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Louise Kjølbæk[1*], Alfonso Benítez-Páez[2*], Eva M. Gómez del Pulgar[2], Lena K. Brahe[1], Gerhard Liebisch[3], Silke Matysik[3], Simone Rampelli[4], Joan Vermeiren[5], Patrizia Brigidi[4], Lesli H. Larsen[1], Arne Astrup[1], Yolanda Sanz[2].

Affiliations:

[1] Department of Nutrition, Exercise and Sports, Faculty of Science, University of Copenhagen, Rolighedsvej 26, 1958 Frederiksberg C, Denmark.

[2] Microbial Ecology, Nutrition & Health Research Unit, Institute of Agrochemistry and Food Technology. Spanish National Research Council (IATA-CSIC), 46980, Paterna, Valencia, Spain.

[3] Institute of Clinical Chemistry and Laboratory Medicine, University of Regensburg, 93053 Regensburg, Germany.

[4] Microbial Ecology of Health Unit, Department of Pharmacy and Biotechnology, University of Bologna.40126 Bologna, Italy

[5] Cargill R&D Centre Europe, Havenstraat 84, B-1800 Vilvoorde, Belgium.

* Both authors equally contributed to this paper and they are the authors to whom address any correspondence:

Louise Kjølbæk, Rolighedsvej 26, 1958 Frederiksberg C, Denmark. Phone: (+45) 35331462. E-mail: louisekjoelbaek@nexs.ku.dk.

Alfonso Benítez-Páez, C/. Catedràtic Agustín Escardino Benlloch 7, 46980 Paterna, Valencia, Spain. Phone: (+34) 963900022 ext 2131. E-mail: abenitez@iata.csic.es.

Running title: AXOS and PUFAs effect on gut microbiota and metabolism

Abbreviations:

ALAT, alanine aminotransferase; ApoB, apolipoprotein B; ASAT, aspartate aminotransferase; AX, arabinoxylan; AXOS, arabinoxylan oligosaccharides; BMI, body mass index; BP, blood pressure; BW, body weight; CHO, cholesterol; CID, clinical investigation day; CPM, counts per minutes; DHA, docosahexaenoic acid; DNA, deoxyribonucleic acid; EPA, eicosapentaenoicacid; E%, energy percentage; HDL, high density lipoprotein; HOMA-β, homeostatic model assessment - beta-cell function; HOMA-IR, homeostatic model assessment -insulin resistance; hsCRP, high sensitive C-reactive protein; LDA, Linear Discriminant Analysis; LDL, low density lipoprotein; LMM, Linear Mixed Model; OTU, Operational Taxonomic Units; PC, principal coordinate; PCoA, Principal Coordinate Analysis; PCR, Polymerase chain reaction; PUFA, polyunsaturated fatty acids; REE, resting energy expenditure; rRNA, ribosomal ribonucleic acid; SFA, saturated fatty acid; TG, triglycerides; VAS, visual analogue scale; VLDL, very low density lipoprotein; WBE, wheat bran extract

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Ethical committee: H-4-2014-052

The Danish Data Protection Agency: 2013-54-0522

Abstract

Background & Aims: Gut microbiota composition is linked to obesity and metabolic syndrome. The nutrients and doses required to modulate the gut microbiota towards beneficially influence components of the metabolic syndrome are unclear. This study aimed to investigate diet-induced effects on the gut microbiota and metabolic markers in overweight individuals with indices of the metabolic syndrome.

Methods: A twelve-week randomized cross-over trial was conducted with two intervention periods separated by a washout period. The dietary intakes of interest were wheat bran extract, rich in arabinoxylan oligosaccharides (AXOS) (10.4 g/d AXOS) and polyunsaturated fatty acids (PUFA) (3.6 g/d n-3 PUFA). Dietary records, fecal and blood samples, as well as anthropometric data, were collected before and after intervention. Anthropometry and gastrointestinal symptoms were evaluated weekly. Gut microbiota composition was analyzed by massive sequencing of 16S ribosomal RNA gene V3-V4 amplicons.

Results: Twenty-seven participants completed the study (90%). Intake of AXOS induced an expected bifidogenic effect on gut microbiota ($p < 0.01$) and increased butyrate-producing bacterial species as well ($p < 0.05$). Beta-diversity analysis indicated that the structure of the gut microbiota only changed as a result of the AXOS intervention (Permanova = 1.90, $p < 0.02$) and no changes in metabolic markers were observed after any of the interventions. **Conclusions:** AXOS intake has bifidogenic effects and also increases butyrate producers in the gut microbiota; even though this type of dietary fiber did not modulate lipid or glucose metabolic parameters related to metabolic syndrome. Four-week PUFA intake did not induce any notable effect on the gut microbiota composition or metabolic risk markers.

Registration: Registered under ClinicalTrials.gov Identifier no.NCT02215343

Keywords: gut microbiota, arabinoxylan oligosaccharide, fiber, fish oil, metabolic syndrome, obesity.

Introduction

Obesity is a global health problem [1] and presents a major health risk, as it can lead to a wide range of diseases including type II diabetes and cardiovascular diseases. The increase in health risk is often attributed to the metabolic syndrome that is a cluster of metabolic risk markers including abdominal obesity, impaired glucose metabolism, dyslipidemia and hypertension [2]. Worldwide, it has been estimated that approximately one-fourth of the adult human population has the metabolic syndrome [3] and that 3.4 million deaths were caused by overweight and obesity in 2010 [4]. Thus, effective strategies to reduce obesity and obesity-related morbidity and mortality are needed in order to be implemented by public health systems.

Gut microbiota is associated with obesity [5–7] as well as type II diabetes [8,9] and cardiovascular disease [10]. Thus a change in the gut microbiota composition may have the potential to confer improvements in host health and to reduce the risk for obesity-associated chronic metabolic diseases. Fecal microbiota transplantation has been suggested to change microbiota composition with concomitant improvements in metabolic markers [11]. Another more feasible method to modulate the gut microbiota is the diet [12,13]. In recent years, a vast amount of studies clearly indicate that diet is one of the main environmental factors modulating the gut microbiota. In particular, dietary fiber exerts a deep impact on gut microbiota structure and function, increasing the abundance of bacteria specialized in the utilization of complex carbohydrates as energy source. Dietary fiber such as wheat bran extract (WBE) is enriched in arabinoxylan oligosaccharides (AXOS), which are conceived as prebiotics given their ability to stimulate the growth of bifidobacteria [14]. The AXOS breakdown to arabinose and xylose monomers occurs through the activity of microbes such as *Bifidobacterium* species [14]. Indeed, previous dietary interventions with AXOS have shown to increase the abundance of the *Bifidobacterium* species in the human gut microbiota [15–

18], which theoretically could mediate beneficial health effects. In addition, the production of short-chain fatty acids (SCFAs) via fermentation of dietary fiber seems important for improving metabolic health. Of SCFAs, butyrate [19], and more recently, propionate production [20] have been suggested to beneficially influence metabolic health. When AXOS is fermented by bifidobacteria acetate is produced as immediate metabolic product but this can be further metabolized to butyrate by other intestinal bacteria (butyrate producing bacteria) via cross-feeding mechanism [21]. Acute intake studies carried out with AX and AXOS have shown that overnight AXOS intake may improve glucose metabolism and AX intake reduces the postprandial glucose peak [22,23].

On the other hand, it is known that intake of polyunsaturated fatty acids (PUFA) especially long-chain n-3 PUFAs (DHA and EPA) are beneficial for human health [24], as reflected in dietary recommendations [25]. Given that digestion and absorption of dietary fat takes place in the small intestine, it has been thought that colonic bacteria may play a minor role in the digestion and absorption of such macronutrient. However, studies in animal models suggest that gut microbiota could influence the absorption of dietary lipids and, thereby, their health-related effects [26,27] and interestingly, a limited number of human studies have indicated that the specific fat subtype could affect microbiota composition [28,29]. However, the precise underlying mechanisms are less well defined.

We hypothesize that changes in diet can modulate the gut microbiota and, thereby, contribute to improving lipid or glucose metabolic dysfunctions in overweight and obese individuals. Consequently, we aimed to test how two different dietary interventions, WBE with a high AXOS content, a recognized prebiotic fiber with notable clinical [22, 23] and technical properties (e.g. high solubility, pH stability, taste, and colour) making it a suitable ingrediet to be used in manufacturing of innovative healthy food products, and long-chain n-3 PUFA enriched fish oil, can modulate the gut microbiota and metabolic risk markers in overweight

individuals with metabolic syndrome. Additionally, the cross-over design combining two different dietary strategies will help to determine the best performance between the macronutrients tested on the same population in terms of the speed and strength of the response.

Materials and Methods

Study design

The study had a cross-over design with two diet periods of 4 weeks each separated by a 4-week washout period (Figure 1). The completing participants were randomized to the sequence of the two diets (ratio 1:1) and all visits were planned to be conducted within a window of ± 3 days. The computer-based randomization list was generated at *randomization.com*. Due to the study design blinding of project staff and participants was not possible. Data were collected on 4 clinical investigation days (CID) during the study: baseline (week 0), after first dietary intervention period (week 4), after washout (before second diet period) (week 8), and after the second dietary intervention period (week 12). Furthermore, the participants had two dietician consultations (week 2 and week 10) to ensure body weight (BW) maintenance. The dietician called the participants in the remaining weeks (week 1, 3, 9 and 11) (Figure 1). Prior to each CID, the participants consumed a standardized dinner in the evening followed by a fasting period of minimum 8 hours. The study was conducted at the Department of Nutrition, Exercise and Sports, University of Copenhagen from August 2014 to June 2015. The study is registered at Clinical Trial (NCT02215343), conducted according to the guidelines laid down in the Declaration of Helsinki and was carried out in accordance with the ethical standards of the responsible regional committee on human experimentation in Denmark, registered as H-4-2014-052, and the Danish Data Protection Agency (2013-54-0522).

Study participants

The participants were recruited through the web-pages (*http://forsøgsperson.dk* and *http://nexs.ku.dk*), social media and newspapers. Informed consent was obtained after the participant had obtained written and spoken information. Participants received either 4,000 d.kr (~$600) or five meetings with a dietician as compensation for their participation. Eligible men and women were 18-60 years and a body mass index (BMI) of 25-40 kg/m^2 at screening. Furthermore, participation required a waist circumference ≥94 cm for men and ≥80 cm for women plus at least one of the following criteria for metabolic syndrome [3]; raised triglycerides (TG) (≥1.7 mmol/L), reduced high density lipoprotein (HDL) cholesterol (CHO) (men: <1.03 mmol/L, women: <1.29 mmol/L), raised fasting plasma glucose (≥5.6 mmol/L) or raised blood pressure (BP) (systolic BP ≥130 mmHg or diastolic BP ≥85 mmHg). At screening, blood measurements were evaluated from a finger prick test (Lipid Pro^{TM}, infopia Co., Ltd). Additionally, a hemoglobin concentration ≥7 mmol/L was a requirement for inclusion. Women were required to be non-pregnant, non-lactating and not planning pregnancy during the study. Exclusion criteria were: use of antibiotics three months prior to and during the study, medication related to dyslipidemia, type II diabetes or elevated BP. Furthermore, individuals were not allowed to take dietary supplements with pro- and/or prebiotics, fiber or fish oil six weeks before the study start. Vegetarian and vegan individuals or with food allergies (e.g. wheat, milk etc.) were also excluded. Other exclusion criteria were smoking and BW change of ± 3 kg two months prior to study start. Elite athletes or those with intensive physical training (>10 hours of strenuous physical activity per week) as well as those donating blood one month before study start were excluded as well. Additionally, individuals with gastrointestinal and liver diseases, chronic inflammatory disorders (excluding obesity), psychiatric disorders including treatment required depression, surgical treatment of obesity as well as abdominal surgery were excluded. Individuals unable to comply with the procedures required by the study protocol were excluded.

Intervention

Each AXOS intervention aimed reaching a fiber intake of ~30 g/d. AXOS was delivered partly as a powder supplement to dissolve in water twice a day and partly as 4 biscuits/crackers per day, nutritional information is provided in Table 1. By providing 15 g WBE per day, 11.2 g of total fiber was administrated to the participants' of which 10.4 g corresponded to AXOS (Table 1). The remaining fiber intake was obtained from the participants' habitual diet and supervised by a dietician. The goal of the PUFA period was to reach a daily PUFA intake of approximately 10 E% by increasing the intake of PUFA including n-3 fatty acids and lowering saturated fatty acid (SFA) intake. The participants' diet was supplemented with fish oil capsules (~228 kJ/d) containing 3.6 g/d n-3 PUFA (1.32 g/d DHA and 1.86 g/d EPA). Furthermore, the dietician provided individual dietary advices based on the habitual intake of the participants. During the two diet periods the participants were instructed to maintain their BW. Thus, the dietician guided weekly the participants to iso-calorically substitute food items from their habitual diet with the dietary supplements and to avoid products containing pro- and prebiotics.

Outcomes

The study primary outcome was to detect changes in the gut microbiota composition. Secondary outcomes to obtain were changes in the metabolic and biochemical parameters listed in Table 3. Anthropometry, energy expenditure, and gastrointestinal function were included as exploratory outcomes as well.

Anthropometry: Participants voided their bladder before anthropometric measurements. BW was measured with the participant in their underwear by a digital scale (Lindells, Malmo, Sweden) approximated to the nearest 0.1 kg. Height was measured twice at screening to the nearest 0.5 cm using a wall mounted stadiometer (Hultafors, Sweden) and the average of the two measurements was recorded. BMI was calculated as: BW / height^2. Waist and hip circumferences were measured twice with a non-elastic tape measure on the skin with a

precision of 0.5 cm, from which an average was calculated. Waist circumference was measured halfway between the lowest rib and iliac crest and the measurement was taken when the participant exhaled. Hip circumference was measured as the largest circumference in the area around the buttock. Sagittal diameter was measured with the participant in a lying position with an abdominal caliper (Holtain-Kahn) with a precision of 0.1 cm when the participant exhaled. Fat mass and lean body mass were determined in underwear by a dual-energy x-ray absorptiometry (DXA) scan (GE Lunar Prodigy).

Blood pressure: After 25 minutes resting in lying position, BP was measured with an automatically inflated cuff (A&D Instruments LTD, Saitama, Japan). BP was measured on the left arm three times. If the last two measurements differed by >5 mmHg, an additional measurement was performed. The average was calculated from the last two measurements.

Microbiota analysis: Fecal collection took place prior to all CIDs. The participant collected a morning fecal sample and it was kept cold and delivered to the Department within 3 hours. The fecal sample was weighted, aliquoted into the EasySampler® kit for stool collection (GP Medical Devices, Denmark), and stored at -80˚C. The fecal DNA was extracted using the QIAamp Fast DNA Stool Mini Kit (Qiagen, Hilden, Germany) according to the manufacturer's instructions with a prior step of bead beating in 2 mL micro centrifuge tubes containing 0.1 mm diameter glass beads, ~200 mg faeces, and 1 mL InhibitEX buffer. Bead beating was carried out in a Mini-Bead Beater apparatus (BioSpec Products, Bartlesville, USA) with two cycles of shaking during 1 min and incubation on ice between cycles. The fecal DNA was measured by UV methods (Nanodrop, Thermo Scientific, Wilmington, USA) and an aliquot of every sample was prepared at 20 ng/µL with nuclease-free water for polymerase chain reaction (PCR). The V3-V4 hypervariable regions of the 16S ribosomal ribonucleic acid (rRNA) gene were amplified using 20 ng DNA (1 µL diluted aliquot) and 25 PCR cycles consisting of the following steps: 95ºC for 20 sec., 55ºC for 20 sec., and 72ºC for

20 sec. Phusion High-Fidelity Taq Polymerase (Thermo Scientific, Wilmington, USA) and the 6-mer barcoded primers, S-D-Bact-0341-b-S-17 (CCTACGGGNGGCWGCAG) and S-D-Bact-0785-a-A-21 (GACTACHVGGGTATCTAATCC) which target a wide range of bacterial 16S rRNA genes [30], were used during PCR. Dual barcoded PCR products, consisting of ~500bp, were purified from triplicate reactions with the Illustra GFX PCR DNA and Gel Band Purification Kit (GE Healthcare, Little Chalfont, UK) and quantified through Qubit 3.0 and the Qubit dsDNA HS Assay Kit (Thermo Fisher Scientific, Waltham, MA, USA). Samples were multiplexed by combining equimolar quantities of amplicon DNA (100 ng per sample) and sequenced in an Illumina MiSeq platform with 2x300 PE configuration (Eurofins Genomics GmbH, Ebersberg, Germany). Raw data were delivered in fastq files and pair ends with quality filtering were assembled using *Flash* software [31]. Sample de-multiplexing was carried out using sequence information from the respective DNA barcodes and *Mothur v1.36.1* suite of analysis [32]. After assembly and barcodes/primers removal, the sequences were processed for chimera removal using *Uchime* algorithm [33] and SILVA reference set of 16S sequences [34]. Alpha diversity was calculated with *Mothur v1.36.1* using default parameters and average method in the clustering step. Consequently, the Chao's richness, Shannon's evenness and Simpson's reciprocal index were computed using a high quality and a normalized subset of 17,750 sequences per sample, randomly selected after shuffling (10,000X) of the original dataset. Taxonomic assessment was performed using the Ribosomal Database Project (RDP) classifier v2.12 [35]. The Operational Taxonomic Unit (OTU)-picking approach was performed with the normalized subset of 17,750 sequences and the *uclust* algorithm implemented in USEARCH v8.0.1623 [36]. Beta-diversity was evaluated using Principal Coordinate Analysis (PCoA) and Bray-Curtis dissimilarity index.

qPCR: absolute quantification of DNA molecules belonging to species of the *Bifidobacterium* genus was evaluated using the primers bifido84f CGGGTGAGTAATGCGTGACC (94%

genus specificity) and bifido194r CGACCCCATCCCATGCCG (98% genus specificity) designed with PrimerProspector [37] and the set of reference sequences of the bacterial 16S rRNA gene from SILVA database (release 110) [34]. The single-stranded DNA (ssDNA), fully covering the region to be amplified (128 nt) was obtained from Isogen Life Science B.V (Utrecht, The Netherlands) where it was synthesized, PAGE-purified, quantified, and used for molecule titration during qPCR. The qPCR reactions were set in 96-well plates using the SYBR Green I Master Mix (Roche Lifesciences), 0.5 μM of forward oligonucleotide, 0.25 μM of reverse oligonucleotide, and 1 μL of the 1:5 diluted in nuclease-free water fecal DNA obtained for amplicon sequencing (final concentration in the qPCR reaction between 3 and 13 ng DNA). All samples were set in duplicate in the plate and amplified at once with standards in a LightCycler 480 II instrument (Roche Lifesciences) with the following cycling profile: initial incubation at 95° for 5 min and 40 cycles of 10 s at 95°, 20 s at 65°, and 15 s at 72°. Finally, the melting curve was set from 65 to 97° with a ramp rate of 0.11°/s. The absolute quantification was assessed with Ct values obtained for every sample and from titration curve (with duplicate measures) using the LightCycler® 480 Software v1.5 (Roche Lifesciences). The number of 16S rRNA gene molecules was normalized against the total DNA concentration (ng/μL) present in the diluted DNA sample measured through high sensitive fluorometric methods such as Qubit 3.0 and the Qubit dsDNA HS Assay Kit (Thermo Fisher Scientific, Waltham, MA, USA). Differential abundance of *Bifidobacterium* species was assessed by the Wilcoxon Rank Sum test for paired samples before and after AXOS intake.

Blood biochemistry: Venous blood samples were drawn at the CIDs after an overnight fast. Blood samples for analyses of insulin, ASAT and ALAT, hsCRP and lipid profile (total CHO, VLDL-CHO, LDL-CHO, HDL-CHO, TG, ApoB) were collected in serum tubes and kept at room temperature for 20 minutes to coagulate. Plasma samples for glucose (in fluoride tube) were put directly on ice and immediately centrifuged. All samples were centrifuged at 2500 x

g for 10 min at 4ºC and stored at -80ºC until processing. Samples for whole blood analyses of hemoglobin and white blood cell were collected in ethylenediaminetetraacetic acid (EDTA) tubes and concentrations were immediately measured (SysmexKX-21, Sysmex Corporation, Kobe, Japan). Insulin was measured by chemiluminescentimmunometric assay (IMMULITE 2000 INSULIN, Siemens Healthcare Diagnostics Inc.) on the IMMULITE2000 INSULIN Analyzer (Siemens Healthcare Diagnostics Products Ltd., UK). Samples with an insulin concentration below the detection limit (14.4 pmol/L) were set to 7.2 pmol/L. Glucose was measured by enzymatic hexokinase method on the Pentra 400 Analyzer (HORIBA ABX, Montpellier, France). The homeostatic model assessment was used to quantify insulin resistance (HOMA-IR) and beta-cell function (HOMA-β) from measurements of fasting insulin and glucose concentrations. HOMA-IR was calculated as: *(insulin (µU/mL)×glucose (mmol/L))/22.5* and HOMA-β as: *(20×insulin (µU/mL))/(glucose (mmol/L)- 3.5*) [38]. HsCRP was measured by immunoturbidimetric method on the Pentra 400 Analyzer (HORIBA ABX, Montpellier, France). ASAT and ALAT were measured on the Pentra 400 Analyzer (HORIBA ABX, Montpellier, France). Lipid profile was analyzed on an auto-analyzer platform DIMENSION VISTA® (Siemens Healthcare Diagnostics Inc., USA). Total CHO and TG were measured by enzymatic methods. LDL-CHO and HDL-CHO were analyzed by the same method but with a disintegration of the other lipoproteins prior to the enzymatic reactions as included in the test scheme. ApoB concentration was measured by nephelometry. Specific antibodies form immunocomplexes with the ApoB proteins, which result in scattering light. Concentration of very low density lipoprotein (VLDL)-CHO was calculated from the values above. All lipids were measured in mg/dL but converted to mmol/L by multiplying with 0.0259 for total-CHO, LDL-CHO, HDL-CHO and VLDL-CHO and multiplying with 0.0113 for TG. ApoB concentration was multiplied with 0.01 for obtaining concentration in g/L.

Breath hydrogen: Fasting breath hydrogen was measured by a hand-held non-invasive Gastro^{+}Gastrolyzer (Bedfont Scientific Ltd, Kent, England).

Energy Expenditure: At all CIDs resting energy expenditure (REE) was measured twice after a minimum of 30 minutes of resting by a ventilated hood system (Jaeger Oxycon PRO, ViasysHealtcare GmbH, Hoechberg, Germany). Each measurement lasted 25 minutes and was separated by a 10-minute rest period. The standardized dinner from the study kitchen at the Department provided prior to each CID contained 3 or 4 MJ, depending on the estimated energy requirements of the participant, and had a macronutrient distribution of 16 E% protein, 31 E% fat and 53 E% carbohydrate. Participants were not allowed to consume alcohol and asked to limit physical activity 48 hours prior to the REE measurement.

Dietary records and physical activity: Prior to the four CIDs (week 0, 4, 8, 12) the participants reported all ingested foods in a 3-day dietary record including information on brand names, cooking and processing. Whenever possible, foods were weighed otherwise household measures were applied. Content of energy, macro and micro nutrients were calculated as an average from the 3-day dietary records. The dietary records were assessed using a computer database of foods from the National Food Agency of Denmark (Dankost Pro, National Food Agency of Denmark, Søborg, Denmark). During the same 3 consecutive days (3 entire 24-hour periods) physical activity was measured using a waist-worn accelerometer (ActiGraph GT3X+, Pensacola, FL, USA). Participants were only allowed to take the accelerometer off during showering and swimming and these non-wear activities were recorded in a diary including sleeping and wake-up time. The participants were instructed to maintain their normal physical activity habits during the study. Data were reintegrated into 60 sec. epochs and analyzed using Actilife v6 software. Before analysis self-reported sleeping and non-wear times were removed and the remaining time was scored in ActiLife6 to evaluate physical activity. Only data for participants with at least two days of measurements, defined as a

minimum of 600 min wear time per day, were considered valid for analysis. Total tri-axial physical activity (counts per minutes (CPM)) was expressed as a vector magnitude of the total tri-axial counts from monitor wear-time, divided by measured monitor wear-time. Sedentary time, light physical activity, and Moderate-to-Vigorous physical activity (MVPA) were defined as ≤99 vertical CPM, 100–2019 vertical CPM, and ≥2020 vertical CPM, respectively [39].

Compliance: The compliance evaluation was based on the number of days during the diet period where the participants did not consume 100% of the provided supplement. Compliance was evaluated as very good (≤4 days), good (>4 and ≤8 days), bad (>8 and ≤12 days) or very bad (>12 days) during the diet period. The compliance degree was reduced one level if information about intake of supplement was missing.

Adverse events: During the two diet periods the participants filled out a weekly 100 mm visual analogue scale (VAS). The VAS was anchored with "no symptoms" (0 mm) and "extreme symptoms" (100 mm) and the following symptoms were evaluated; stomach pain, abdominal distension, flatulence, constipation, diarrhea, nausea, oily faces, wind break and frequent rectal tenesmus. During the 3-day diet registration, the participants recorded all time points of defecation and evaluated stool consistency using the Bristol stool scale. Average defecation frequency was calculated as the number of stools divided by the 3 days of recording. Average stool consistency was calculated as the sum of Bristol stool scale divided by the total number of registered stools. The Bristol composite measure i.e. a parameter of defecation frequency and stool consistency was calculated as the sum of Bristol stool scale divided by the 3 days of recording. Concomitant medication and adverse events were registered at all CIDs and all mid-visits. An adverse event was evaluated by intensity (mild, moderate or severe) and the project staff evaluated whether the adverse event was related to the intervention (plausible, likely, perhaps, unlikely, impossible).

Statistical analyses

The number of participants was calculated before study start according to previous studies with AXOS interventions [16,17]. Sample size calculation was based on the expected primary outcome "increase in amount of bifidobacteria" during the AXOS intervention period, however based on another analysis method than used in the current study. By including 30 participants (24 completers), this study would have a statistical power of 80% to detect a difference of 0.35 log10 cell/g dry weight feces (SD of 0.6), allowing for a 20% dropout at a 0.05 significance level.

The level of significance was set to $p \leq 0.05$. Statistical analyses on metabolic, physical activity, gastrointestinal symptoms, anthropometry, taxonomy categories, and dietary outcomes were performed using SPSS v24. The effects of the dietary intervention on all outcomes were analyzed using a linear mixed model (LMM) with repeated measures. The model included a treatment (AXOS vs. PUFA) × time (before vs. after intervention) interaction and adjustment for age, gender, recruiting BMI, and order of treatments. Data not normally distributed were log-transformed before analysis by LMM. Data are presented as means ± SD unless stated otherwise. To investigate the effect of the treatment we compared the before versus after points, within and between treatments (AXOS and PUFA). Additionally to LMM methods, statistical analyses on microbiome outcomes were also performed in R v3.2.3 (http://cran.r.project.org). Non-parametric Wilcoxon Rank-Sum test for the paired samples, the Wilcoxon Signed-Rank test for the unpaired samples, and Linear Discriminant Analysis (LDA) [40] were performed to measure differences among fecal microbial communities at different taxonomic levels as a result of the different interventions with AXOS or PUFAs. Structural changes in the gut microbial community associated with diet were assessed by beta diversity analysis based on Bray-Curtis dissimilarity index and permutation based test (Permanova) using *qiime* v1.9.1 suite of analysis [41]. Pairwise

Spearman's rank correlation coefficient between principal coordinate (PC) and OTU abundance were conducted to investigate particular changes in OTU abundances during the diet period. Similarly, pairwise Spearman's rank correlations between OTU abundance (OTUs with a LDA score > 3) and biochemical parameters were determined. The *post hoc* False Discovery Rate (FDR) was used to adjust for multiple comparisons in the correlation tests.

Results

Dietary assessment and compliance

Three of out the thirty recruited participants did not complete the study; one dropped out for personal reasons during the study, thus getting the effective number of 28 and 27 participants for AXOS and PUFA interventions, respectively (Supplementary Figure 1). Five participants could not manage the visit window of ±3 days, thus four had a longer washout period and one had a longer second diet period. The characteristics of the 29 participants who completed the baseline visit are shown in Supplementary Table 1. A comparison of participants in the two interventions (AXOS-I and PUFA-I) during the first diet period showed no baseline differences (Supplementary Table 2).

At baseline, the participants had a fiber intake of 24.5 ±12.0 g/d and this was increased to 31.2 ± 7.94 g/d during the AXOS intervention (Table 2). The self-reported compliance showed that of the 28 participants, who completed the AXOS intervention, 21 had a very good compliance, 6 had a good compliance and 1 participant (randomized to PUFA during the first period and AXOS during the second period) had a very bad compliance. The baseline intakes of total fat, SFA, monounsaturated fatty acid (MUFA) and PUFA are shown in Table 2 and Supplementary Table 3 shows before and after intakes for each intervention. During the PUFA intervention, PUFA intake increased from 6.19 E% to 7.77 E%. The self-reported

compliance showed that of the 27 participants, 25 had very good compliance and 3 had a good compliance.

Anthropometry and physiology evaluation

The results from LMM analysis on anthropometric measurements, blood pressure, blood biochemistry and metabolism are found in Table 3. Neither AXOS nor PUFA intakes had any effect on these outcomes, even when outcomes were analyzed separately in the first or second periods of respective interventions (Supplementary Table 4). However, flatulence was significantly associated with AXOS intake (Table 3). Adverse events were registered throughout the study. None of the adverse events were characterized as serious and they were evaluated as unlikely or impossibly related to the interventions. The majority of adverse events were seasonal diseases such as sore throat (5 events), common cold (13 events), influenza (4 events) and fever (1 event) in addition to headache (10 events) and gastrointestinal symptoms (28 events). Flatulence was reported more frequently during AXOS intake, compared to PUFA intake, and vice versa for reflux. Otherwise none of the adverse events occurred more frequently during a specific diet period.

Dietary intervention impact on gut microbiota

The diet-induced microbial community changes were analyzed by comparing the different diet periods of the respective interventions individually to discern a possible carry-over effect (Figure 1). An initial assessment indicated that AXOS and PUFA intake did not lead to significant changes in any alpha-diversity parameter analyzed (Chao's richness, Simpson's reciprocal index, Shannon evenness) (Supplementary Figure 2). Moreover, beta-diversity analyses were also conducted to evaluate significant shifts in the microbial communities as a result of the AXOS consumption. Using the Bray-Curtis dissimilarity index as descriptor in a PCoA, we depicted a uniform pattern of variation in all subjects after AXOS intake (first

intervention period, AXOS-I) (Figure 2). Graphically, the microbial composition shifts towards the lower left corner of the PCoA plot. This was further supported by a permutation based analysis, which indicated that from all categorical variables analyzed (i.e. gender) only AXOS intake explained the changes in the microbial community structure (Permanova = 1.90, $p < 0.0111$). In order to disclose additional OTUs driving the shift in the microbial structure in response to AXOS, we performed linear correlations among OTU abundances and PC values. Several phylotypes were enriched or reduced in response to AXOS intake (Supplementary Table 5).

We further performed the comparisons at several taxonomy levels including phylum and family distribution and OTUs to identify the possible bacterial species modified by the respective diets. At phylum level, we found that AXOS intake only increased the proportion of Actinobacteria in the combined data from both diet periods of the AXOS intervention (LDA = 4.13, $p < 0.0012$). Such effect was basically due to the response in the AXOS-I group (LDA = 4.62, $p < 0.0015$) (AXOS intake during first diet period) given than no effect was observed in the AXOS-II group (Figure 3A). Similar results were obtained following the LMM analysis (results not shown). We did not detect differences in microbiota composition at baseline between the AXOS-I and PUFA-I participants and a comparative analysis of the microbiota after the washout period (i.e. before the second diet period) between the AXOS-I and PUFA-I subjects did not reveal differences either. The results of further analysis to determine the effects of AXOS on lower taxonomic bacterial categories are reported only for the AXOS-I participants since for the AXOS-II participants no differences were detected. At family level, AXOS increased abundance of the Bifidobacteriaceae (LDA = 4.41, $p <0.0014$) and Coriobacteriacea (LDA = 4.22, $p < 0.0041$) families of the Actinobacteria phylum, whereas the abundances of Rikenellaceae (LDA = 4.37, $p < 0.0238$) and Porphyromonadaceae (LDA = 3.91, $p < 0.0450$) belonging to the phylum Bacteroidetes were

reduced (Figure 3B). Abundance analysis of OTUs showed that 11 phylotypes increased following AXOS intake (Table 4). As expected, three OTUs were assigned to the genus *Bifidobacterium* and the remaining OTUs were identified as potential members of bacteria groups that include butyrate producers such as *Eubacterium rectale, Eubacterium hallii, Faecalibacterium prautsnitzii, Dorea longicatena, Blautia luti* and *Blautia wexlerae* (all from the phylum Firmicutes and order Clostridales). A NMR-based metabolomics analysis performed only in the AXOS-I plasma and fecal samples positively correlated the concentration of SCFAs to known butyrate producer bacterial species as described elsewhere [42]. We also detected decreased abundance of three OTUs that could not be properly identified at genus or species level, but appear to be phylotypes associated with the Ruminococcaceae and Erysipelotrichaceae families of the phylum Firmicutes (Table 4). We confirmed the bifidogenic effect observed in the AXOS-I subjects by absolute qPCR quantification (Supplementary Figure 3).

The PUFA intervention did not result in detectable microbiota changes at phylum or family levels, in abundances of OTUs or in alpha-diversity parameters (Supplementary Figure 2), neither using pooled samples from both diet periods or separately. Multidimensional analysis showed a heterogeneous response to the PUFA intervention among the subjects that drastically differed from the more homogeneous response that was observed following the AXOS intervention (at least in the first intervention period) (Figure 2). Beta diversity analysis (using samples of single or both intervention periods) based on Bray-Curtis dissimilarity index showed no shifts in the microbial community structure when paired samples were compared before and after the PUFA intervention (Permanova = 0.56, $p<0.9601$). A longitudinal evaluation of the microbiota at the genus level across the 4 time-point assessments carried out in every subject included in this study revealed that PUFA-I response seemed to be much lighter than the AXOS-I response (Figure 4). As expected, the delta

values Δ_1 (explaining the changes during the AXOS-I period) and Δ_2 (explaining the changes during washout period) in the group of subjects that started with the AXOS intervention retrieved similar results as those using LDA methods (Figure 3) and non-parametric correlation using multidimensional data (Supplementary Table 5). This analysis of delta values also expanded the potential set of microbial groups mostly affected by AXOS intake (Figure 4B). Thus, fast positive response (increased) to AXOS was observed in *Bifidobacterium* ($p < 0.0001$) and *Blautia* ($p < 0.0029$), and fast negative response (decreased) was observed in *Oscillibacter* ($p < 0.0199$), *Alistipes* ($p < 0.0068$), *Bacteroides* ($p< 0.0020$), and *Parabacteroides* ($p < 0.0060$) species. The proportion of these species also showed a rapid return to their baseline values at the end of washout period, further supporting that their shifts were the result of the dietary intervention. Conversely, we observed no significant changes when Δ_1 and Δ_2 values where compared in the group of subject starting with the PUFA intervention. However, we did observed some differences ($p < 0.05$) when Δ_{1+2} were compared to Δ_3, which explain the changes induced by the intervention with AXOS (AXOS-II period) (Figure 4C). The results of this longitudinal analysis suggest that PUFA-I response could be slow and persist during the washout period and it was only slightly changed by exposure to the second intervention with AXOS.

Correlation between gut microbiota features and biochemical parameters

Given that AXOS modified the gut microbiota by increasing the abundance of potential beneficial bacterial species, correlations between the OTU abundances and physiological and biochemical data were analyzed for the AXOS intervention during the first diet period (AXOS-I). More than 170 correlations between OTU abundance and blood biochemistry parameters, based on Spearman's *rho* parameter and FDR ≤ 0.1, were found. Notably, there were a large proportion of positive correlations (~60%) between OTUs abundance and concentration of insulin, TG, LDL-CHO and VLDL-CHO, ApoB, and total CHO. Focusing

on those OTUs that repeatedly showed correlations with markers related to similar functions, we could identify three OTUs that exhibited the largest number of positive correlations with biomarkers of lipid metabolism (VLDL, ApoB, total CHO, TG), liver function (ALAT), and glucose metabolism (insulin, HOMA-IR, HOMA-β) (Supplementary Table 6). Those OTUs were certainly identified as *Paraprevotella clara* (OTU93, 98% identity), *Eubacterium contortum* (OTU435, 100% identity), and a Lachnoclostridium member of the Lachnospiraceae family (OTU278, >95% identity). Other species showing positive correlations specifically with plasma lipid concentrations (except for HDL-CHO) included *Prevotellamassilia timonensis* (OTU138, 100% identity) and *Mitsuokella jalaludinii* (OTU263, 99% identity). Strikingly, the OTU116, whose identity could be not well solved by Blast or SINA-based comparisons, showed the largest amount of negative correlations with concentrations of lipid metabolic biomarkers such as ApoB (*rho* -0.59; $p < 0.005$), total CHO (-0.60; $p < 0.005$) and LDL-CHO (-0.62; $p < 0.005$).

Moreover, we found a large set of tentative microbial species (OTUs) positively associated with glucose metabolic markers such as fasting insulin, HOMA-IR and HOMA-β values, although we obtained reliable identifications only in few cases including *Intestinimonas butyriciproducens* (OTU172, 100% identity), *Desulfovibrio piger* (OTU97, 99% identity) and *Coprobacter fastidiosus* (OTU99, 99% identity).

We also found negative correlations between the abundances of OTUs and concentrations of glucose or insulin (essentially lower HOMA values) for *Dialister succinatiphilus* (OTU102, 100% identity), *Turcibacter sanguinis* (OTU249, 99% identity) and *Alloprevotella* spp. (OTU281, >95% identity). Additionally, we detected positive correlations between HDL-CHO concentration and the abundances of *Eubacterium coprostanoligenes* group (OTU151, > 95% identity) and a Clostridium from Family XIII *Ihubacter* spp. (OTU926, > 95% identity).

Discussion

The present study reports that AXOS intake exerts a bifidogenic effect confirming previous results obtained in human controlled interventions [15–18]. Moreover, we found that AXOS intake also increased the abundance of butyrate producing bacteria by the use of massive 16S sequencing methods, that enable the evaluation of the gut microbiota composition as a whole instead of quantifying a restricted number of taxonomy groups targeted by specific primers or probes (e.g. qPCR and hybridization approaches) [15–18]. For the first time, we have reported that AXOS intake reduces significantly the proportion of both Rikenellaceae and of Porphyromonadaceae species, which has been associated with inflammatory processes in patients with cirrhosis [43]. In particular, the OTUs analysis showed an increased abundance of the species *B. adolescentis* and *B. longum*, which have been shown to be able to hydrolyze AXOS in an *in vitro* study [44]. Additionally, members of the genera *Faecalibacterium, Ruminococcus, Dorea,* and *Eubacterium* increased during the AXOS intervention. An increase in bifidobacteria may increase acetate production, which in turn can be metabolized by butyrate producing bacteria, thus stimulating their growth [21]. This cross-feeding process could explain the increased abundance of bacteria belonging to the Clostridia class, particularly *E. rectale, F.prausnitzii* and *E. hallii* found in our study. Furthermore, the observed shift in the entire gut microbial community following AXOS intake was associated with increased abundances in *Roseburia, Coprococcus* and *Anaerostipes* species, which are butyrate producers as well [21]. The remaining OTUs (all belonging to phylum Firmicutes and order Clostridales) that increased in abundance were *Blautia* and *Fusicantenibacter* genera, which have not previously been reported to change in response to AXOS intake [15–18,45].

It was shown that 10 g/d AXOS increased the abundance of the species *B. longum* and *D. longicatena*, but did not affect fasting glucose metabolism, as observed in previous AXOS interventions [16–18]. Conversely, the increasing in abundance of the species *B. longum* and

D. longicatena, as a consequence of the regular consumption of dietary fiber, has been previously associated with reduced insulin resistance [46]. Although AXOS has a lower viscosity than AX, which reduces postprandial glucose [47], beneficial dose-dependent effects on overnight glucose metabolism has been already suggested [22]. For the lipid profile, we found no major effects in any parameter analyzed, similarly to what reported in other AXOS intervention studies [16,17,48]. By contrast, another study showed that 15 g/d AX consumption over 6 weeks decreased the fasting serum glucose, TG, and the apolipoprotein A1 concentrations, compared to placebo treatment [49]. The above differences regarding the glucose and lipid metabolism could be related to the duration of the study and the specific type of dietary fiber used in the intervention. In fact, García *et al.* [49] observed changes in glucose and lipid metabolism by using a 6-week long intervention and using AX, whereas others studies reporting no effects were done with AXOS administration from 2 up to 4 weeks.

Prior to the study initiation, a limited number of human studies indicated that fat type could affect microbiota composition [28,29] and the main support for our initial hypothesis was evidence from animal studies. We did not observe any effect of PUFA intake on the gut microbiota composition, but results from a few human studies suggest that n-3 PUFA supplementation reduces *Faecalibacterium* and increases *Lachnospiraceae* species [50]. In a cohort study with 876 women, Menni *et al.* [51] observed associations between circulating levels of total and various types of PUFAs and greater microbiome diversity. Positive associations between serum DHA and 36 OTUs were observed, of them, 21 OTUs belonged to the *Lachnospiraceae* family, which have the possibility of degrading complex polysaccharides generating SCFAs. Moreover, the association of DHA serum levels with such microbes was still present when data was adjusting by fiber intake information. A recent study in humans by Pu *et al.* [52] investigated effects of MUFAs, PUFAs, and canola oil-

enriched diets on the microbiota and found that few microbial changes occurred at genus level without effects on higher taxonomic levels after 30 days of dietary intervention. An increase of *Bifidobacterium, Oscillospira, Lachnospira, Coprococcus,* and *Faecalibacterium* was observed in a recent human cross-over intervention using PUFAs administered in drinks or capsules, in two different intervention periods during 8-week each and with a 12-week washout [53]. In our study, the lack of effect on the gut microbiota may be explained by several reasons that should be considered for future research. First, the increase in PUFA intake did not cause a reduction in SFA intake as we aimed for. Second, another possibility is that the change in PUFAs was too small to exert measurable effects on microbial composition in relatively short time. Via the fish oil supplement, we provided a dose of EPA and DHA (1.32 and 1.86 g/d, respectively) lower than that reported to have adverse events (5 g/d) [54] and lower than that showing changes in certain microbial genera (2 g/d EPA and 2 g/d DHA) [53]. However, it resulted in a much smaller difference in PUFA intake (1.84 percentage point) compared to the difference between monounsaturated fatty acid (MUFA) and PUFA interventions in the study by Pu *et al.* [52] where PUFA intake differed by 7.2-9.4 percentage points (6.7-9.1 E% vs 16.3 E%). Third, the duration of the intervention is a factor important to consider since interesting results have been obtained following longer intervention with an ample washout period to demonstrate reversible effects after the PUFA intake [53]. Although some extreme dietary changes can shift the gut microbiota composition within a few days, the response to some nutrients could be slower and depending on the concentration and overall dietary intake pattern. Pu *et al.* observed effects after a 30-day intervention but the changes in fat quality were larger, total fat intake was constant and all consumed meals were provided during the intervention periods [52]. According to our longitudinal analysis of delta values (changes between the assessments of the four different sampling points) a longer dietary intervention might have been needed to detect PUFA effects on gut microbiota. We also observed that the slight effects of the PUFA intervention (PUFA-I period), persisted to some

591 extent during the washout period and potentially beyond the AXOS intervention (AXOS-II
592 period) with potential carry-over effects, which could explain the lack of significant effects of
593 AXOS in this subjects group. Therefore, the design of future PUFA-based studies should
594 consider the need of doing longer intervention and washout periods to find detectable and
595 meaningful effects. Fourth, our sample size calculation was based on bifidogenic fiber effects
596 as data from human interventions with PUFA intake on gut microbiota modulation were not
597 available when the study was designed. Thus, the power to observe effects on gut microbiota
598 composition in relation to PUFA intake could be too low.

599 Regarding the metabolic effects of the interventions, these are strongly dependent on the
600 whole diet composition. For example, effects of fat intake on metabolic markers reported in
601 previous studies depend on whether one type of fat is replaced by other types of fat (or
602 macronutrients), and fat quality may be more important for health than fat quantity. In the
603 current study, the participants did increased PUFA intake at expense of a reduction in SFA
604 intake, which make the comparison to other studies difficult. Also, our study was not powered
605 to obtain significant changes in metabolic outcomes. In spite of this, we observed a none
606 statistical significant reduction of 5.5-6.0 and 1.75-4.3 mmHg in systolic and diastolic blood
607 pressures, respectively, in agreement with a meta-analysis of RCT showing that intake of
608 EPA+DHA in a dose similar as that of our intervention reduced systolic and diastolic blood
609 pressures [55]. For cholesterol concentrations, Schwarb *et al*. [56] concluded in a systematic
610 review that total and LDL cholesterol concentrations were lower on a PUFA-rich diet,
611 compared to a SFA-rich diet. Although we instructed the participants to increase PUFA intake
612 and reduce SFA intake, this was not the case which may explain why we did not observe
613 beneficial metabolic effects on cholesterol levels either. Also a limitation of our study was
614 that the participants were less metabolically challenged than we aimed for. This problem
615 seems to be related to screening methods as fewer participants had low HDL-CHO

concentration in their venous blood sample at baseline compared to HDL-CHO concentration measured by the finger prick test at screening. This may also explain why dietary effects on metabolic markers were limited. Effect observed in the AXOS-I group but not in the AXOS-II group could be explained by the potential carry-over effects above discussed.

Furthermore, different studies have suggested that individuals, depending on their initial gut microbiota, could respond differently to a dietary intervention. Zeevi *et al.* showed that an algorithm including information on gut microbiota composition could predict postprandial glycemic response to a wide range of foods consumed in real-life settings [57]. Thus, the glycemic response to a food was affected by the individual gut microbiota composition and surprisingly, what normally is accepted as healthy and unhealthy food did not cause the same glucose response in all individuals. Currently, there is no definition that can distinguish individuals as responders or non-responders to a specific dietary intervention but it is possible that interventions with few participants, as in our study, have a skewed or limited distribution of responders and non-responders which makes it more difficult to observe both microbial and metabolic effects and disentangle their possible connection.

Conclusions

Intake of AXOS changed the gut microbiota composition. Higher abundance of bifidobacteria and butyrate producing bacteria were the main contributors to this change. Multiple correlations were established between specific OTUs and biochemical markers that could be beneficial for metabolic health (e.g. lower HOMA, higher HDL CHO) and should be further explored since limitations in the duration of this study could have precluded the detection of significant beneficial effects on these end-points. PUFA intake did not affect gut microbiota composition or any metabolic marker likely because it requires longer time than AXOS to drive significant changes. Further studies are needed to disentangle the role played by the

640 individual’s microbiota in predicting the health related effects in response to dietary
641 interventions.

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Statement of Authorship

LK, LKB, AA, JV, YS and LHL designed the study. JV designed the WBE study products. LK, LKB, EMGP, ABP, GL, and SM, conducted the experimental research. LK, LKB, ABP, PB, SR, YS and LHL analyzed data. LK and ABP wrote the paper, LK and ABP have the primary responsibility for final content. All authors critically reviewed the manuscript and approved the final manuscript.

Conflict of Interest Statement and Funding sources

All the authors declare to have no conflict of interest.

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Availability of data and material

The raw fasta sequences generated from the 16S amplicon sequencing of fecal DNA are publicly available at the MG-RAST server [58] upon the project accession number mgp84629.

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Figure and Table legends

Figure 1: Diagram of the study design.

AXOS, arabinoxylan oligosaccharides; PA, physical activity; PUFA, polyunsaturated fatty acids; REE: resting energy expenditure, Wk, week

Figure 2: Change in beta-diversity visualized by plots from Principal Coordinate Analysis. Comparison among the three main principal coordinate PC1, PC2, and PC3 for AXOS intervention (upper part) and PUFA intervention (lower part). This analysis is depicted particularly for the first intervention periods of both interventions (AXOS-I and PUFA-I). Small blue filled circles correspond with samples before intervention and small red filled circles correspond with samples after intervention. Greater filled circles correspond with respective centroids calculated from median of the PCs plotted. AXOS, arabinoxylan oligosaccharides; PC, Principal Coordinate; PUFA, polyunsaturated fatty acids.

Figure 3: Gut microbiota change during AXOS intervention (N=28). A) AXOS intake increased abundance of phylum Actinobacteria (AXOS I+II, $p < 0.0016$; AXOS I , $p < 0.0015$; AXOS-II, $p < 0.1825$). Normalized read count (before versus after AXOS intervention) was analyzed by non-parametric LDA analysis. B) AXOS intake during first diet period increased abundance of Bifidobacteriaceae ($p < 0.0014$) and Coribacteriaceae ($p < 0.0041$) families of Actinobacteria and decreased abundance of Rikenellaceae ($p < 0.0238$) and Porphyromonadaceae ($p < 0.0450$) families of Bacteroidetes. Changes in abundance (before versus after intervention) was analyzed by non-parametric LDA analysis. AXOS, arabinoxylan oligosaccharides; LDA, Linear Discriminant Analysis.

Figure 4: Longitudinal data analysis of delta values obtained with the four time-point assessments (week 0, 4, 8 and 12) of each of the two randomization orders (the first one starting with the AXOS intervention and the second starting with the PUFA intervention). A) The global schema of the

longitudinal delta analysis. Calculation of respective delta values is depicted across the full intervention timeline as well as the type of response predicted. The black dashed line indicates the microbiota response in those participants starting the study with AXOS intake (AXOS-I → PUFA-II). The grey dashed line shows the microbiota response in those participants starting the study with PUFA intake (PUFA-I → AXOS-II), suggesting a minor but persistent effect of PUFAs beyond the washout period. B) Bacterial genera with significantly different trajectory between AXOS-I period and the washout (Δ_1 vs Δ_2). C) Bacterial genera with significantly different trajectory between the PUFA-I+washout periods and the AXOS-II intervention (Δ_{1+2} vs Δ_3). The light-grey lines in plots correspond to longitudinal trajectories per subject in each cohort, whereas the black solid line indicates the median from the respective observations.

Supplementary Figure 1: Flow chart of the recruiting process.

Supplementary Figure 2: Alpha diversity analysis of fecal microbiota. Three common descriptors, including Chao's richness, Shannon's evenness, and Simpson's reciprocal index, were used to assess changes in the gut microbiota diversity of subjects enrolled in the AXOS and PUFA cross-over intervention. The distribution of respective metrics (boxplots arranged in row fashion) is drawn across the different subgroups according to the dietary intervention periods (boxplots arranged in column fashion). The p-values were computed by pairwise comparisons between groups using the non-parametric Wilcoxon test and stated inside respective boxplots.

Supplementary Figure 3: Absolute qPCR quantification of *Bifidobacterium* species. Fecal DNA samples from AXOS-I subjects were used to measure the number of 16S rRNA gene molecules of DNA belonging to *Bifidobacterium* species. The absolute number of 16S rRNA gene molecules obtained was normalized against the total DNA concentration present in 1 µL sample used for qPCR (fluorometric methods). Absolute quantification is shown in $\log_{10}$ scale. Wilcoxon Rank-Sum test for the paired samples was used to assess statistical differences due to AXOS intake and the *p*-value supporting the rejection of null hypothesis is shown above the boxplot.

Tables

Table 1: Characterization of WBE and AXOS supplements consumed during the AXOS intervention

	WBE (per 100g)	**Powder (5g WBE)**	**Crackers (per piece)**	**Biscuits (per piece)**	**Total daily intake[1]**
Energy (kJ)	812[2]	41[2]	125	162	655
Protein (g)	0.7	0.04	0.6	0.5	2.3
Fat (g)	0	0	0.7	1.8	5.0
Carbohydrates (g)	19.7	1.0	4.8	4.8	21.2
Of which sugar (g)	3.8	0.2	0.7	1.7	5.2
Fiber (g)	72.0[3]	3.6	1.0	1.0	11.2
WBE (g)	100	5	1.3	1.2	15.0
AXOS[4] (g)	69	3.5	0.9	0.8	10.4

[1]Total daily intake: 2 powder, 2 crackers and 2 biscuits

[2]Calculated value

[3]Measured by methods approved by Association of the Official Analytical Chemists (AOAC) 2009.01 that nearly reproduces the human physiological digestion and measures the total dietary fiber content as currently defined by Codex Alimentarius.

[4]Average degree of polymerization was 5, Arabinose/Xylose ratio was 0.24, ash content 0.20% and moisture 3.4%
AXOS, arabinoxylan oligosaccharides; WBE, wheat bran extract

Table 2: Dietary intake at baseline, and after each intervention (mean ±SD)

	Baseline (N=29)	**After AXOS (N=28)**	**After PUFA (N=27)**	**Treatment *p*[1]**	**Time *p*[1]**	**Treatment × Time *p*[1]**
Energy intake (kJ/d)	8,843 ±2,771	8,836 ±2,383	8,859 ±2,799	0.673	0.796	0.807
Carbohydrate (E%)	45.8 ±6.82	48.0 ±6.53	43.5 ±7.36	0.561	0.864	0.333
Protein (E%)	17.7 ±4.43	17.4 ±4.01	17.3 ±4.43	0.392	0.999	0.370
Fat (E%)	35.0 ±5.41	33.3 ±4.68	36.8 ±4.37	0.202	0.703	0.616
PUFA (E%)	6.19 ±1.70	5.29 ±1.45	7.77 ±1.88	0.002	0.229	0.004
MUFA (E%)	11.9 ±3.13	11.2 ±2.39	11.4 ±2.91	0.360	0.689	0.342
SFA (E%)	12.0 ±3.14	11.8 ±2.61	11.9 ±3.20	0.473	0.851	0.780
Fiber (g/d)	24.5 ±12.0	31.2 ±7.94	20.9 ±6.97	0.008	0.009	0.001

[1]Based on LMM analysis and adjusting by covariates (recruiting BMI) and fixed effects (age, gender, and order of treatments). AXOS, arabinoxylan oligosaccharides, E%, energy percentage; MUFA, mono unsaturated fatty acids; PUFA, poly unsaturated fatty acids; SFA, saturated fatty acids.

Table 3: Outcome related to time points; baseline, after washout and after each intervention (mean ±SD)

	Baseline (N=29)	**After AXOS (N=28)**	**After PUFA (N=27)**	**Treatment *p*[1]**	**Time *p*[1]**	**Treatment × Time *p*[1]**
Anthropometric						
Body weight (kg)	88.0 ±13.7	88.7 ±13.8	88.8 ±14.2	0.931	0.929	0.995
WC (cm)	96.5 ±8.82	97.3 ±8.80	96.6 ±9.18	0.511	0.663	0.617
HC (cm)	111 ±6.31	111 ±6.45	111 ±6.45	0.776	0.762	0.741
Sagittal height (cm)	22.0 ±2.53	22.0 ±2.38	21.9 ±2.65	0.776	0.944	0.948
Blood pressure						
Systolic (mmHg)	120 ±15.1	121 ±16.6	117 ±14.8	0.900	0.456	0.118
Diastolic (mmHg)	77.9 ±9.51	77.0 ±9.90	74.4 ±10.4	0.424	0.304	0.486
Pulse (beats/min)	59.3 ±8.14	60.0 ±7.20	60.1 ±7.93	0.644	0.703	0.916
Lipid profile						
Total CHO (mmol/L)	5.05 ±0.94[2]	5.17 ±1.01[4]	5.01 ±0.85[4]	0.321	0.984	0.933
HDL-CHO (mmol/L)	1.40 ±0.40[2]	1.37 ±0.35[4]	1.44 ±0.46[4]	0.687	0.984	0.548
LDL-CHO (mmol/L)	3.06 ±0.87[2]	3.17 ±0.90[4]	2.94 ±0.85[4]	0.352	0.891	0.712
ApoB (g/L)	0.95 ±0.24[2]	0.96 ±0.26[4]	0.93 ±0.25[4]	0.453	0.899	0.882
VLDL-CHO (mmol/L)	0.59 ±0.21[2]	0.64 ±0.24[4]	0.63 ±0.18[4]	0.935	0.147	0.754
Triglycerides (mmol/L)	1.24 ±0.47[2]	1.38 ±0.61[4]	1.11 ±0.43[4]	0.129	0.971	0.150
Glucose metabolism						
Glucose (mmol/L)	5.48 ±0.41[2]	5.56 ±0.44[4]	5.61 ±0.38[4]	0.945	0.117	0.318
Insulin (pmol/L)	43.6 ±30.3[2]	48.5 ±34.9[4]	50.3 ±34.1[4]	0.953	0.965	0.598
HOMA-IR	1.88 ±1.37[2]	2.01 ±1.50[4]	2.13 ±1.39[4]	0.983	0.892	0.483
HOMA-β	78.3 ±55.8[2]	79.6 ±53.0[4]	81.9 ±55.1[4]	0.935	0.954	0.725
Inflammation markers						
hsCRP (mg/L)	2.71 ±3.31[2]	2.77 ±5.43[4]	2.73 ±2.99[4]	0.848	0.525	0.352
Hb (mmol/L)	8.66 ±0.76[2]	8.59 ±0.84[4]	8.66 ±0.83[4]	0.425	0.874	0.936
WBC (10^9/L)	5.38 ±1.34[2]	5.41 ±1.18[4]	5.29 ±1.58[4]	0.398	0.505	0.680
Liver markers						
ASAT (U/L)	29.8 ±37.3[2]	23.6 ±8.03[4]	23.9 ±9.02[4]	0.772	0.473	0.792
ALAT (U/L)	31.1 ±33.6[2]	25.3 ±16.5[4]	27.0 ±17.3[4]	0.437	0.431	0.754
ASAT/ALAT	1.05 ±0.31[2]	1.10 ±0.39[4]	1.05 ±0.39[4]	0.663	0.552	0.998
Other						
Flatulence	17.1 ±17.1	30.2 ±19.5	17.5 ±22.4	0.033	0.064	0.103
Bristol	3.78 ±1.34	4.15 ±1.18	3.5 ±1.23	0.037	0.681	0.303
Breath hydrogen (ppm)	20.2 ±25.1	31.9 ±32.0	22.2 ±44.9	0.113	0.559	0.252
PA vector (CPM)	575 ±189	547 ±177	618 ±205	0.393	0.449	0.214
REE (kJ/d)	6,317 ±1,181	6,418 ±1,133	6,388 ±1,209	0.646	0.584	0.599
Respiratory quotient	0.805 ±0.03	0.811 ±0.05	0.802 ±0.04	0.806	0.560	0.544

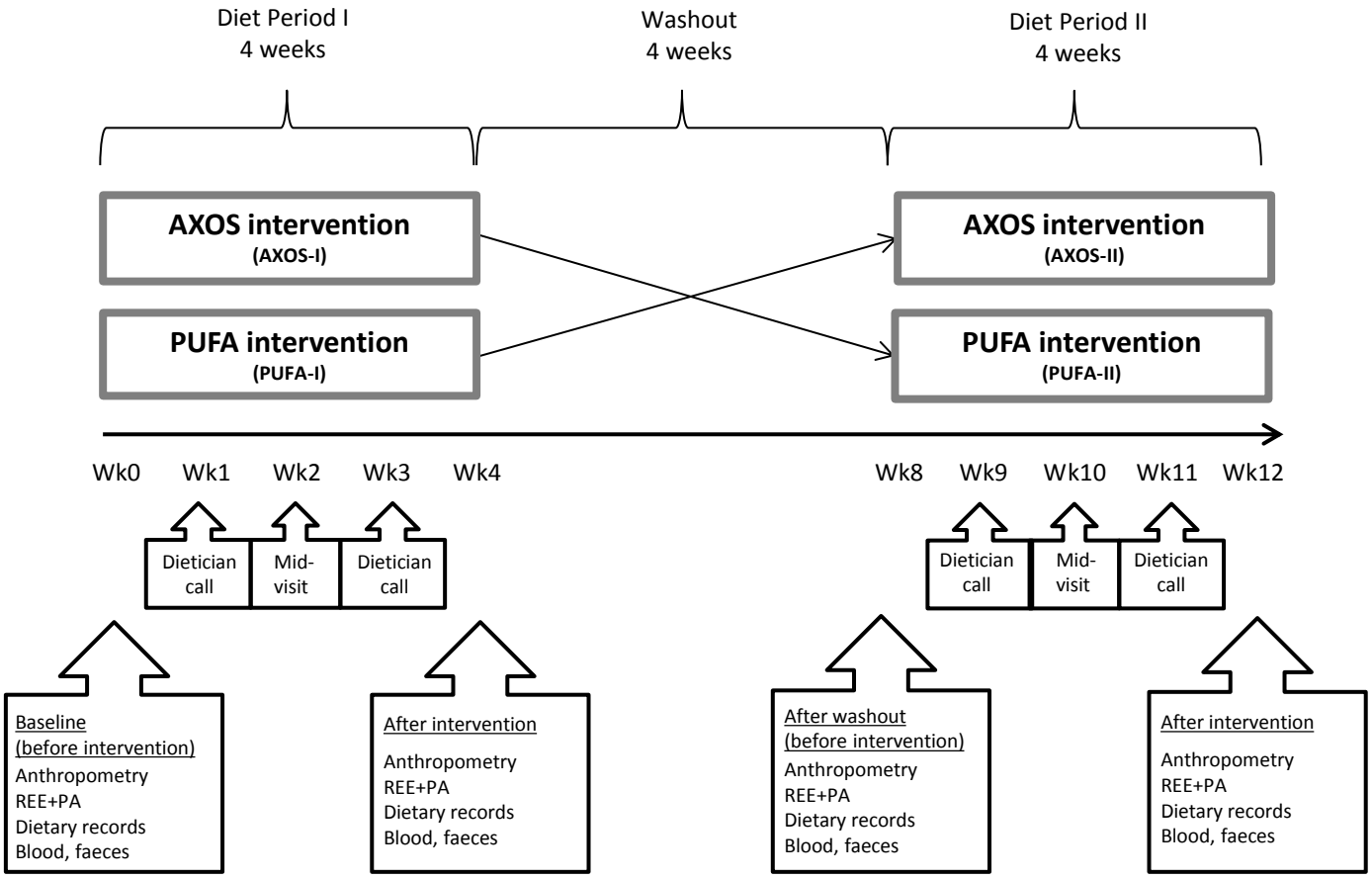
[1]Based on LMM analysis and adjusting by covariates (recruiting BMI) and fixed effects (age, gender, and order of treatments).
[2]N=28,
[3]N=26
[4]N=27

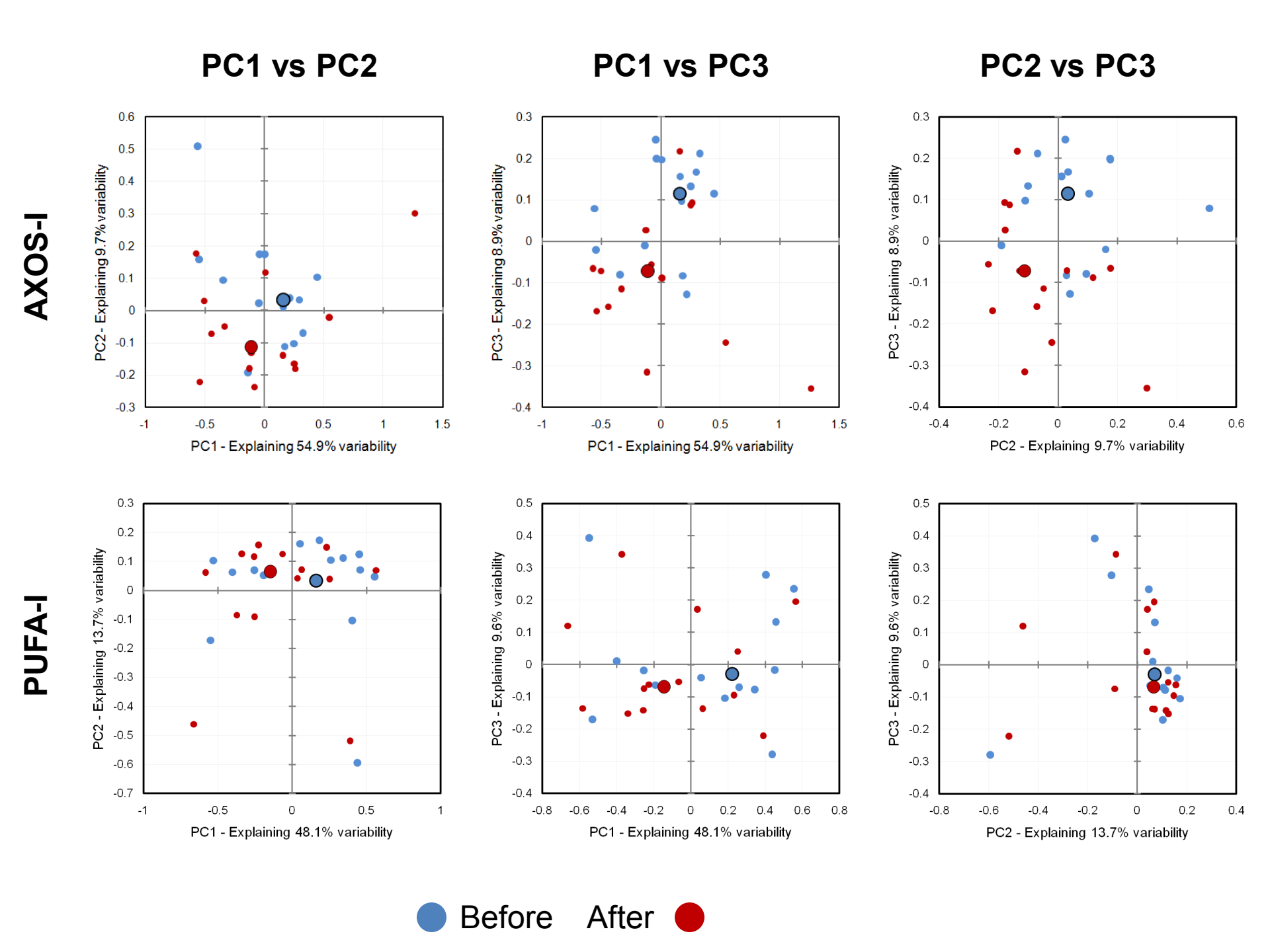
ALAT, alanine aminotransferase; ApoB, apolipoprotein B; ASAT, aspartate aminotransferase; AXOS, arabinoxylan oligosaccharides; CHO, cholesterol; CPM, counts per minutes; Hb, hemoglobin; HC, hip circumference; HDL, high density lipoprotein; HOMA-β, homeostatic model assessment - beta-cell function; HOMA-IR, homeostatic model assessment- insulin resistance; hsCRP, high sensitive C-reactive protein; LDL, low density lipoprotein; REE, resting energy expenditure; PA, physical activity, PUFA, poly unsaturated fatty acids; VLDL, very low density lipoprotein; WBC, white blood cell count; WC, waist circumference.

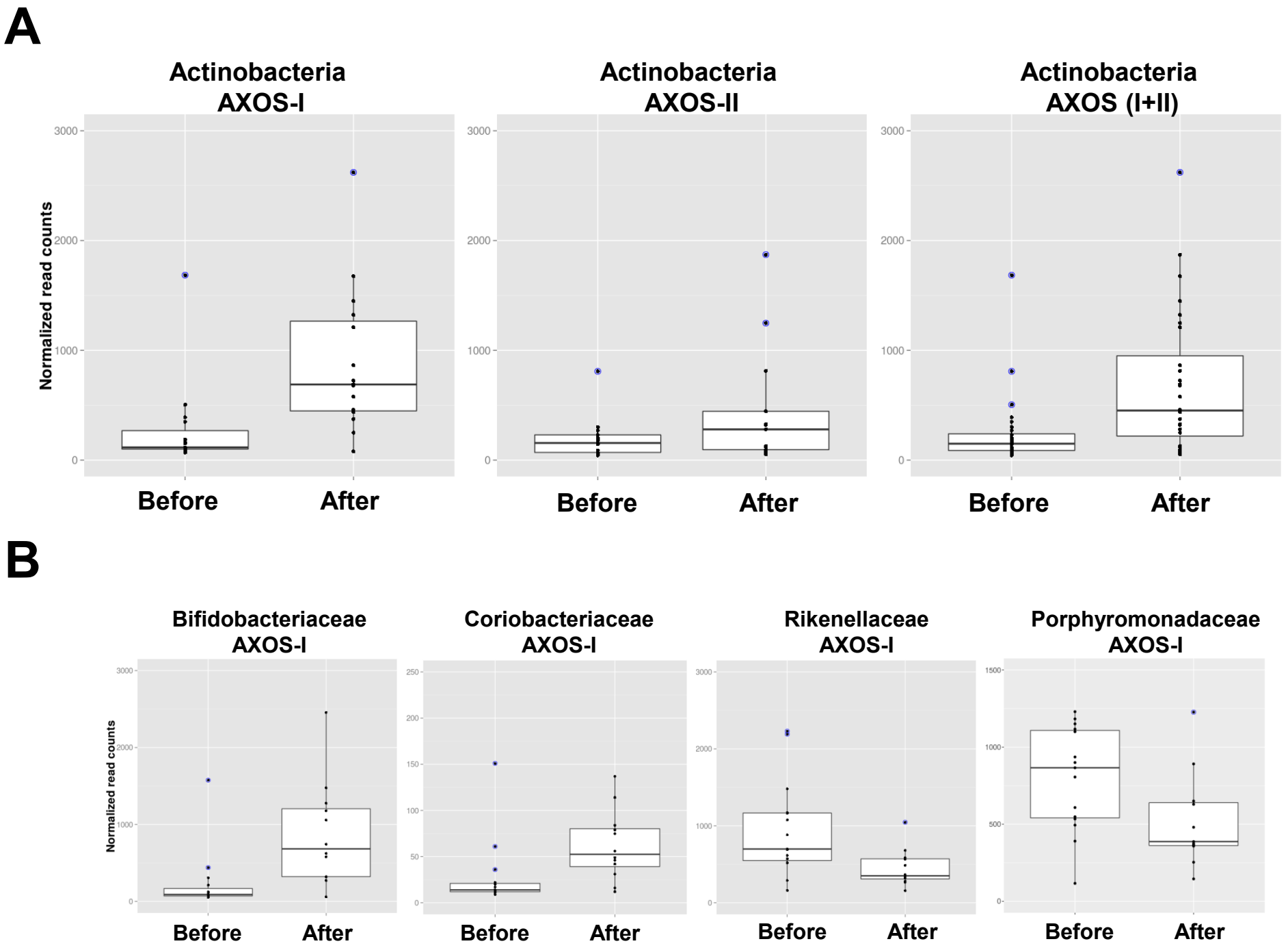
Table 4: OTU changes as a result of the AXOS intervention in the first diet period (n=15)

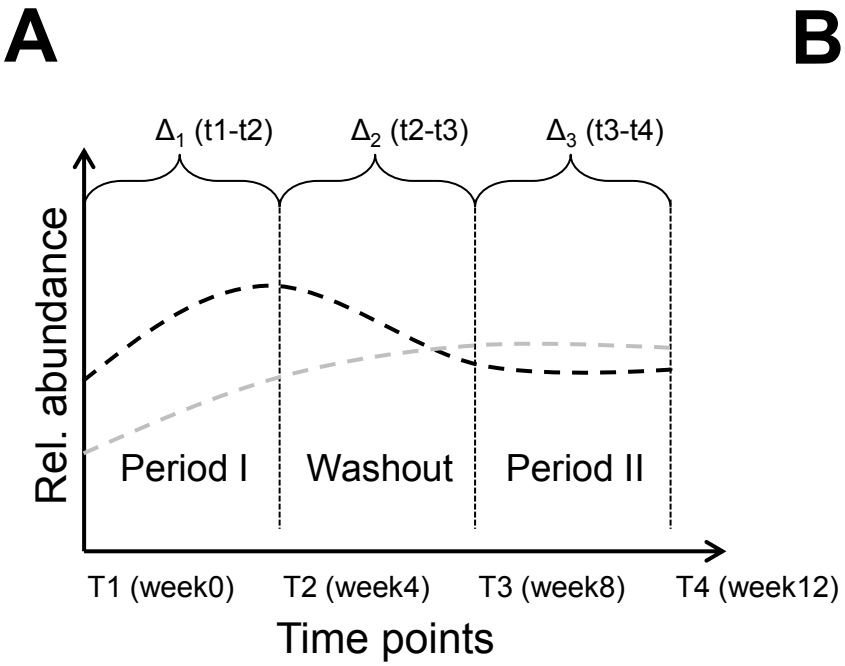
OTU	Blast 16S database[1]	id%[2]	LDA score	*p*-value[3]
Increased abundance				
4	*Eubacterium rectale*	100	4.34	0.029
5	*Faecalibacterium prausnitzii*	99	4.09	0.033
14	*Bifidobacterium faecale, Bifidobacterium stercoris, Bifidobacteriuma dolescentis*	100	3.93	0.044
26	*Blautia wexlerae*	100	3.80	0.001
770	*Bifidobacterium angulatum, Bifidobacterium merycicum, Bifidobacterium pseudocatenulatum, Bifidobacterium catenulatum*	99	3.67	0.019
27	*Fusicatenibacter saccharivorans*	100	3.60	0.036
52	*Bifidobacterium longum*	100	3.31	0.008
534	*Ruminococcus obeum*	99	3.30	0.012
44	*Dorea longicatena*	99	3.26	0.008
78	*Eubacterium hallii*	99	3.21	0.036
54	*Blautia luti*	99	3.19	0.019
Decreased abundance				
751	*Clostridium methylpentosum*	94	3.15	0.035
764	*Anaerotruncus colihominis*	92	3.10	0.035
688	*Erysipelothrix rhusiopathiae*	85	3.09	0.035

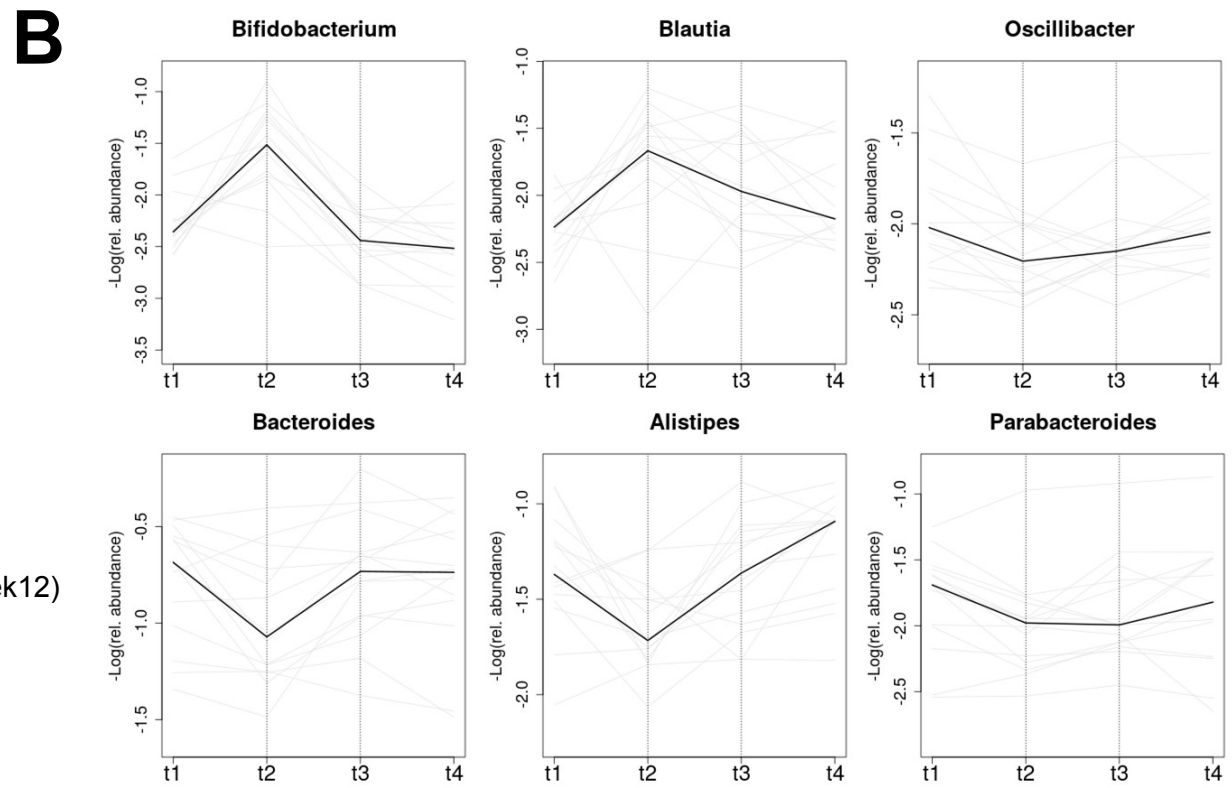
[1]Bacterial species/strain matching the OTU sequence according to best hit in a Blast-based search.
[2]Percentage of sequence identity supporting the taxonomic assignation of the respective OTU through the Blast-based search (alignment length percentage were 100 for all the OTUs presented in the table).
[3]Changes in OTU abundance in the microbiota of subjects before to after the first AXOS intervention was compared by non-parametric LDA (only OTUs with a LDA-score above 3are shown in the table). Differences were considered statistically significant at p-values < 0.05.
AXOS, arabinoxylan oligosaccharides; LDA, Linear Discrimination Analysis; OTU, Operational Taxonomic Unit.

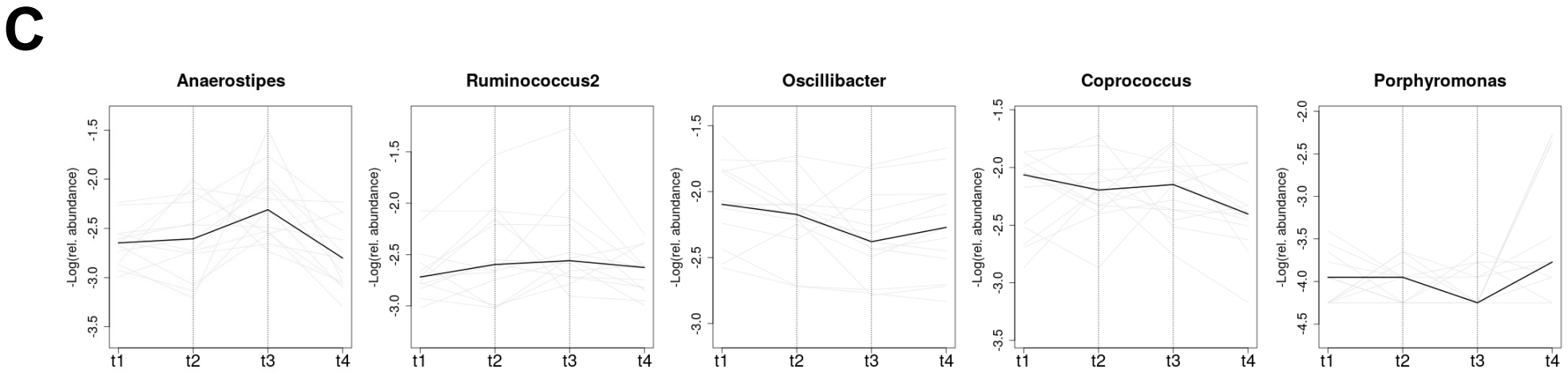

Diet Period I
4 weeks
Washout
4 weeks
Diet Period II
4 weeks
AXOS intervention
(AXOS-I)
PUFA intervention
(PUFA-I)
AXOS intervention
(AXOS-II)
PUFA intervention
(PUFA-II)
Wk0
Wk1
Wk2
Wk3
Wk4
Wk8
Wk9
Wk10
Wk11
Wk12
Dietician call
Mid-visit
Dietician call
Dietician call
Mid-visit
Dietician call
Baseline
(before intervention)
Anthropometry
REE+PA
Dietary records
Blood, faeces
After intervention
Anthropometry
REE+PA
Dietary records
Blood, faeces
After washout
(before intervention)
Anthropometry
REE+PA
Dietary records
Blood, faeces
After intervention
Anthropometry
REE+PA
Dietary records
Blood, faeces


PC1 vs PC2
PC1 vs PC3
PC2 vs PC3
AXOS-I
PUFA-I
PC2- Explaining 9.7% variability
PC1 - Explaining 54.9% variability
PC3- Explaining 8.9% variability
PC1 - Explaining 54.9% variability
PC3 - Explaining 8.9% variability
PC2 - Explaining 9.7% variability
PC2 - Explaining 13.7% variability
PC1 - Explaining 48.1% variability
PC3 - Explaining 9.6% variability
PC1 - Explaining 48.1% variability
PC3 - Explaining 9.6% variability
PC2 - Explaining 13.7% variability
Before
After


A
Actinobacteria
AXOS-I
Actinobacteria
AXOS-II
Actinobacteria
AXOS (I+II)
Normalized read counts
3000
2000
1000
0
Before
After
B
Bifidobacteriaceae
AXOS-I
Coriobacteriaceae
AXOS-I
Rikenellaceae
AXOS-I
Porphyromonadaceae
AXOS-I
250
200
150
100
50
1500
500


A
Δ1 (t1-t2)
Δ2 (t2-t3)
Δ3 (t3-t4)
Rel. abundance
Period I
Washout
Period II
T1 (week0)
T2 (week4)
T3 (week8)
T4 (week12)
Time points


B
Bifidobacterium
Blautia
Oscillibacter
Bacteroides
Alistipes
Parabacteroides
-Log(rel. abundance)
t1
t2
t3
t4


C
Anaerostipes
Ruminococcus2
Oscillibacter
Coprococcus
Porphyromonas
-Log(rel. abundance)
t1
t2
t3
t4