, compared to placebo. Secondary outcomes include the effect of 6 wks AX supplementation versus placebo on gene transcription and protein expression of TJ, fecal microbiota composition and activity, cytokine production by PBMCs, metabolic markers and on GI tolerance. Sample size was determined for the primary outcome of the study, using a significance level alpha = 0.017 and a power of 80%. A study in diet-induced obese mice, investigating the effect of a dietary fiber on GI permeability [21], was used for calculating the sample size. To detect a difference in intestinal permeability of $0.3 \pm 0.25 \ \mu g/ml$ (mean \pm SD) a total of 45 subjects would be required. Baseline characteristics are displayed as mean (SD) for numerical variables. Baseline values in age and BMI between overweight and obese individuals and lean controls were compared using independent t-test, and baseline numbers in sex were evaluated with chi-square test. Differences in gut barrier (function), fecal pH, relative abundance of main phylo of the fecal microbiota, alpha diversity metrics, fecal SCFA concentrations, immune response, blood parameters and digestive tolerance between placebo and 7.5 g AX and placebo and 15 g AX were assessed using linear mixed models with group (placebo, 7.5 g AX and 15 g AX), time (0, 3 and 6 wks for AX effect) and group*time as fixed factors, where an unstructured covariance structure was used for repeated measures. The linear mixed model accounts for the correlation between repeated measures and missing data, where a likelihood approach was used assuming data missing at random.

GI permeability data were not normally distributed, as assessed by Shapiro–Wilk Test. Mann–Whitney *U* test was used to evaluate the difference in baseline GI permeability between healthy lean and overweight and obese individuals. Statistical analysis was performed using IBM SPSS Statistics for Windows (version 21.0, Armonk, NY, USA).

R language for statistical computing [http://www.R-project.org] was used for normalizing sequencing depth, calculating rarefaction curves and biodiversity indices. It was also used to generate exploratory heat maps, using the heatmap.2 function from the R package gplots on logarithmic transformed data. To determine the effect of intervention on the shared diversity between samples, beta diversity metrics (BC dissimilarity) were calculated for placebo versus 7.5 g AX and placebo versus 15 g AX. A Principal coordinate analysis (PCoA), based on the BC similarity distances, was used in order to visualize the microbial variation among placebo, 7.5 g AX and 15 g AX. A two-way crossed analysis of similarity (ANOSIM) was used to determine if the microbial communities were significantly different between treatment groups. These analyses were performed using PAleontological STatistics version 3.11 (PAST, Øyvind Hammer, Natural History Museum, University of Oslo). For all comparisons, two-sided p-values <0.05 were considered statistically significant.

3. Results

3.1. Study subjects

A total of 47 healthy volunteers were enrolled in the study; 14 subjects were assigned to the placebo group, 16 subjects to the AX 7.5 g group and 17 subjects to the AX 15 g group. Forty-five volunteers completed the entire study protocol. One participant dropped out during the intervention period because of a pneumonia and consequent antibiotic treatment. The second dropout developed gallstones during the study period and had to undergo surgery. From these participants only baseline characteristics were available (Online Supplemental Material Fig. 1). Sigmoidoscopy was performed in 9 participants in the placebo group, 9 participants in the 7.5 g AX group and 8 participants in the 15 g AX group. Baseline characteristics are presented in Table 1.

Data of a group of 33 lean age and sex matched controls, derived from Mujagic et al. [13], were used to evaluate differences in GI permeability between lean and overweight/obese subjects.

3.2. Gastrointestinal permeability

Baseline GI permeability was significantly increased in our healthy, but overweight population, compared to an age and sex matched healthy, lean population (all P < 0.001, Table 2).

3.2.1. Six weeks effect

Table 3 presents the effect of 6 wks intervention on GI permeability. Gastroduodenal permeability, as indicated by the sucrose excretion in the 0-5 h urine fraction, did not significantly change after both 7.5 g AX and 15 g AX supplementation compared to

Table 2

| Gastrointestinal permeability, reflected by urinary sugar excretion (µmol) and ratios |
|---|
| of excreted sugars (in 0-5, 5-24 and 0-24 h fraction) in lean, and overweight and |
| obese subjects. |

| Sugar excretion | Lean $(n = 33)$ | Overweight & obese $(n = 45)$ | P value |
|------------------|----------------------|-------------------------------|---------|
| 0–5 h sucrose | 2.06 [0.51; 6.91] | 7.75 [4.68; 13.91] | <0.001 |
| 0–5 h L/R ratio | 0.016 [0.009; 0.036] | 0.050 [0.030; 0.108] | <0.001 |
| 5–24 h S/E ratio | 0.009 [0.006; 0.015] | 0.018 [0.014; 0.023] | <0.001 |
| 0–24 h S/E ratio | 0.010 [0.005; 0.014] | 0.020 [0.014; 0.024] | <0.001 |

Differences between lean subjects and overweight and obese subjects tested with Mann–Whitney *U* test. Data are given as median [IQR, i.e. Q1; Q3]. L/R, lactulose/L-rhamnose. S/E, sucralose/erythritol.

placebo (all $P \ge 0.224$). Neither was the 0–5 h urinary L/R ratio, reflecting small intestine permeability, altered after the AX interventions compared to placebo (all $P \ge 0.219$). Colonic and whole gut permeability, respectively reflected by the S/E ratio in 5–24 h and 0–24 h urine fractions, did not show significant differences among AX and placebo groups after 6 wks supplementation (all $P \ge 0.257$).

3.3. Gene transcription of TJ and associated proteins

Results regarding the effects of AX administration on gene transcription of TJ and associated proteins are given in Table 4. Transcription of claudin-3 and claudin-4 was significantly upregulated in participants receiving daily 15 g AX for 6 wks, compared to participants receiving placebo (P = 0.012 and P = 0.046 respectively). Furthermore, a trend towards upregulation of the TJ protein occludin was seen after 15 g AX intake compared to placebo (P = 0.057). In the 7.5 g AX group we observed a significant upregulation of occludin compared to placebo (P = 0.031). No significant changes in gene transcription for MLCK and ZO-1 between both AX groups and the placebo group were observed (all $P \ge 0.087$).

3.4. Protein concentration of claudin-3, ZO-1 and occludin

At baseline, protein concentrations of claudin-3 were 6.10 ± 0.61 (mean \pm SEM), 4.92 ± 0.41 and 7.02 ± 1.49 pg claudin-3 per ug protein for placebo, 7.5 g AX and 15 g AX respectively. Protein concentration of ZO-1 and occludin in biopsies were quantified by calculating an AUC. At baseline, AUCs of ZO-1 were 159 ± 34 (mean \pm SEM), 126 ± 33 and 177 ± 41 and of occludin 112 ± 19 , 142 ± 35 and 140 ± 29 for placebo, 7.5 g AX and 15 g AX respectively. No significant differences were observed in sigmoid protein concentrations of claudin-3, ZO-1 and occludin between placebo and AX interventions after 6 wks supplementation (all $P \ge 0.144$, data not shown).

3.5. Microbiota composition and activity

From 37 study participants (placebo: n = 12; 7.5 g AX: n = 9; 15 g AX: n = 15) gut microbiota composition data were available. Data of

Table 1

Baseline characteristics in the overweight and obese study population, and lean controls.

| | Overall cohort $(n = 47)$ | Placebo $(n = 14)$ | AX 7.5 g (<i>n</i> = 16) | AX 15 g (<i>n</i> = 17) | Lean controls $(n = 33)$ |
|--------------------------|---------------------------|--------------------|------------------------------|-----------------------------|---|
| Age (y) | 48 ± 16 | 49 ± 17 | 49 ± 17 | 47 ± 15 | $ \begin{array}{r} 42 \pm 3 \\ 13/20 \\ 22.0 \pm 0.31 \end{array} $ |
| Sex (M/F) | 25/22 | 8/6 | 10/6 | 7/10 | |
| BMI (kg/m ²) | 31.0 ± 2.4 | 31.4 ± 3.1 | 30.2 ± 1.9 | 31.5 ± 2.2 | |

Differences in age and BMI between overall cohort and lean controls tested with independent t-test, differences in sex between overall cohort and lean controls tested with chi-square test. Data are given as mean \pm SD. P < 0.001 overall cohort vs. lean controls. AX, arabinoxylans.

Table 3

Gastrointestinal permeability, reflected by urinary sugar excretion (μ mol) and ratios of excreted sugars (in 0–5, 5–24 and 0–24 h fraction) at baseline and after 6 wks supplementation.

| Sugar excretion | Intervention | Baseline | End | P value |
|------------------|--------------|----------------------|----------------------|---------|
| 0–5 h sucrose | Placebo | 5.43 [3.97; 8.74] | 7.10 [4.99; 11.75] | |
| | 7.5 g AX | 10.76 [8.39; 21.89] | 12.52 [8.38; 17.55] | 0.256 |
| | 15 g AX | 7.05 [3.38; 13.24] | 7.88 [4.99; 9.29] | 0.224 |
| 0—5 h L/R ratio | Placebo | 0.045 [0.030; 0.073] | 0.065 [0.038; 0.170] | |
| | 7.5 g AX | 0.070 [0.040; 0.120] | 0.060 [0.048; 0.135] | 0.464 |
| | 15 g AX | 0.050 [0.030; 0.115] | 0.065 [0.040; 0.095] | 0.219 |
| 5–24 h S/E ratio | Placebo | 0.020 [0.014; 0.025] | 0.018 [0.016; 0.023] | |
| | 7.5 g AX | 0.016 [0.012; 0.020] | 0.020 [0.013;0.024] | 0.766 |
| | 15 g AX | 0.020 [0.014; 0.026] | 0.017 [0.013; 0.024] | 0.810 |
| 0–24 h S/E ratio | Placebo | 0.020 [0.015; 0.025] | 0.017 [0.015; 0.020] | |
| | 7.5 g AX | 0.018 [0.014; 0.022] | 0.018 [0.013; 0.025] | 0.257 |
| | 15 g AX | 0.020 [0.014; 0.024] | 0.016 [0.014; 0.024] | 0.986 |

Differences between placebo, 7.5 g AX and 15 g AX tested with linear mixed model with correction for baseline values. Data are given as median [IQR, i.e. Q1; Q3]. AX, arabinoxylans. L/R, lactulose/L-rhamnose. S/E, sucralose/erythritol.

Table 4

Gene transcription of TJ and associated proteins (expressed as normalised expression ratios) at baseline and after 6 wks supplementation.

| Gene transcription | Intervention | Baseline | | End | | Difference in means | 95% CI | P value |
|--------------------|--------------|----------|------|------|------|---------------------|-------------|---------|
| | | Mean | SD | Mean | SD | | | |
| Claudin-3 | Placebo | 1.13 | 0.06 | 1.12 | 0.04 | | | |
| | 7.5 g AX | 1.09 | 0.04 | 1.13 | 0.03 | 0.02 | -0.01, 0.05 | 0.225 |
| | 15 g AX | 1.12 | 0.05 | 1.16 | 0.04 | 0.05 | 0.01, 0.08 | 0.012 |
| Claudin-4 | Placebo | 1.13 | 0.04 | 1.11 | 0.03 | | | |
| | 7.5 g AX | 1.10 | 0.04 | 1.12 | 0.03 | 0.02 | -0.01, 0.05 | 0.152 |
| | 15 g AX | 1.10 | 0.05 | 1.13 | 0.04 | 0.03 | 0.00, 0.06 | 0.046 |
| MLCK | Placebo | 1.08 | 0.06 | 1.08 | 0.03 | | | |
| | 7.5 g AX | 1.06 | 0.03 | 1.10 | 0.03 | 0.03 | 0.00, 0.06 | 0.087 |
| | 15 g AX | 1.06 | 0.06 | 1.09 | 0.05 | 0.02 | -0.01, 0.05 | 0.236 |
| OCLN | Placebo | 1.14 | 0.05 | 1.14 | 0.02 | | | |
| | 7.5 g AX | 1.13 | 0.03 | 1.17 | 0.03 | 0.04 | 0.00, 0.07 | 0.031 |
| | 15 g AX | 1.15 | 0.07 | 1.17 | 0.05 | 0.03 | -0.01, 0.07 | 0.057 |
| ZO-1 | Placebo | 1.17 | 0.05 | 1.17 | 0.04 | | | |
| | 7.5 g AX | 1.15 | 0.04 | 1.19 | 0.04 | 0.02 | -0.01, 0.05 | 0.197 |
| | 15 g AX | 1.14 | 0.05 | 1.17 | 0.04 | 0.01 | -0.02, 0.05 | 0.516 |

Differences between placebo, 7.5 g AX and 15 g AX tested with linear mixed model. The presented difference in means is corrected for baseline differences. AX, arabinoxylans. MLCK, myosin light-chain kinase. OCLN, occludin. ZO-1, zonula occludens.

8 participants were not available because there was no amplification in sequencing or data were removed due to quality control issues.

3.5.1. Phylum relative abundance

16S targeted DNA-based Illumina with MiSeq platform allowed to obtain at least 30.000 usable reads per sample. The dominant phyla across all baseline fecal samples were Firmicutes (mean 52.5%, SD 10.5%) and Bacteroidetes (mean 36.0%, SD 10.1%), followed by Proteobacteria (mean 7.7%, SD 7.6%) and Actinobacteria (mean 3.0%, SD 2.9%). The relative abundance of these four dominant phyla did not change significantly after 6 weeks intake of 7.5 g AX or 15 g AX, compared to placebo (all $P \ge 0.061$; Fig. 1).

3.5.2. Fecal samples alpha diversity

Six wks treatment with the two dosages AX did not change the Simpson evenness index, compared to placebo (all $P \ge 0.063$, Table 5). However, 6 wks intake of 15 g AX but not 7.5 g AX significantly reduced the observed richness and lowered Shannon diversity, compared to placebo (P = 0.029 and P = 0.036 respectively, Table 5). Six wks treatment with 7.5 g AX or 15 g AX versus placebo did not change the Simpson evenness index.

3.5.3. Microbiota composition and taxon distribution

The PCoA analyses, based on the BC (dis)similarity matrix of the bacterial community sequence data, per test day are shown as

Online Supplemental Material Figs. 2 and 3 and 4. In order to see if treatment during study period significantly altered fecal microbial composition, a two-way ANOSIM was performed. Two-way ANO-SIM applying the BC coefficient showed no significant differences for treatment (P = 0.746) using time as factor. The heat map analysis showed that variations observed were not linked to a specific dosage of the test product and were masked by the interindividual variability among subjects. The cluster dendrogram showed that,



Fig. 1. Relative abundance of the main phyla according the 16S-targeted Illumina data for placebo, 7.5 g AX and 15 g AX at baseline (0), after 3 wks supplementation (3) and after 6 wks supplementation (6). No significant differences in relative abundance between placebo, 7.5 g AX and 15 g AX were during study period observed, as tested with linear mixed model with correction for baseline values. AX, arabinoxylans.

| Table 5 | |
|--|--|
| Observed richness, Shannon diversity and Simpson evenness indices at baseline and after 6 wks supplementation. | |

| Indices | Intervention | Baseline | | End | | Difference in means | 95% CI | P value |
|-------------------|--------------|----------|------|-------|------|---------------------|--------------|---------|
| | | Mean | SD | Mean | SD | | | |
| Observed richness | Placebo | 295.3 | 21.4 | 285.2 | 44.9 | | | |
| | 7.5 g AX | 301.4 | 49.3 | 287.8 | 33.1 | -1.9 | -39.1, 35.3 | 0.918 |
| | 15 g AX | 272.4 | 40.3 | 235.8 | 62.5 | -36.7 | -69.3, -4.0 | 0.029 |
| Shannon diversity | Placebo | 4.13 | 0.28 | 4.05 | 0.33 | | | |
| - | 7.5 g AX | 4.28 | 0.37 | 4.03 | 0.31 | -0.11 | -0.41, 0.20 | 0.473 |
| | 15 g AX | 4.08 | 0.25 | 3.75 | 0.43 | -0.29 | -0.55, -0.02 | 0.036 |
| Simpson evenness | Placebo | 0.96 | 0.02 | 0.96 | 0.02 | | | |
| | 7.5 g AX | 0.97 | 0.02 | 0.94 | 0.03 | -0.01 | -0.04, 0.01 | 0.278 |
| | 15 g AX | 0.96 | 0.01 | 0.94 | 0.03 | -0.02 | -0.04, 0.00 | 0.063 |

Differences between placebo, 7.5 g AX and 15 g AX tested with linear mixed model. The presented difference in means is corrected for baseline differences. AX, arabinoxylans.

with a few exceptions mainly related to the 15 g AX, samples from the same donor cluster together at baseline, after 3 wks intervention and end of the study, irrespective of the treatment received (data not shown).

3.5.4. Fecal pH, ammonium and SCFA concentrations

Data regarding fecal pH, ammonium and SCFA concentrations are depicted in Table 6. Fecal pH was significantly reduced after 6 wks 7.5 g AX supplementation, compared to placebo (P = 0.012). Six wks daily intake of both 7.5 g and 15 g AX increased the concentration of total SCFA, compared to a decrease in the placebo group, the difference being statistically significant (7.5 g AX: P = 0.012; 15 g AX: P = 0.006). The same was observed for fecal concentrations of acetate (7.5 g AX: P = 0.038; 15 g AX: P = 0.016) and butyrate (7.5 g AX: P = 0.001; 15 g AX: P = 0.030). Fecal propionate concentrations significantly increased in the 15 g AX group, compared to placebo (P = 0.026) AX intake versus placebo did not affect fecal ammonium concentrations (7.5 g AX: P = 0.111; 15 g AX: P = 0.370). No significant differences between interventions were seen in fecal pH, ammonium and SCFA concentrations after 3 wks supplementation (all $P \ge 0.082$, data not shown).

3.6. Production of cytokines by stimulated peripheral blood mononuclear cells

Production of the pro-inflammatory cytokine TNFα by stimulated PBMCs was significantly reduced after 6 weeks 15 g AX intake

Table 6

Fecal pH, ammonium (mg/L) and short-chain fatty acid concentrations ($\mu mol/g$ feces) at baseline and after 6 wks supplementation.

| | Intervention | Baseline | End | P value |
|------------------|--------------|-------------------|-------------------|---------|
| рН | Placebo | 7.3 [7.0; 7.6] | 7.7 [7.3; 8.1] | |
| | 7.5 g AX | 7.1 [6.8; 7.5] | 7.0 [6.9; 7.4] | 0.013 |
| | 15 g AX | 7.2 [6.9; 7.9] | 7.4 [6.9; 7.7] | 0.086 |
| Ammonium | Placebo | 58.5 [46.1; 74.5] | 44.8 [34.7; 59.9] | |
| | 7.5 g AX | 53.8 [45.8; 69.7] | 55.4 [41.9; 91.4] | 0.111 |
| | 15 g AX | 43.4 [28.2; 60.8] | 51.8 [31.5; 88.1] | 0.370 |
| SCFA: total | Placebo | 67.4 [46.4; 97.0] | 59.1 [31.7; 63.8] | |
| | 7.5 g AX | 63.6 [50.0; 86.2] | 71.6 [54.7; 88.3] | 0.012 |
| | 15 g AX | 49.3 [33.7; 72.5] | 59.2 [41.8; 80.3] | 0.006 |
| SCFA: acetate | Placebo | 34.8 [29.2; 49.9] | 31.2 [17.4; 35.4] | |
| | 7.5 g AX | 35.6 [28.6; 44.7] | 35.0 [25.4; 53.3] | 0.038 |
| | 15 g AX | 27.8 [17.9; 40.1] | 34.0 [23.0; 44.5] | 0.016 |
| SCFA: propionate | Placebo | 11.1 [7.7; 18.4] | 9.8 [6.1; 12.2] | |
| | 7.5 g AX | 11.5 [8.7; 17.0] | 12.5 [9.0; 15.5] | 0.229 |
| | 15 g AX | 10.2 [6.4; 17.0] | 12.7 [8.4; 17.6] | 0.026 |
| SCFA: butyrate | Placebo | 9.0 [6.1; 27.4] | 6.7 [3.7; 9.9] | |
| - | 7.5 g AX | 10.9 [8.7; 14.3] | 11.9 [9.4; 17.0] | 0.001 |
| | 15 g AX | 6.9 [4.5; 15.2] | 7.4 [5.2; 14.6] | 0.030 |

Differences between placebo, 7.5 g AX and 15 g AX tested with linear mixed model with correction for baseline values. Data are given as median [IQR, i.e. Q1; Q3]. AX, arabinoxylans. SCFA, short-chain fatty acids.

(P = 0.035; Table 7) compared to placebo. No significant differences were found regarding the production of the other measured cytokines (all $P \ge 0.176; \text{Table 7})$.

3.7. Blood lipids, glucose and insulin

At baseline, total cholesterol, LDL- and HDL-cholesterol, triglycerides, free fatty acids, glucose and insulin lied within normal blood value ranges. These parameters were not significantly altered by either 3 wks or 6 wks supplementation with 7.5 g or 15 g AX compared to placebo (all $P \ge 0.083$; Table 8). Subsequently, no change between intervention groups was observed regarding insulin sensitivity (all $P \ge 0.768$, Table 8).

3.8. Gastrointestinal tolerance

No significant differences between intervention groups regarding reported GI symptoms were observed during the study (all $P \ge 0.118$, data not shown). Mean scores remained under 2 during all treatments, which means that the participants during intervention experienced no troublesome symptoms. Neither 7.5 g nor 15 g AX significantly affected stool frequency or consistency throughout the entire study period (all $P \ge 0.271$, data not shown).

4. Discussion

In this study, we have shown that GI permeability is compromised in overweight and obese individuals when compared to lean controls. Six wks intake of 7.5 g or 15 g AX per day did not affect GI permeability, as assessed by the multi-sugar test. Although no differences were found in the relative abundance of the dominant phyla, intake of 15 g AX resulted in reduced microbial richness and diversity, when compared to placebo. AX supplementation increased fecal SCFA concentrations. Furthermore, 7.5 g AX intake significantly lowered fecal pH. This modulation of the intestinal luminal environment may positively affect the gut barrier, as reflected by upregulation in the transcription of different TJ proteins in sigmoid biopsies, although we did not detect significant changes in TJ protein expression. The decreased TNF α production by stimulated PBMCs in the high dose (15 g) AX group points to downstream positive anti-inflammatory effects.

Evidence indicating that obesity is associated with an impaired gut barrier is mainly derived from animal models [3]. Human studies on gut barrier and obesity are scarce, and results contradictory [22,23]. Here, we showed that gut permeability is increased in obese compared to lean healthy volunteers. AX treatment did not affect gastroduodenal, small intestinal, colonic or whole gut permeability, as assessed with a multi-sugar test. However, interpretation of these results must be done with caution as permeability testing with sugars might be influenced by individual

Table 7

Table 8

Cytokine production by stimulated PBMCs (ng/mL) at baseline and after 6 wks supplementation.

| Cytokine production | Intervention | Baseline | End | P value |
|---------------------|--------------|--------------------|--------------------|---------|
| IFNγ | Placebo | 29.8 [5.0; 75.5] | 48.8 [12.6; 419.2] | |
| | 7.5 g AX | 14.2 [9.3; 59.3] | 38.7 [2.5; 104.5] | 0.176 |
| | 15 g AX | 54.9 [26.5; 85.3] | 49.5 [10.7; 87.7] | 0.211 |
| II-2 | Placebo | 15.2 [0.9; 38.2] | 7.5 [1.2; 23.6] | |
| | 7.5 g AX | 0.3 [0.1; 7.3] | 5.3 [0.2; 41.1] | 0.427 |
| | 15 g AX | 10.7 [0.4; 53.4] | 1.5 [0.2; 49.9] | 0.489 |
| Il-10 | Placebo | 6.7 [2.6; 18.6] | 12.8 [11.4; 27.1] | |
| | 7.5 g AX | 3.8 [1.5; 10.1] | 5.3 [3.2; 18.4] | 0.205 |
| | 15 g AX | 8.3 [5.0; 18.3] | 11.5 [5.6; 17.2] | 0.402 |
| Il-12p40 | Placebo | 0.02 [0.01; 0.16] | 0.05 [0.02; 0.17] | |
| | 7.5 g AX | 0.02 [0.01; 0.03] | 0.04 [0.02; 0.06] | 0.210 |
| | 15 g AX | 0.02 [0.01; 0.07] | 0.02 [0.00; 0.06] | 0.861 |
| TNFα | Placebo | 72.9 [44.5; 147.6] | 97.2 [52.4; 119.3] | |
| | 7.5 g AX | 56.9 [28.6; 103.4] | 61.9 [34.0; 138.7] | 0.708 |
| | 15 g AX | 83.0 [50.5; 96.0] | 60.9 [27.4; 77.6] | 0.035 |

Differences between placebo, 7.5 g AX and 15 g AX tested with linear mixed model with correction for baseline values. Data are given as median [IQR, i.e. Q1; Q3]. AX, arabinoxylans. IFN, interferon. TNF, tumor necrosis factor.

| Blood lipids (mmol/L), glucose (r | nmol/L), insulin (pmol/L) and | QUICKI at baseline and after | 6 wks supplementation. |
|-----------------------------------|-------------------------------|------------------------------|------------------------|

| Metabolic markers | Intervention | Baseline | | End | | Difference in means | 95% CI | P value |
|-------------------|--------------|----------|-------|------|------|---------------------|-------------|---------|
| | | Mean | SD | Mean | SD | | | |
| Total cholesterol | Placebo | 5.39 | 1.00 | 5.37 | 1.24 | | | |
| | 7.5 g AX | 5.83 | 1.00 | 5.71 | 1.05 | -0.11 | -0.49, 0.27 | 0.563 |
| | 15 g AX | 5.59 | 0.80 | 5.46 | 0.99 | -0.12 | -0.49, 0.25 | 0.524 |
| LDL-C | Placebo | 3.26 | 0.77 | 3.29 | 0.95 | | | |
| | 7.5 g AX | 3.67 | 0.83 | 3.56 | 0.99 | -0.14 | -0.52, 0.23 | 0.442 |
| | 15 g AX | 3.39 | 0.75 | 3.16 | 0.90 | -0.26 | -0.62, 0.10 | 0.148 |
| HDL-C | Placebo | 1.42 | 0.31 | 1.46 | 0.35 | | | |
| | 7.5 g AX | 1.44 | 0.41 | 1.41 | 0.41 | -0.06 | -0.20, 0.08 | 0.357 |
| | 15 g AX | 1.60 | 0.36 | 1.64 | 0.45 | 0.00 | -0.14, 0.13 | 0.972 |
| Triglycerides | Placebo | 1.57 | 0.58 | 1.37 | 0.51 | | | |
| | 7.5 g AX | 1.79 | 1.32 | 2.06 | 2.11 | 0.41 | -0.08, 0.90 | 0.102 |
| | 15 g AX | 1.32 | 0.64 | 1.47 | 0.82 | 0.42 | -0.06, 0.89 | 0.083 |
| Glucose | Placebo | 5.38 | 0.49 | 5.39 | 0.66 | | | |
| | 7.5 g AX | 5.54 | 0.90 | 5.36 | 0.50 | -0.08 | -0.45, 0.28 | 0.659 |
| | 15 g AX | 5.26 | 0.54 | 5.12 | 0.38 | -0.24 | -0.59, 0.12 | 0.186 |
| Insulin | Placebo | 75.8 | 29.8 | 62.4 | 26.3 | | | |
| | 7.5 g AX | 124.8 | 250.5 | 68.3 | 33.9 | 3.74 | -25.4, 32.9 | 0.797 |
| | 15 g AX | 70.7 | 35.9 | 68.4 | 48.7 | 6.26 | -22.4, 34.9 | 0.661 |
| QUICKI | Placebo | 0.26 | 0.01 | 0.27 | 0.02 | | | |
| | 7.5 g AX | 0.26 | 0.02 | 0.27 | 0.02 | -0.0018 | -0.01, 0.01 | 0.768 |
| | 15 g AX | 0.27 | 0.02 | 0.27 | 0.02 | 0.0003 | -0.01, 0.01 | 0.963 |

Differences between placebo, 7.5 g AX and 15 g AX tested with linear mixed model. The presented difference in means is corrected for baseline differences. AX, arabinoxylans. QUICKI, quantitative insulin sensitivity check index.

differences in gastric emptying and intestinal transit time, parameters we did not measure in these participants. Furthermore, although we aimed to examine a homogenous study population, possible confounders (i.e. lifestyle, sex) could have affected permeability results. Prebiotics, such as inulin, were shown to improve GI permeability in obese subjects after dietary intervention for 9 wks [24]. The discrepancy in results between our and above study might be explained by various factors, such as differences in method of measuring GI permeability, sample size and duration of study product intake. Furthermore, we assessed the effects of AX intake during weight maintenance, while they investigated the effect during a weight loss intervention [24]. Some animal studies have shown beneficial effects of AX on an impaired gut barrier [9,10]. This is the first human study analysing the effects of a (putative) prebiotic on the expression of TJ proteins in sigmoid mucosa in overweight subjects. From a subset of our study population we obtained mucosal samples from sigmoid colon and despite the small sample size, 7.5 g AX showed to significantly upregulate gene transcription of the TJ protein occludin, while 15 g AX significantly upregulated claudin-3 and claudin-4. These proteins are structural transmembrane components, which organize complex TJ protein systems, determining paracellular permeability. These results are in line with the preclinical data in obese mice, where AX significantly increased mRNA concentrations of TJ proteins expression [9]. No significant changes in sigmoid TJ protein concentrations were observed after AX treatment.

Modulating gut microbiota composition by prebiotic compounds, and thereby influencing its effect on intestinal homeostasis, appears to be a promising strategy to treat and prevent chronic diseases. This is the first human study investigating the effect of a high molecular weight AX on the microbial community composition in overweight and obese individuals. *In vitro* and mice studies have shown that long-chain AX can be efficiently fermented, leading to specific stimulation of bifidobacterial species and beneficial fermentation profiles [9,11]. While in previous studies the effect of this particular AX concentrate on the microbiota was assessed by selective qPCR protocols [10–12], in the current trial we made use of a metagenomic approach. Here, we observed a decrease in microbial richness and diversity after 15 g AX supplementation, but no change in overall microbiota composition was seen. Intestinal microbiota convert indigestible food components into metabolites, such as SCFA, that can be processed by the host. SCFA are able to exert antioxidant, anti-inflammatory and immunomodulating effects [25]. Furthermore, the SCFA butyrate functions as the principal energy source for the proliferation and differentiation of colonic epithelial cells and is essential in establishing and maintaining the gut barrier by regulating the expression of TI proteins [25]. We demonstrated that the fecal concentration of the individual and total SCFA were increased upon intake of AX compared to a decrease in the placebo group. Our findings confirm previous data from in vitro and in vivo studies. Two different in vitro models, often used to assess modulation of the gut microbiome (SHIME, TIM-2), demonstrated that long-chain AX specifically increase propionate concentrations [11]. In germ-free rats, inoculated with human feces, long-chain AX increased the concentrations of acetate, propionate, butyrate and total amount of SCFA [12]. In a human study, medium-chain AX significantly increased fecal concentrations of both propionic and butyric acid after 4 wks intake [26]. Other prebiotics, such as inulin and fructooligosaccharides, showed less pronounced effects on SCFA production in humans [27,28]. Concomitantly, we found a significant decrease in fecal pH in the 7.5 g AX group. A more acidic luminal environment may contribute to the reduction of pathogenic bacteria and creates a more favorable environment for the growth of beneficial bacteria.

To assess the effect of AX on immune response we applied an ex vivo model using PHA-stimulated whole blood to evaluate the capacity to produce cytokines. We found a significant decrease in production of the pro-inflammatory cytokine TNFa after supplementation with 15 g AX, suggesting a capacity to reduce the intensity of an acute pro-inflammatory reaction. In obese mice AX showed to have an anti-inflammatory effect [9]. Human data regarding the effect of prebiotics on immune modulation, assessed via the current ex vivo model, are limited. One study in healthy subjects showed that a prebiotic mixture had the capacity to reduce the pro-inflammatory pathway and to stimulate anti-inflammatory pathways [26]. Pro-inflammatory cytokines are associated with the development of systemic IR, β -cell dysfunction and CVD [29]. One important function of SCFA is suppressing the production of such cytokines [25]. In the present human trial we observed an increase in fecal SCFA concentrations after AX treatment, providing a possible explanation for the decrease in TNFa production by PBMC.

Obesity is typically associated with a wide cluster of metabolic alterations, including glucose homeostasis disorders and dyslipidemia. AX showed to improve glycemic control and blood triglycerides concentrations in subjects with an impaired glucose tolerance or DM2 [30]. In the present study, supplementation with AX in healthy overweight and obese subjects did not affect systemic glucose, insulin and lipid concentrations. This can be explained by the fact that the participants had normal values from beginning of the study.

The daily ingestion of AX did not influence bowel habits, confirming previous observations in prebiotics intervention studies [28]. The low prevalence of GI complaints during AX supplementation provides evidence that AX is well-tolerated by humans.

This study followed a unique approach to assess the gut barrier, by combining functional analyses (multi-sugar test) with TJ analyses in mucosal tissue specimens. Some potential shortcomings should be considered. Firstly, we measured fecal SCFA concentrations to assess the microbiota fermentation capacity. SCFA are efficiently absorbed from the gut, with only 5%–10% being excreted via the feces. Fecal SCFA concentrations hence do not accurately reflect the exact intestinal SCFA concentration. However, previous studies showed that the ratio between the respective SCFA, measured in feces and colon, is comparable to each other [31]. Thus,

changes in fecal SCFA concentration reflect relative changes in intestinal concentrations of the separate SCFA. Furthermore, as we choose to focus on the effects of AX on gut barrier function and gut microbiota, we did not obtain information regarding hepatic health of our study subjects. Prebiotics target multiple metabolic impairments associated with obesity-related non-alcohol fatty liver disease, and thus the effects of AX on hepatic health in this study population may be another interesting objective to assess. We did not control the diet and exercise pattern of our participants, as we aimed to evaluate the effects of AX when supplementing the habitual diet, which is in line with future applications. Furthermore, in this study we observed potentially advantageous findings regarding gut barrier and immune function, however these findings are still theoretical and were not translated into clinically positive outcomes. Future research is required to assess whether these findings can improve gut barrier function and immune health.

In conclusion, we have shown that 6 wks AX supplementation resulted in a more beneficial fermentation (SCFA) profile in overweight and obese subjects. In addition, an increased gene transcription of several transmembrane proteins was observed, although no changes in TJ proteins expressions or GI permeability were detected. Furthermore, we showed that 15 g AX might have an anti-inflammatory potential and that the intake of AX was welltolerated.

Statement of authorship

The authors' responsibilities were as follows: BNS, FJT, SP and AAM designed research; BNS, FJT, EW and AAM conducted research; BNS, FJT, PT, MM, RVV, DHP, RJ, TW and AAM analysed data or performed statistical analysis; BNS and EW had responsibility for participant care and selection; BNS and SP wrote the paper; BNS, FJT, MM, TW, SP and AAM had primary responsibility for final content. All authors read and approved the final manuscript.

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Conflict of interest statement

None.

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Appendix A. Supplementary data

Supplementary data related to this article can be found at http://dx.doi.org/10.1016/j.clnu.2017.01.024.

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