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1 **Arabinoxylan oligosaccharides and polyunsaturated fatty acid effects on gut microbiota**  
2 **and metabolic markers in overweight individuals with signs of metabolic syndrome: a**  
3 **randomized cross-over trial**

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

25 Abbreviations:

26 ALAT, alanine aminotransferase; ApoB, apolipoprotein B; ASAT, aspartate

27 aminotransferase; AX, arabinoxylan; AXOS, arabinoxylan oligosaccharides; BMI, body mass

28 index; BP, blood pressure; BW, body weight; CHO, cholesterol; CID, clinical investigation

29 day; CPM, counts per minutes; DHA, docosahexaenoic acid; DNA, deoxyribonucleic acid;

30 EPA, eicosapentaenoic acid; E%, energy percentage; HDL, high density lipoprotein; HOMA-

31  $\beta$ , homeostatic model assessment - beta-cell function; HOMA-IR, homeostatic model

32 assessment -insulin resistance; hsCRP, high sensitive C-reactive protein; LDA, Linear

33 Discriminant Analysis; LDL, low density lipoprotein; LMM, Linear Mixed Model; OTU,

34 Operational Taxonomic Units; PC, principal coordinate; PCoA, Principal Coordinate

35 Analysis; PCR, Polymerase chain reaction; PUFA, polyunsaturated fatty acids; REE, resting

36 energy expenditure; rRNA, ribosomal ribonucleic acid; SFA, saturated fatty acid; TG,

37 triglycerides; VAS, visual analogue scale; VLDL, very low density lipoprotein; WBE, wheat

38 bran extract

39 Clinical trial registry (<https://www.clinicaltrials.gov/>): NCT02215343

40 Ethical committee: H-4-2014-052

41 The Danish Data Protection Agency: 2013-54-0522

## 42 **Abstract**

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

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

55 **Results:** Twenty-seven participants completed the study (90%). Intake of AXOS induced an  
56 expected bifidogenic effect on gut microbiota ( $p < 0.01$ ) and increased butyrate-producing  
57 bacterial species as well ( $p < 0.05$ ). Beta-diversity analysis indicated that the structure of the  
58 gut microbiota only changed as a result of the AXOS intervention (Permanova = 1.90,  $p <$   
59 0.02) and no changes in metabolic markers were observed after any of the interventions.

60 **Conclusions:** AXOS intake has bifidogenic effects and also increases butyrate producers in  
61 the gut microbiota; even though this type of dietary fiber did not modulate lipid or glucose  
62 metabolic parameters related to metabolic syndrome. Four-week PUFA intake did not induce  
63 any notable effect on the gut microbiota composition or metabolic risk markers.

64 **Registration:** Registered under ClinicalTrials.gov Identifier no.NCT02215343

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

## 67 **Introduction**

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

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

92 18], which theoretically could mediate beneficial health effects. In addition, the production of  
93 short-chain fatty acids (SCFAs) via fermentation of dietary fiber seems important for  
94 improving metabolic health. Of SCFAs, butyrate [19], and more recently, propionate  
95 production [20] have been suggested to beneficially influence metabolic health. When AXOS  
96 is fermented by bifidobacteria acetate is produced as immediate metabolic product but this  
97 can be further metabolized to butyrate by other intestinal bacteria (butyrate producing  
98 bacteria) via cross-feeding mechanism [21]. Acute intake studies carried out with AX and  
99 AXOS have shown that overnight AXOS intake may improve glucose metabolism and AX  
100 intake reduces the postprandial glucose peak [22,23].

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

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

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

## 121 **Materials and Methods**

### 122 *Study design*

123 The study had a cross-over design with two diet periods of 4 weeks each separated by a 4-  
124 week washout period (Figure 1). The completing participants were randomized to the  
125 sequence of the two diets (ratio 1:1) and all visits were planned to be conducted within a  
126 window of  $\pm 3$  days. The computer-based randomization list was generated at  
127 *randomization.com*. Due to the study design blinding of project staff and participants was not  
128 possible. Data were collected on 4 clinical investigation days (CID) during the study: baseline  
129 (week 0), after first dietary intervention period (week 4), after washout (before second diet  
130 period) (week 8), and after the second dietary intervention period (week 12). Furthermore, the  
131 participants had two dietician consultations (week 2 and week 10) to ensure body weight  
132 (BW) maintenance. The dietician called the participants in the remaining weeks (week 1, 3, 9  
133 and 11) (Figure 1). Prior to each CID, the participants consumed a standardized dinner in the  
134 evening followed by a fasting period of minimum 8 hours. The study was conducted at the  
135 Department of Nutrition, Exercise and Sports, University of Copenhagen from August 2014  
136 to June 2015. The study is registered at Clinical Trial (NCT02215343), conducted according  
137 to the guidelines laid down in the Declaration of Helsinki and was carried out in accordance  
138 with the ethical standards of the responsible regional committee on human experimentation in  
139 Denmark, registered as H-4-2014-052, and the Danish Data Protection Agency (2013-54-  
140 0522).

### 141 *Study participants*

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

167 *Intervention*

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

## 182 *Outcomes*

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

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

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

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

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

218 20 sec. Phusion High-Fidelity Taq Polymerase (Thermo Scientific, Wilmington, USA) and  
219 the 6-mer barcoded primers, S-D-Bact-0341-b-S-17 (CCTACGGGNGGCWGCAG) and S-D-  
220 Bact-0785-a-A-21 (GACTACHVGGGTATCTAATCC) which target a wide range of  
221 bacterial 16S rRNA genes [30], were used during PCR. Dual barcoded PCR products,  
222 consisting of ~500bp, were purified from triplicate reactions with the Illustra GFX PCR DNA  
223 and Gel Band Purification Kit (GE Healthcare, Little Chalfont, UK) and quantified through  
224 Qubit 3.0 and the Qubit dsDNA HS Assay Kit (Thermo Fisher Scientific, Waltham, MA,  
225 USA). Samples were multiplexed by combining equimolar quantities of amplicon DNA (100  
226 ng per sample) and sequenced in an Illumina MiSeq platform with 2x300 PE configuration  
227 (Eurofins Genomics GmbH, Ebersberg, Germany). Raw data were delivered in fastq files and  
228 pair ends with quality filtering were assembled using *Flash* software [31]. Sample de-  
229 multiplexing was carried out using sequence information from the respective DNA barcodes  
230 and *Mothur v1.36.1* suite of analysis [32]. After assembly and barcodes/primers removal, the  
231 sequences were processed for chimera removal using *Uchime* algorithm [33] and SILVA  
232 reference set of 16S sequences [34]. Alpha diversity was calculated with *Mothur v1.36.1*  
233 using default parameters and average method in the clustering step. Consequently, the Chao's  
234 richness, Shannon's evenness and Simpson's reciprocal index were computed using a high  
235 quality and a normalized subset of 17,750 sequences per sample, randomly selected after  
236 shuffling (10,000X) of the original dataset. Taxonomic assessment was performed using the  
237 Ribosomal Database Project (RDP) classifier v2.12 [35]. The Operational Taxonomic Unit  
238 (OTU)-picking approach was performed with the normalized subset of 17,750 sequences and  
239 the *uclust* algorithm implemented in USEARCH v8.0.1623 [36]. Beta-diversity was evaluated  
240 using Principal Coordinate Analysis (PCoA) and Bray-Curtis dissimilarity index.

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

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

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

268 g for 10 min at 4°C and stored at -80°C until processing. Samples for whole blood analyses of  
269 hemoglobin and white blood cell were collected in ethylenediaminetetraacetic acid (EDTA)  
270 tubes and concentrations were immediately measured (SysmexKX-21, Sysmex Corporation,  
271 Kobe, Japan). Insulin was measured by chemiluminescent immunometric assay (IMMULITE  
272 2000 INSULIN, Siemens Healthcare Diagnostics Inc.) on the IMMULITE2000 INSULIN  
273 Analyzer (Siemens Healthcare Diagnostics Products Ltd., UK). Samples with an insulin  
274 concentration below the detection limit (14.4 pmol/L) were set to 7.2 pmol/L. Glucose was  
275 measured by enzymatic hexokinase method on the Pentra 400 Analyzer (HORIBA ABX,  
276 Montpellier, France). The homeostatic model assessment was used to quantify insulin  
277 resistance (HOMA-IR) and beta-cell function (HOMA-β) from measurements of fasting  
278 insulin and glucose concentrations. HOMA-IR was calculated as:  $(\text{insulin } (\mu\text{U/mL}) \times \text{glucose}$   
279  $(\text{mmol/L})) / 22.5$  and HOMA-β as:  $(20 \times \text{insulin } (\mu\text{U/mL})) / (\text{glucose } (\text{mmol/L}) - 3.5)$  [38]. HsCRP  
280 was measured by immunoturbidimetric method on the Pentra 400 Analyzer (HORIBA ABX,  
281 Montpellier, France). ASAT and ALAT were measured on the Pentra 400 Analyzer  
282 (HORIBA ABX, Montpellier, France). Lipid profile was analyzed on an auto-analyzer  
283 platform DIMENSION VISTA® (Siemens Healthcare Diagnostics Inc., USA). Total CHO  
284 and TG were measured by enzymatic methods. LDL-CHO and HDL-CHO were analyzed by  
285 the same method but with a disintegration of the other lipoproteins prior to the enzymatic  
286 reactions as included in the test scheme. ApoB concentration was measured by nephelometry.  
287 Specific antibodies form immunocomplexes with the ApoB proteins, which result in  
288 scattering light. Concentration of very low density lipoprotein (VLDL)-CHO was calculated  
289 from the values above. All lipids were measured in mg/dL but converted to mmol/L by  
290 multiplying with 0.0259 for total-CHO, LDL-CHO, HDL-CHO and VLDL-CHO and  
291 multiplying with 0.0113 for TG. ApoB concentration was multiplied with 0.01 for obtaining  
292 concentration in g/L.

293 Breath hydrogen: Fasting breath hydrogen was measured by a hand-held non-invasive  
294 Gastro<sup>+</sup>Gastrolyzer (Bedfont Scientific Ltd, Kent, England).

295 Energy Expenditure: At all CIDs resting energy expenditure (REE) was measured twice after  
296 a minimum of 30 minutes of resting by a ventilated hood system (Jaeger Oxycon PRO,  
297 ViasysHealthcare GmbH, Hoechberg, Germany). Each measurement lasted 25 minutes and  
298 was separated by a 10-minute rest period. The standardized dinner from the study kitchen at  
299 the Department provided prior to each CID contained 3 or 4 MJ, depending on the estimated  
300 energy requirements of the participant, and had a macronutrient distribution of 16 E% protein,  
301 31 E% fat and 53 E% carbohydrate. Participants were not allowed to consume alcohol and  
302 asked to limit physical activity 48 hours prior to the REE measurement.

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

318 minimum of 600 min wear time per day, were considered valid for analysis. Total tri-axial  
319 physical activity (counts per minutes (CPM)) was expressed as a vector magnitude of the total  
320 tri-axial counts from monitor wear-time, divided by measured monitor wear-time. Sedentary  
321 time, light physical activity, and Moderate-to-Vigorous physical activity (MVPA) were  
322 defined as  $\leq 99$  vertical CPM, 100–2019 vertical CPM, and  $\geq 2020$  vertical CPM, respectively  
323 [39].

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

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

343 *Statistical analyses*

344 The number of participants was calculated before study start according to previous studies  
345 with AXOS interventions [16,17]. Sample size calculation was based on the expected primary  
346 outcome “increase in amount of bifidobacteria” during the AXOS intervention period,  
347 however based on another analysis method than used in the current study. By including 30  
348 participants (24 completers), this study would have a statistical power of 80% to detect a  
349 difference of 0.35 log<sub>10</sub> cell/g dry weight feces (SD of 0.6), allowing for a 20% dropout at a  
350 0.05 significance level.

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

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

## 373 **Results**

### 374 *Dietary assessment and compliance*

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

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

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

### 393 *Anthropometry and physiology evaluation*

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

### 406 *Dietary intervention impact on gut microbiota*

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

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

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

440 reduced (Figure 3B). Abundance analysis of OTUs showed that 11 phylotypes increased  
441 following AXOS intake (Table 4). As expected, three OTUs were assigned to the genus  
442 *Bifidobacterium* and the remaining OTUs were identified as potential members of bacteria  
443 groups that include butyrate producers such as *Eubacterium rectale*, *Eubacterium hallii*,  
444 *Faecalibacterium prausnitzii*, *Dorea longicatena*, *Blautia luti* and *Blautia wexlerae* (all from  
445 the phylum Firmicutes and order Clostridiales). A NMR-based metabolomics analysis  
446 performed only in the AXOS-I plasma and fecal samples positively correlated the  
447 concentration of SCFAs to known butyrate producer bacterial species as described elsewhere  
448 [42]. We also detected decreased abundance of three OTUs that could not be properly  
449 identified at genus or species level, but appear to be phylotypes associated with the  
450 Ruminococcaceae and Erysipelotrichaceae families of the phylum Firmicutes (Table 4). We  
451 confirmed the bifidogenic effect observed in the AXOS-I subjects by absolute qPCR  
452 quantification (Supplementary Figure 3).

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

465 values  $\Delta_1$  (explaining the changes during the AXOS-I period) and  $\Delta_2$  (explaining the changes  
466 during washout period) in the group of subjects that started with the AXOS intervention  
467 retrieved similar results as those using LDA methods (Figure 3) and non-parametric  
468 correlation using multidimensional data (Supplementary Table 5). This analysis of delta  
469 values also expanded the potential set of microbial groups mostly affected by AXOS intake  
470 (Figure 4B). Thus, fast positive response (increased) to AXOS was observed in  
471 *Bifidobacterium* ( $p < 0.0001$ ) and *Blautia* ( $p < 0.0029$ ), and fast negative response (decreased)  
472 was observed in *Oscillibacter* ( $p < 0.0199$ ), *Alistipes* ( $p < 0.0068$ ), *Bacteroides* ( $p < 0.0020$ ),  
473 and *Parabacteroides* ( $p < 0.0060$ ) species. The proportion of these species also showed a  
474 rapid return to their baseline values at the end of washout period, further supporting that their  
475 shifts were the result of the dietary intervention. Conversely, we observed no significant  
476 changes when  $\Delta_1$  and  $\Delta_2$  values were compared in the group of subject starting with the  
477 PUFA intervention. However, we did observe some differences ( $p < 0.05$ ) when  $\Delta_{1+2}$  were  
478 compared to  $\Delta_3$ , which explain the changes induced by the intervention with AXOS (AXOS-  
479 II period) (Figure 4C). The results of this longitudinal analysis suggest that PUFA-I response  
480 could be slow and persist during the washout period and it was only slightly changed by  
481 exposure to the second intervention with AXOS.

#### 482 *Correlation between gut microbiota features and biochemical parameters*

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

490 on those OTUs that repeatedly showed correlations with markers related to similar functions,  
491 we could identify three OTUs that exhibited the largest number of positive correlations with  
492 biomarkers of lipid metabolism (VLDL, ApoB, total CHO, TG), liver function (ALAT), and  
493 glucose metabolism (insulin, HOMA-IR, HOMA- $\beta$ ) (Supplementary Table 6). Those OTUs  
494 were certainly identified as *Paraprevotella clara* (OTU93, 98% identity), *Eubacterium*  
495 *contortum* (OTU435, 100% identity), and a Lachnoclostridium member of the  
496 Lachnospiraceae family (OTU278, >95% identity). Other species showing positive  
497 correlations specifically with plasma lipid concentrations (except for HDL-CHO) included  
498 *Prevotellamassilia timonensis* (OTU138, 100% identity) and *Mitsuokella jalaludinii*  
499 (OTU263, 99% identity). Strikingly, the OTU116, whose identity could be not well solved by  
500 Blast or SINA-based comparisons, showed the largest amount of negative correlations with  
501 concentrations of lipid metabolic biomarkers such as ApoB ( $\rho$  -0.59;  $p < 0.005$ ), total CHO  
502 (-0.60;  $p < 0.005$ ) and LDL-CHO (-0.62;  $p < 0.005$ ).

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

508 We also found negative correlations between the abundances of OTUs and concentrations of  
509 glucose or insulin (essentially lower HOMA values) for *Dialister succinatiphilus* (OTU102,  
510 100% identity), *Turcibacter sanguinis* (OTU249, 99% identity) and *Alloprevotella* spp.  
511 (OTU281, >95% identity). Additionally, we detected positive correlations between HDL-  
512 CHO concentration and the abundances of *Eubacterium coprostanoligenes* group (OTU151, >  
513 95% identity) and a Clostridium from Family XIII *Ihubacter* spp. (OTU926, > 95% identity).

## 514 Discussion

515 The present study reports that AXOS intake exerts a bifidogenic effect confirming previous  
516 results obtained in human controlled interventions [15–18]. Moreover, we found that AXOS  
517 intake also increased the abundance of butyrate producing bacteria by the use of massive 16S  
518 sequencing methods, that enable the evaluation of the gut microbiota composition as a whole  
519 instead of quantifying a restricted number of taxonomy groups targeted by specific primers or  
520 probes (e.g. qPCR and hybridization approaches) [15–18]. For the first time, we have reported  
521 that AXOS intake reduces significantly the proportion of both Rikenellaceae and of  
522 Porphyromonadaceae species, which has been associated with inflammatory processes in  
523 patients with cirrhosis [43]. In particular, the OTUs analysis showed an increased abundance  
524 of the species *B. adolescentis* and *B. longum*, which have been shown to be able to hydrolyze  
525 AXOS in an *in vitro* study [44]. Additionally, members of the genera *Faecalibacterium*,  
526 *Ruminococcus*, *Dorea*, and *Eubacterium* increased during the AXOS intervention. An  
527 increase in bifidobacteria may increase acetate production, which in turn can be metabolized  
528 by butyrate producing bacteria, thus stimulating their growth [21]. This cross-feeding process  
529 could explain the increased abundance of bacteria belonging to the Clostridia class,  
530 particularly *E. rectale*, *F.prausnitzii* and *E. hallii* found in our study. Furthermore, the  
531 observed shift in the entire gut microbial community following AXOS intake was associated  
532 with increased abundances in *Roseburia*, *Coprococcus* and *Anaerostipes* species, which are  
533 butyrate producers as well [21]. The remaining OTUs (all belonging to phylum Firmicutes  
534 and order Clostridiales) that increased in abundance were *Blautia* and *Fusicantennibacter*  
535 genera, which have not previously been reported to change in response to AXOS intake [15–  
536 18,45].

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

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

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

565 enriched diets on the microbiota and found that few microbial changes occurred at genus level  
566 without effects on higher taxonomic levels after 30 days of dietary intervention. An increase  
567 of *Bifidobacterium*, *Oscillospira*, *Lachnospira*, *Coprococcus*, and *Faecalibacterium* was  
568 observed in a recent human cross-over intervention using PUFAs administered in drinks or  
569 capsules, in two different intervention periods during 8-week each and with a 12-week  
570 washout [53]. In our study, the lack of effect on the gut microbiota may be explained by  
571 several reasons that should be considered for future research. First, the increase in PUFA  
572 intake did not cause a reduction in SFA intake as we aimed for. Second, another possibility is  
573 that the change in PUFAs was too small to exert measurable effects on microbial composition  
574 in relatively short time. Via the fish oil supplement, we provided a dose of EPA and DHA  
575 (1.32 and 1.86 g/d, respectively) lower than that reported to have adverse events (5 g/d) [54]  
576 and lower than that showing changes in certain microbial genera (2 g/d EPA and 2 g/d DHA)  
577 [53]. However, it resulted in a much smaller difference in PUFA intake (1.84 percentage  
578 point) compared to the difference between monounsaturated fatty acid (MUFA) and PUFA  
579 interventions in the study by Pu *et al.* [52] where PUFA intake differed by 7.2-9.4 percentage  
580 points (6.7-9.1 E% vs 16.3 E%). Third, the duration of the intervention is a factor important to  
581 consider since interesting results have been obtained following longer intervention with an  
582 ample washout period to demonstrate reversible effects after the PUFA intake [53]. Although  
583 some extreme dietary changes can shift the gut microbiota composition within a few days, the  
584 response to some nutrients could be slower and depending on the concentration and overall  
585 dietary intake pattern. Pu *et al.* observed effects after a 30-day intervention but the changes in  
586 fat quality were larger, total fat intake was constant and all consumed meals were provided  
587 during the intervention periods [52]. According to our longitudinal analysis of delta values  
588 (changes between the assessments of the four different sampling points) a longer dietary  
589 intervention might have been needed to detect PUFA effects on gut microbiota. We also  
590 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

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

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

## 631 **Conclusions**

632 Intake of AXOS changed the gut microbiota composition. Higher abundance of bifidobacteria  
633 and butyrate producing bacteria were the main contributors to this change. Multiple  
634 correlations were established between specific OTUs and biochemical markers that could be  
635 beneficial for metabolic health (e.g. lower HOMA, higher HDL CHO) and should be further  
636 explored since limitations in the duration of this study could have precluded the detection of  
637 significant beneficial effects on these end-points. PUFA intake did not affect gut microbiota  
638 composition or any metabolic marker likely because it requires longer time than AXOS to  
639 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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## 653 **Statement of Authorship**

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

## 659 **Conflict of Interest Statement and Funding sources**

660 All the authors declare to have no conflict of interest.

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668 **Availability of data and material**

669 The raw fasta sequences generated from the 16S amplicon sequencing of fecal DNA are  
670 publicly available at the MG-RAST server [58] upon the project accession number  
671 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 ( $\Delta_1$  vs  $\Delta_2$ ). C) Bacterial genera with significantly different trajectory between the PUFA-I+washout periods and the AXOS-II intervention ( $\Delta_{1+2}$  vs  $\Delta_3$ ). 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  $\mu$ L 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

	<b>WBE (per 100g)</b>	<b>Powder (5g WBE)</b>	<b>Crackers (per piece)</b>	<b>Biscuits (per piece)</b>	<b>Total daily intake<sup>1</sup></b>
Energy (kJ)	812 <sup>2</sup>	41 <sup>2</sup>	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 <sup>3</sup>	3.6	1.0	1.0	11.2
WBE (g)	100	5	1.3	1.2	15.0
AXOS <sup>4</sup> (g)	69	3.5	0.9	0.8	10.4

<sup>1</sup>Total daily intake: 2 powder, 2 crackers and 2 biscuits

<sup>2</sup>Calculated value

<sup>3</sup>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.

<sup>4</sup>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  $\pm$ SD)

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

<sup>1</sup>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  $\pm$ SD)

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

<sup>1</sup>Based on LMM analysis and adjusting by covariates (recruiting BMI) and fixed effects (age, gender, and order of treatments).

<sup>2</sup>N=28,

<sup>3</sup>N=26

<sup>4</sup>N=27

ALAT, alanine aminotransferase; ApoB, apolipoprotein B; ASAT, aspartate aminotransferase; AXOS, arabinosyloxylan oligosaccharides; CHO, cholesterol; CPM, counts per minutes; Hb, hemoglobin; HC, hip circumference; HDL, high density lipoprotein; HOMA- $\beta$ , 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)

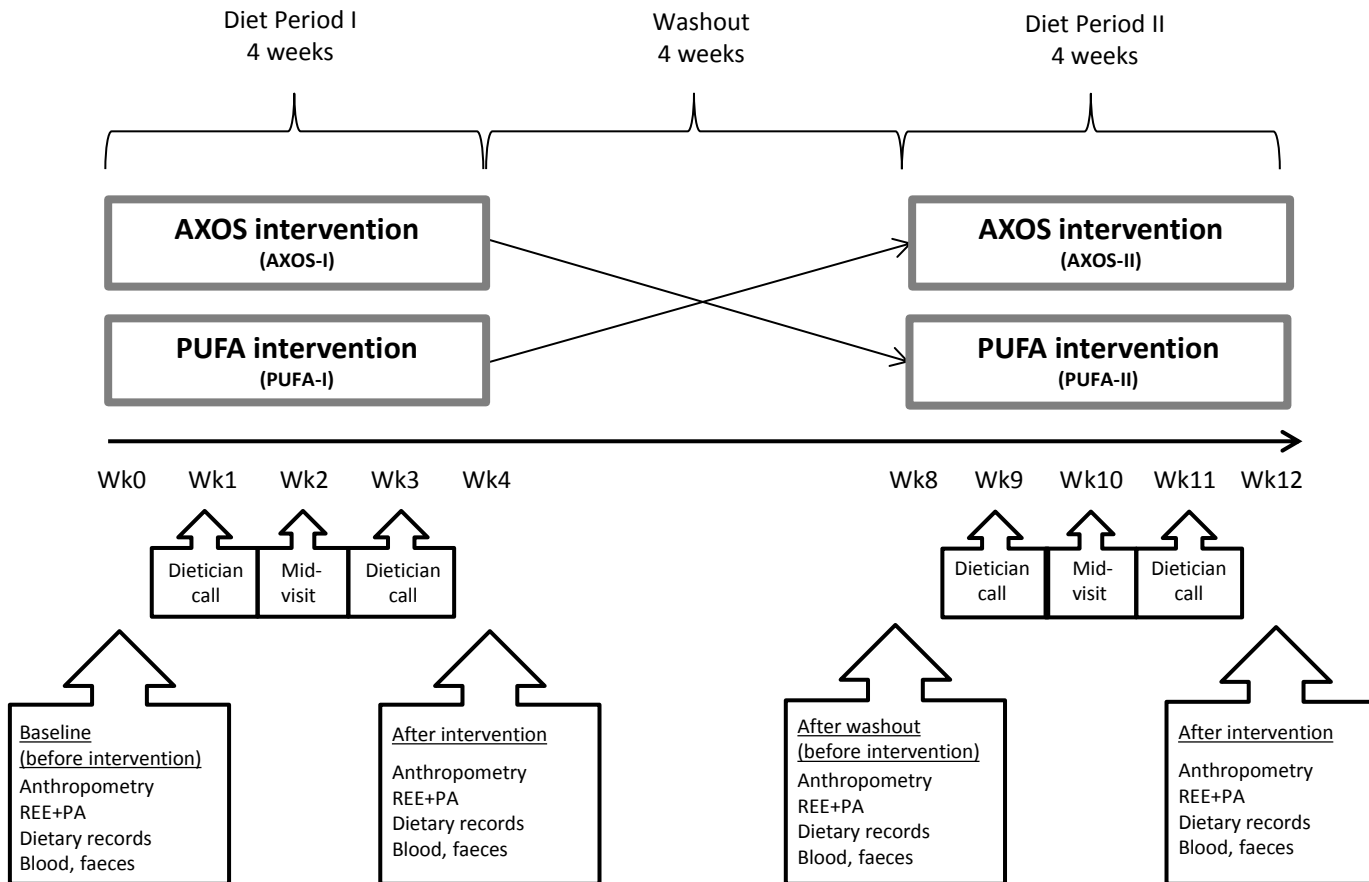
OTU	Blast 16S database <sup>1</sup>	id% <sup>2</sup>	LDA score	p-value <sup>3</sup>
<b>Increased abundance</b>				
4	<i>Eubacterium rectale</i>	100	4.34	0.029
5	<i>Faecalibacterium prausnitzii</i>	99	4.09	0.033
14	<i>Bifidobacterium faecale</i> , <i>Bifidobacterium stercoris</i> , <i>Bifidobacteriuma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

<sup>1</sup>Bacterial species/strain matching the OTU sequence according to best hit in a Blast-based search.

<sup>2</sup>Percentage of sequence identity supporting the taxonomic assignment of the respective OTU through the Blast-based search (alignment length percentage were 100 for all the OTUs presented in the table).

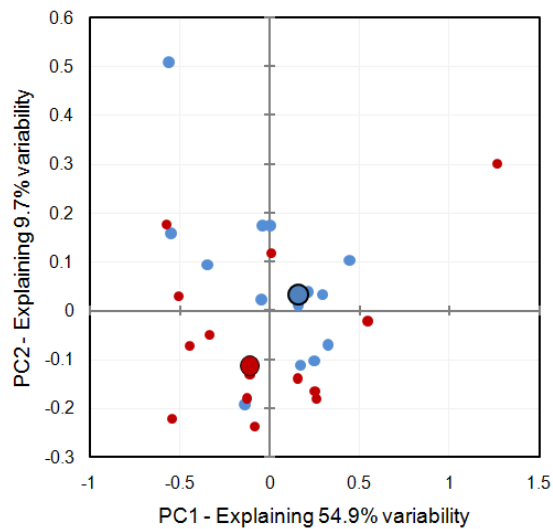
<sup>3</sup>Changes in OTU abundance in the microbiota of subjects before to after the first AXOS intervention was compared by non-parametric LDA (only OTUs with a LDA-score above 3 are shown in the table). Differences were considered statistically significant at p-values < 0.05.

AXOS, arabinoxylan oligosaccharides; LDA, Linear Discrimination Analysis; OTU, Operational Taxonomic Unit.

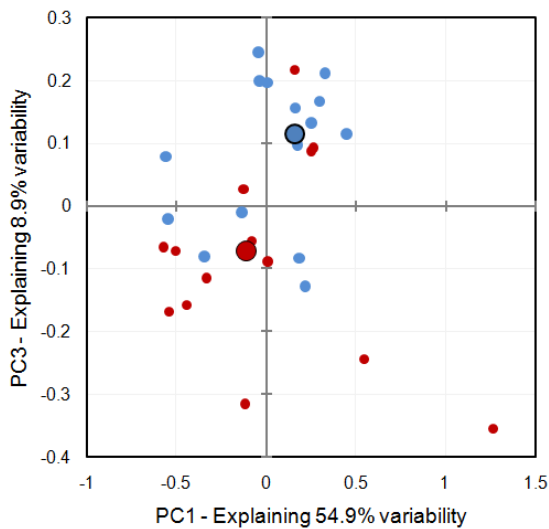


AXOS-I

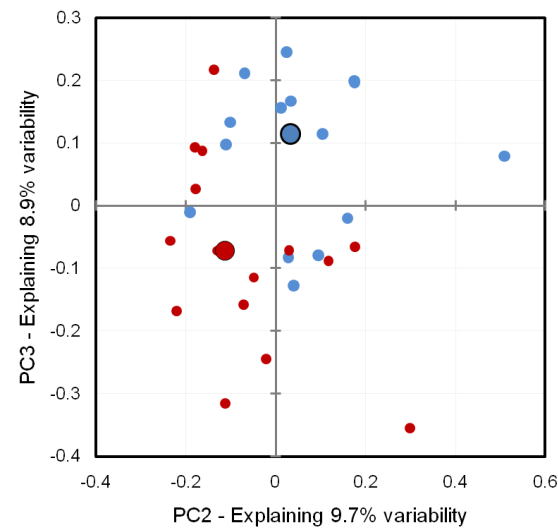
PC1 vs PC2



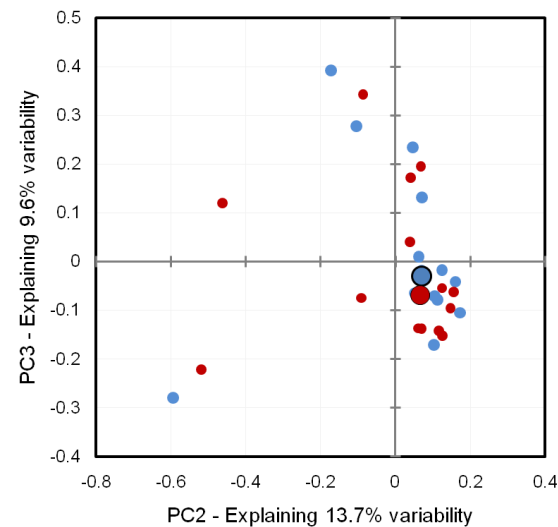
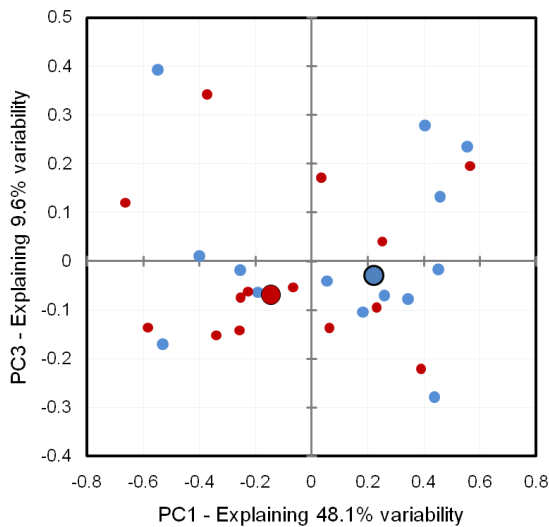
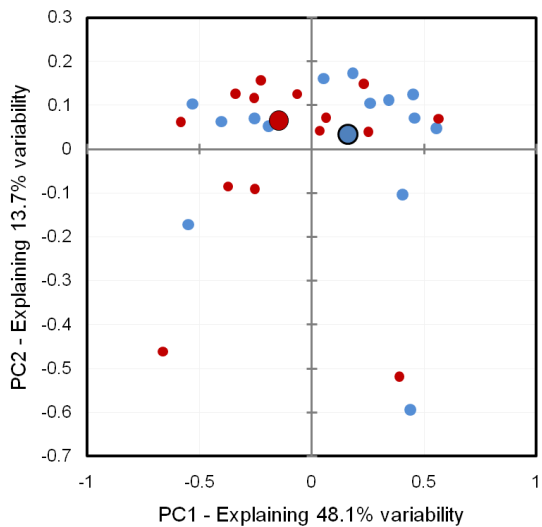
PC1 vs PC3



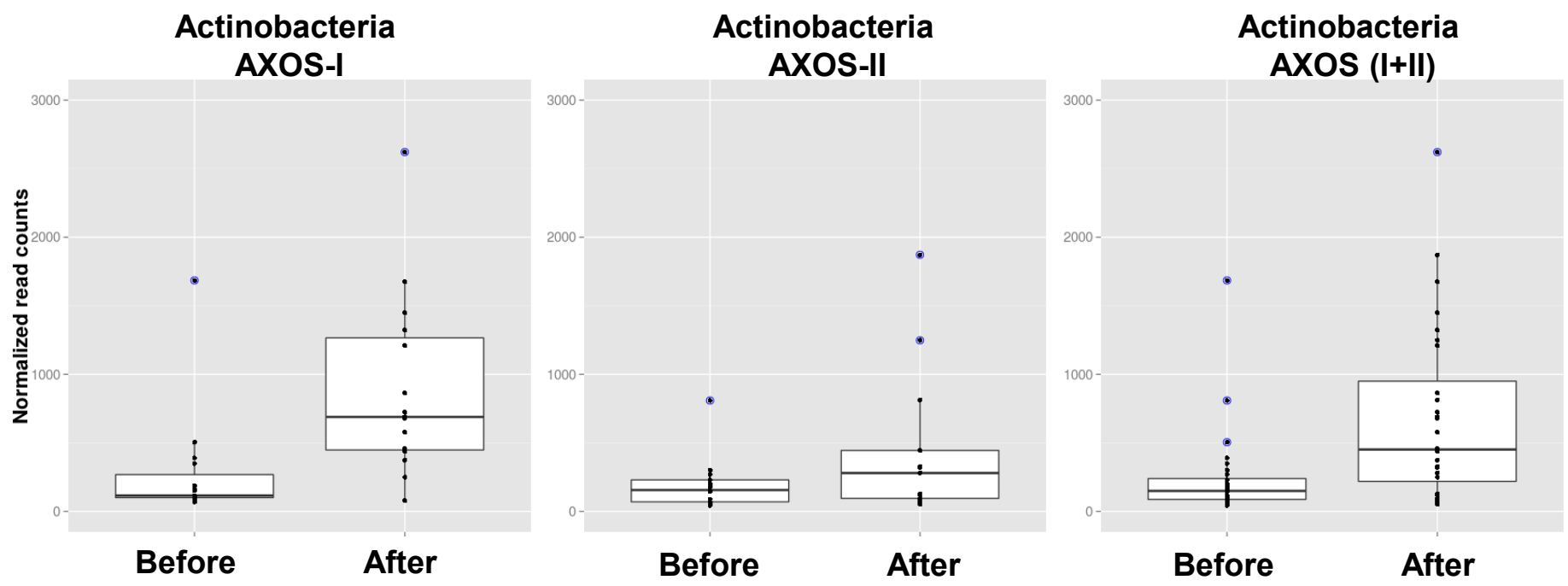
PC2 vs PC3



PUFA-I



● Before ● After

**A****B**