British Journal of Nutrition (2010), **103**, 703–713
doi:10.1017/S0007114509992248

Tolerance of arabinoxylan-oligosaccharides and their prebiotic activity in healthy subjects: a randomised, placebo-controlled cross-over study

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(Received 26 March 2009 – Revised 28 August 2009 – Accepted 1 September 2009 – First published online 10 December 2009)

The tolerance and prebiotic effect following oral intake by healthy human subjects of arabinoxylan-oligosaccharides (AXOS), produced by partial enzymic hydrolysis of the wheat fibre arabinoxlyan, were studied. A total of twenty healthy subjects participated in the present randomised, placebo-controlled cross-over study. They consumed 10 g AXOS or placebo per d each for 3 weeks with a 4-week wash-out period in between. Before and immediately after each intake period, blood samples were taken to measure haematological and clinical chemistry parameters and the subjects completed a questionnaire about gastrointestinal symptoms. Additionally, urine was collected over 48 h for analysis of *p*-cresol and phenol content by GC–MS, and faeces were collected over 72 h for analysis of microbiota using real-time PCR. Of the subjects, ten also performed a urine and faeces collection 2 weeks after the start of intake (during intervention). A limited number of tested blood parameters were influenced in a statistically significantly way by either AXOS or placebo intake, but these changes remained within the normal range. Blood lipids remained unchanged. AXOS had no statistically significant effect on the range of gastrointestinal symptoms, except for a mild increase in flatulence. Urinary *p*-cresol excretion, an indicator of protein fermentation, was significantly decreased after 2 weeks of AXOS intake. The levels of bifidobacteria were significantly increased after 2 and 3 weeks of AXOS intake as well as after 3 weeks of placebo. However, the effect of AXOS on bifidobacteria was more pronounced than that of placebo. In conclusion, AXOS are a well-tolerated prebiotic at the dose of 10 g/d. AXOS intake increases faecal bifidobacteria and reduces urinary *p*-cresol excretion.

Arabinoxylan-oligosaccharides: Prebiotics: Protein fermentation: Tolerance

Arabinoxylan-oligosaccharides (AXOS) are produced by partial enzymic hydrolysis of the cereal fibre arabinoxylan (AX) and have recently been proposed as a new prebiotic substrate[1]. To classify a food component as a prebiotic, it needs to fulfil three criteria[2].

The first criterion is resistance to gastric acid, gastrointestinal enzymes and absorption in the small bowel. *In vitro* studies have shown that AXOS resist a pH value of 2·0 and are less acid-sensitive than xylo-oligosaccharides and fructo-oligosaccharides (FOS)[3,4]. It has also been reported that xylobiose (xylosyl-β-1,4-xylose) is not degraded to xylose upon addition of either saliva, pepsin-containing artificial gastric juice, a preparation of pancreatic enzymes or intestinal mucosal homogenate[5] and similar data have been obtained for AXOS[6]. Furthermore, studies with ileostomy patients demonstrated that AX from cereals is not or only poorly digested in the ileum[7–9].

The second criterion is the fermentability of the candidate prebiotic by intestinal bacteria. An *in vitro* study with human faecal slurries demonstrated that AXOS are metabolised within 24 h, with production of acetic acid and lactic acid, and subsequent generation of propionic acid and butyric acid[10]. An increase in intestinal acetic acid concentration has also been observed in mice and rats after the intake of 2·5 and 5·0 % AXOS for 4 weeks[3]. Administration of 4·0 % AXOS with a low average degree of polymerisation (avDP) (≤5) to rats for 14 d significantly increased colonic acetic acid and butyric acid but not propionic acid production[11]. Taking into account a food consumption of 20 g/d and a mean body weight of 450 g, these doses of AXOS consumed correspond to an intake of approximately 1–2 g/d per kg body weight.

The third criterion to classify components as prebiotics is the selective stimulation of gastrointestinal microbial populations

Abbreviations: avDP, average degree of polymerisation; AX, arabinoxylan; AXOS, arabinoxylan-oligosaccharides; FOS, fructo-oligosaccharides; IQR, interquartile range.

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with health-promoting properties. Particularly, bifidobacteria, lactobacilli and some enterococci have been generally recognised as beneficial bacteria. In chickens, the levels of caecal bifidobacteria significantly increased after a 2-week intake period of 0·25 % AXOS with an avDP of 9 and an average degree of arabinose substitution of 0·34, whereas no effects were seen on caecal enterobacteriaceae and lactobacilli[12]. The levels of caecal bifidobacteria were also significantly and selectively increased in chickens following intake of 0·17 % AXOS with an avDP of 15 and an average degree of arabinose substitution of 0·26 for 2 weeks[13]. In rats, after a treatment period of 2 weeks, the bifidogenic effect of 4·0 % AXOS with an avDP of ≤ 5 was also demonstrated[11].

In healthy human subjects, the supplementation of 2·2 g AXOS with an avDP of 9 to the diet for 2 weeks significantly increased the levels of faecal bifidobacteria, suggesting the prebiotic potential of AXOS in humans[14]. The colonic ammonia metabolism of healthy subjects was beneficially modulated after the administration of a single dose of 2·2 and 4·9 g AXOS[15].

In the present study, tolerance of a higher dose of AXOS, 10·0 g/d, was investigated by measuring its influence on haematological and clinical chemistry parameters and gastrointestinal symptoms. The prebiotic potential of AXOS was determined through studying their influence on the composition of faecal microbiota, and on the colonic protein fermentation metabolism through measurement of urinary phenolic compounds.

Methods

Subjects

A total of twenty healthy subjects (fourteen women and six men; mean age 24 (SD 5) years, mean BMI 20·9 (SD 2·3) kg/m^2) participated in the study. Exclusion criteria were gastrointestinal complaints, the intake of antibiotics or medication influencing gut transit and/or the microbiota during the previous 3 months, abdominal surgery and pregnancy. During the study, the subjects were allowed to eat their usual diets but were asked to have a regular eating pattern (three meals per d). The intake of food substances containing probiotics and/or prebiotics was forbidden, whereas the consumption of food containing natural small amounts of inulin and/or FOS such as chicory, artichoke, garlic, onions, green banana, leek and soyabeans was limited to once per week. At the time of inclusion, all subjects were informed about pro- and prebiotics and the food products containing pro- and/or prebiotics. They were asked to read food labels carefully to check whether pro- and/or prebiotics had been added. The present study was conducted according to the guidelines laid down in the Declaration of Helsinki and all procedures involving human subjects were approved by the Ethics Committee (Institutional Review Board) of the Katholieke Universiteit Leuven. Written informed consent was obtained from all subjects.

Substrates

The AXOS preparation used in the present study had an avDP of 6 and an average degree of arabinose substitution of 0·26 and was obtained from commercial wheat bran treated with α-amylase to hydrolyse starch. Subsequently, the bran residue was washed and re-suspended in water for treatment with a *Bacillus subtilis* endoxylanase preparation (Grindamyl H640; Danisco, Copenhagen, Denmark) and with an *Aspergillus oryzae* endoxylanase preparation (Novozymes, Bagsvaerd, Denmark). After clarification using decanters and disc centrifuges and after heat inactivation of the enzyme, the supernatant fraction was filtered and purified with strong base anion exchange and strong cation exchange resins. Finally, the solution was spray-dried. Table 1 presents the characteristics of the AXOS-preparation. We administered 2 × 6·95 g of the crude AXOS product, corresponding to 2 × 5·0 g per d pure AXOS after correction, taking into account the content in polymeric xylose and arabinose (70·6 %).

Maltodextrin (Maldex 150; Syral Belgium, Aalst, Belgium) was used as the placebo. The subjects daily ingested 2 × 6·95 g maltodextrin.

Both substrates were dissolved in an orange juice drink (1 litre consisted of 41·7 g substrate (i.e. AXOS or placebo), 125·0 g water and 833·0 g orange juice). During each 3-week intake period, the subjects drank 333 ml of the prepared orange juice drink per d, which was equally divided between morning and evening servings.

Study design

Figure 1 presents a schematic overview of the present randomised, placebo-controlled cross-over study. All subjects collected dedicated samples before the start of each intake period (i.e baseline and wash-out) and on the first day after each 3-week intake period. Of the twenty participants, ten were subjected to additional sample collection on day 15 of the intake period. Here, substrate ingestion continued during the 3 d sample collection, in contrast to the sample collection performed after the 3-week intake period. The latter test was performed to investigate whether changes in the composition of the faecal microbiota were linked to modulations in protein fermentation.

In the morning, after an overnight fast, the subjects came to the laboratory and collected a basal urine sample. For the tests

Table 1. Characterisation of the arabinoxylan-oligosaccharide (AXOS) preparation

	AXOS-6-0·26
Moisture (%)	2·7
Ash (% DM)	0·22
Protein content (% DM)	3·5
Monosaccharide composition after hydrolysis (% DM)	
L-Arabinose	16·6
D-Xylose	63·6
D-Mannose	0·2
D-Galactose	0·6
D-Glucose	11·7
AXOS content (% DM)*	70·6
Average degree of arabinose substitution†	0·26
Average degree of polymerisation‡	6

* AXOS content = 0·88 × (% L-arabinose + % D-xylose).
† Average degree of arabinose substitution = % L-arabinose:% D-xylose ratio.
‡ Average degree of polymerisation = (% L-arabinose + % D-xylose)/% reducing end xylose.

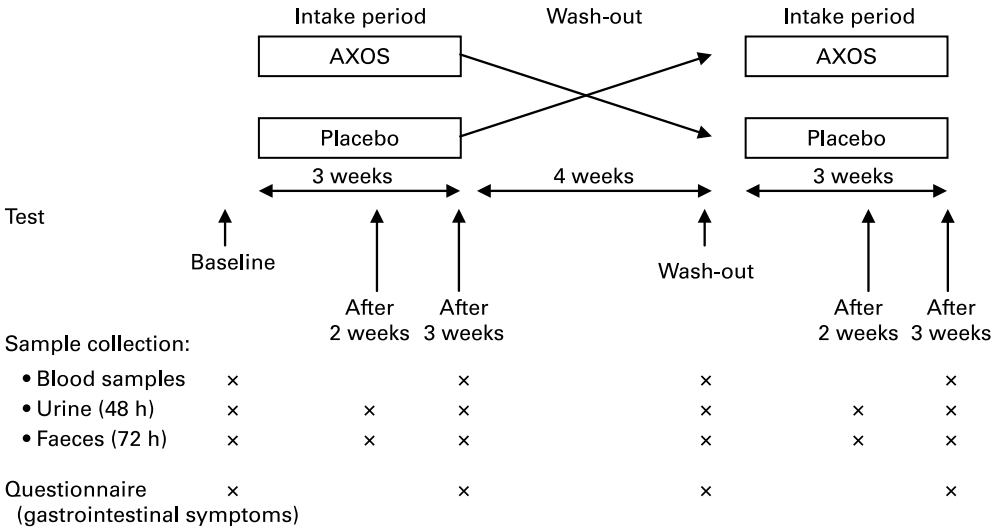

Fig. 1. Schematic presentation of the study design. Arabinoxylan-oligosaccharides (AXOS) and placebo were administered at a dose of 10·0 g/d to twenty healthy subjects. All subjects performed a test before (baseline and wash-out) and after each 3-week intake period. Of the twenty subjects, ten also performed a urine and faeces collection after 2 weeks of intake (during intervention). The dedicated samples were collected at different time points. The subjects also completed a questionnaire about the gastrointestinal symptoms before and after each 3 weeks of intake.

performed before and after each 3-week intake period, blood samples were taken and the subjects completed a questionnaire about possible gastrointestinal symptoms. Thereafter, the subjects took a capsule containing 185 kBq [^{3}H]polyethylene glycol (NEN Life Science Products, Boston, MA, USA) to measure total gastrointestinal transit. From then, urine and faeces were collected for 48 and 72 h, respectively.

Sample collection

Blood samples. Venous blood samples were collected in BD Vacutainer® Heparin or K_2EDTA tubes to obtain plasma and BD Vacutainer® SST tubes to obtain serum (Becton Dickinson Vacutainer Systems, Plymouth, Devon, UK).

Urine. Urine was collected for 48 h in dedicated receptacles to which 500 mg neomycine was added to prevent bacterial growth. The volume of all urine fractions was measured and samples were stored at −20°C until further analysis.

Faecal samples. Immediately after receiving faecal samples (72 h collection stored by subjects at +4°C), a fraction of approximately 5·0 g of fresh faeces was stored at −20°C for microbiological analysis. The remaining part of the stool collection was weighed and stored at −20°C. For further analysis, all stools from each 3 d collection were combined, diluted with water and homogenised. A fraction of this suspension was freeze-dried and weighed again. Weighed samples from the dried material were used for analysis of ^{3}H.

Analytical procedures

Urinary creatinine. The creatinine content of each urine sample was measured using a standard laboratory protocol. The ratio of the observed to expected creatinine excretion was calculated using the formula ((24 h creatinine (mmol) × 113 g/mol)/(24 mg/kg × body weight (kg))) for males and ((24 h creatinine (mmol) × 113 g/mol)/(21 mg/kg × body weight (kg))) for females[16]. If the ratio was lower than 0·7, the urine collection was considered as incomplete[16,17].

Blood samples. Haematological parameters, clinical chemistry parameters (including liver enzymes and parameters of kidney function) and blood lipids were analysed using standard laboratory techniques to assess the safety of AXOS administration. In addition, the effect of AXOS administration on minerals and vitamins was assessed.

Recording of gastrointestinal symptoms. Gastrointestinal symptoms were monitored before and after each intake period. The subjects were asked to grade the following seven symptoms: gastrointestinal discomfort which diminishes after defecation, gastrointestinal pain which diminishes after defecation, gastrointestinal discomfort concomitant with constipation or diarrhoea, gastrointestinal pain concomitant with constipation or diarrhoea, flatulence, cramps and bloating. The severity of the symptoms was graded on a four-step scale ranging from no (0), mild (1), moderate (2) to severe (3). A score for each symptom was obtained by calculating mean and standard deviation. The total score was the sum of the seven individual scores.

^{3}H recovery in faecal samples. The [^{3}H]polyethylene glycol content in stool samples was measured by liquid-scintillation counting (Packard Tri-carb 2100 TR Liquid Scintillation Spectrometer, model 3375; Packard Instruments, Downers Grove, IL, USA) after oxidation to ^{3}H-labelled water (Packard Sample Oxidizer, model 307; Packard Instruments). The ^{3}H recovery was expressed as a percentage of the administered dose recovered over 72 h. The intra- and inter-assay CV (n 3) for ^{3}H measurement were 1·36 and 3·99 %, respectively.

Urinary p-cresol and phenol content. Total *p*-cresol and phenol content in urinary samples was assessed by GC–MS[18]. Briefly, the pH of 950 µl urine was adjusted to 1·0 by the addition of 50 µl concentrated H_2SO_4 (Merck KaA, Darmstadt, Germany). After the addition of 50 µl 2,6-dimethylphenol solution (20 mg/100 ml; Sigma-Aldrich, Steinheim, Germany) as internal standard, the solution was heated for 30 min at 90°C to deproteinise and hydrolyse

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Table 2. Sequence and concentrations of primers and probes used in the quantitative real-time PCR assays

Target organisms	Reference	Primers and probes	Sequence (5′–3′)	Fluorophores	Final concentration (nM)
Bifidobacterium species	19	Forward primer	GCGTGCTTAACACATGCAAGTC		450
		Reverse primer	CACCCGTTTCCAGGAGCTATT		450
		Probe	TCACGCATTACTCACCCGTTCGCC	VIC/MGB	100
Bifidobacterium adolescentis	20, 21	Forward primer	ATAGTGGACGCGAGCAAGAGA		300
		Reverse primer	TTGAAGAGTTTGGCGAAATCG		300
Total bacteria	22	Forward primer	TGGAGCATGTGGTTTAATTCGA		500
		Reverse primer	TGCGGGACTTAACCCAACA		500
		Probe	CACGAGCTGACGACARCCATGCA	FAM/TAMRA	150
Lactobacillus species	23	Forward primer	TGGAAACAGRTGCTAATACCG		300
		Reverse primer	GTCCATTGTGGAAGATTCCC		300
Roseburia–Eubacterium rectale species	24	Forward primer	GCGGTRCGGCAAGTCTGA		600
		Reverse primer	CCTCCGACACTCTAGTMCGAC		600
Enterobacteria	25	Forward primer	GTTAATACCTTTGCTCATTGA		600
		Reverse primer	ACCAGGGTATCTAATCCTGTT		600

VIC/MGB, reporter applied systems dye/quencher minor groove binder; FAM/TAMRA, reporter 6-carboxy fluorescein/quencher 6-carboxytetramethylrhodamine.

conjugated *p*-cresol and phenol. After cooling down, *p*-cresol and phenol were extracted into 1·0 ml ethyl acetate (Sigma-Aldrich). The water content of the ethyl acetate layer was removed using anhydrous sodium sulfate and finally 0·5 µl was analysed on a GC–MS (Trace GC–MS; Thermofinnigan, San José, CA, USA). The analytical column was a 30 m × 0·25 mm internal diameter, 0·50 µm film thickness Rxi™-5-MS (Restek, Bellefonte, PA, USA). The carrier gas used was He (GC grade) with a constant flow of 1·3 ml/min. The oven temperature was programmed from 55°C (isothermal for 5 min), and increased by 10°C/min to 160°C and by 20°C/min to 280°C. Mass spectrometric detection was performed in electron impact full scan mode from *m/z* 59 to *m/z* 590 at two scans/s. Results were expressed as mg *p*-cresol/24 h and mg phenol/24 h. The intra- and inter-assay inter-day CV (*n* 6) for urinary phenolics were 1·16 and 8·97 %, respectively.

Microbiological analysis. Faecal samples (first sample of each 72 h collection) were analysed to evaluate the influence of AXOS and placebo on the composition of the microbiota. Bacterial DNA from faecal samples was obtained by extraction of 180–220 mg of faeces using a commercially available kit (QIAamp DNA Stool Mini Kit; Qiagen Benelux, Venlo, The Netherlands). DNA of faecal samples was extracted within 4 months after the time of collection. The standard protocol was slightly modified as follows. The temperature of the initial step was increased from 75°C to 95°C and the isolated DNA was eluted from the spin column with 2 × 100 µl buffer instead of 1 × 200 µl to increase the concentration of DNA. The amount of DNA was quantified by measurement of UV absorption (NanoDrop spectrophotometer; NanoDrop Technologies, Wilmington, DE, USA). Finally, DNA was stored at −80°C until PCR analysis.

Real-time PCR was used to quantify total amounts of *Bifidobacterium* species, *Bifidobacterium adolescentis*, total bacteria, *Lactobacillus* species, *Roseburia–Eubacterium rectale* species, and enterobacteria. Total amounts of *Bifidobacterium* species and total bacteria were analysed with real-time PCR TaqMan, whereas *Bifidobacterium adolescentis*, *Lactobacillus* species, *Roseburia–Eubacterium rectale* species and enterobacteria were analysed with real-time PCR SYBR Green technology[19–25]. All real-time PCR amplification reactions were performed in duplicate in 25 µl reaction mixtures. DNA of samples was diluted 100 × for analysis of *Bifidobacterium* species, *Bifidobacterium adolescentis*, total bacteria and *Lactobacillus* species, and 10 × for analysis of *Roseburia–Eubacterium rectale* species and enterobacteria to avoid PCR inhibition. TaqMan PCR-mixtures consisted of 1 × TaqMan qPCR Mastermix (Eurogentec, Seraing, Belgium), appropriate concentrations of primers and probes (Table 2), 2·5 µl of 2·0 % bovine serum albumin and 2·5 µl of template DNA. For *Bifidobacterium adolescentis* and *Lactobacillus* species, PCR reactions contained 1 × SYBR Green PCR Master Mix (Applied Biosystems, Warrington, Cheshire, UK), appropriate concentrations of primers (Table 2) and 5 µl of template DNA. For *Roseburia–Eubacterium rectale* species and enterobacteria, PCR reactions contained 1 × SYBR Green PCR Master Mix (Applied Biosystems), appropriate concentrations of primers (Table 2) and 2·5 µl of template DNA. All reactions were performed and analysed in ABI PRISM Optical ninety-six-well reaction plates on an ABI PRISM 7000 Sequence Detection System using 7000 SDS 1.2.3 software (Applied Biosystems). The reaction conditions were 2 min at 50°C and 10 min at 95°C, followed by forty cycles of 15 s at 95°C and 1 min at 60°C. Each real-time PCR run included a negative DNA template control (double-distilled water) and a triplicate serial 10-fold dilution of a positive control to generate standard curves. DNA of *Bifidobacterium animalis lactis* BB12 (DN-173010), *Escherichia coli* (LMG 2092), *Lactobacillus plantarum* (LMG 9211), *Bifidobacterium adolescentis* and *Roseburia intestinalis* (DSM 14 610) ranging from 1 to 1×10^6 genome copies per reaction well were used as positive controls for the analysis of *Bifidobacterium* species, total bacteria, enterobacteria, *Lactobacillus* species, *Bifidobacterium adolescentis* and *Roseburia–Eubacterium rectale* species, respectively. All standard curves had an R^2 exceeding 0·95. PCR amplification efficiency (E) was calculated using the slope of the standard curve ($E = 10^{-1/\text{slope}} - 1$) and ranged between 96 and 100 % for all runs.

Statistics

All results were expressed as median and interquartile range (IQR) except for the results of gastrointestinal scores which

Table 3. Influence of arabinoxylan-oligosaccharides (AXOS) and placebo on blood parameters (*n* 20)

(Medians and interquartile ranges (IQR))

	Before AXOS		After 3 weeks AXOS			Before placebo		After 3 weeks placebo		
	Median	IQR	Median	IQR	*P**	Median	IQR	Median	IQR	*P**
Haematological parameters										
Hb (g/l)	133·5	128·3–147·5	137·0	130·0–146·0	NS	137·0	130·0–142·0	138·5	128·3–146·5	NS
Packed cell volume (%)	39·7	39–43	41·6	40–44	0·033	41·2	40–45	41·0	40–43	NS
Erythrocyte count (10^{12}/l)	4·51	4·30–4·92	4·67	4·44–4·89	0·048	4·67	4·33–4·83	4·74	4·38–4·95	NS
MCV (fl)	88·95	86·93–92·23	89·40	86·83–92·05	NS	88·20	85·90–90·20	87·95	85·68–91·85	NS
MCH (pg)	29·70	29·08–31·03	29·85	28·88–30·45	NS	29·80	29·30–30·50	29·65	28·55–30·90	NS
MCHC (g/l)	336·0	325·5–340·0	330·0	326·0–341·8	NS	338·0	332·0–344·0	335·5	326·5–340·8	0·008
RDW (%)	12·80	12·45–13·38	12·65	12·30–13·28	NS	12·80	12·50–13·70	12·70	12·30–13·45	NS
PLT (10^9/l)	258	226–279	268	251–298	NS	265	189–277	274	244–306	0·012
MPV (fl)	10·30	9·93–10·88	10·55	9·83–10·98	NS	10·40	9·80–10·90	10·50	9·93–11·18	NS
Leucocyte count (10^9/l)	5·85	4·93–7·20	6·10	5·63–7·20	NS	6·10	5·00–7·10	6·15	5·35–8·18	NS
Clinical chemistry parameters										
Urea (mg/l)	260·0	210·0–345·0	275·0	252·5–300·0	NS	265·0	202·5–337·5	255·0	220·0–277·5	NS
Creatinine (mg/l)	7·8	7·1–8·8	7·9	7·3–9·0	NS	7·9	7·3–9·7	8·5	6·9–9·5	NS
Alkaline phosphatase (U/l)	132·5	108·5–160·0	129·0	107·5–151·0	NS	134·5	107·8–163·0	140·5	106·5–157·0	NS
AST (U/l)	21·0	18·3–23·8	20·5	17·3–23·0	NS	20·5	17·5–21·8	19·5	17·3–22·0	NS
ALT (U/l)	16·0	13·0–19·8	16·5	13·3–20·8	NS	16·5	13·3–20·8	15·5	12·5–19·0	NS
GGT (U/l)	12·5	10·0–16·0	13·5	10·0–15·0	NS	13·5	10·0–16·0	14·0	9·3–15·8	NS
Bilirubin (mg/l)	4·7	3·6–7·6	5·5	4·0–7·1	NS	4·9	4·0–6·8	4·7	3·7–6·5	NS
CK (U/l)	104·5	78·5–147·8	90·5	73·0–181·0	NS	97·5	79·0–128·5	82·5	75·0–113·0	NS
LDH (U/l)	325·0	307·0–357·0	287·5	268·5–328·8	0·014	291·0	274·0–311·0	259·0	243·5–311·3	0·017
Total protein (g/l)	75·0	72·0–79·8	77·0	75·3–79·0	0·030	76·0	74·0–78·8	76·5	74·0–78·8	NS
Albumin (g/l)	44·9	42·8–47·5	46·4	45·1–48·1	NS	45·5	44·2–49·2	44·4	43·4–47·4	0·019
K (mmol/l)	3·98	3·80–4·24	3·75	3·51–3·96	0·013	3·78	3·66–3·98	3·86	3·65–4·01	NS
Na (mmol/l)	139·6	138·9–141·1	140·1	138·7–141·4	NS	140·4	138·5–141·4	139·9	138·2–141·6	NS
Ca (mg/l)	91·0	89·3–94·0	93·0	90·6–95·3	NS	92·6	89·7–93·9	92·6	90·7–95·5	NS
Mg (mg/l)	20·7	19·9–21·4	20·7	20·3–22·2	NS	20·0	19·4–21·0	20·6	20·0–21·1	NS
Fe (μg/l)	845·0	582·5–1220·0	930·0	782·5–1230·0	NS	805·0	612·5–1123·0	935·0	702·5–1155·0	NS
Cl (mmol/l)	105·3	103·9–106·1	105·2	103·0–106·1	NS	105·3	104·3–106·6	105·9	103·8–107·3	NS
Bicarbonate (mmol/l)	24·25	23·48–25·33	24·70	23·50–27·25	NS	23·55	22·40–25·78	23·95	22·65–25·28	NS
Anion gap (mEq/l)	14·35	13·40–15·35	13·20	12·30–14·98	NS	14·40	13·05–15·40	13·90	12·78–15·63	NS
Vitamin A (μg/l)	607·0	525·3–746·0	645·5	607·3–752·3	NS	605·0	570·8–703·0	700·0	582·0–732·5	NS
Folate (μg/l)	8·95	6·13–9·90	8·90	7·08–10·58	NS	9·60	7·50–11·98	9·55	7·76–10·93	NS
Glucose (mg/l)	785	743–840	815	763–888	NS	815	763–888	845	803–900	NS

MCV, mean corpuscular volume; MCH, mean content of Hb; MCHC, mean corpuscular Hb concentration; RDW, random distribution of erythrocyte weight; PLT, platelet count; MPV, mean platelet volume; AST, aspartate aminotransferase; ALT, alanine aminotransferase; GGT, γ-glutamyl transpeptidase; CK, creatinine kinase; LDH, lactate dehydrogenase.

* Wilcoxon test.

Table 4. Scores of gastrointestinal symptoms (graded from 0 to 3) before and after the intake period of arabinoxylan-oligosaccharides (AXOS) and placebo (*n* 20)

(Mean values and standard deviations)

	Before AXOS		After 3 weeks AXOS			Before placebo		After 3 weeks placebo		
	Mean	SD	Mean	SD	*P**	Mean	SD	Mean	SD	*P**
Gastrointestinal discomfort which diminishes after defecation	0·25	0·55	0·70	0·73	NS	0·00	0·00	0·00	0·00	NS
Gastrointestinal pain which diminishes after defecation	0·15	0·49	0·15	0·37	NS	0·15	0·37	0·15	0·49	NS
Gastrointestinal discomfort concomitant with constipation or diarrhoea	0·30	0·57	0·35	0·59	NS	0·20	0·41	0·30	0·57	NS
Gastrointestinal pain concomitant with constipation or diarrhoea	0·20	0·52	0·15	0·37	NS	0·00	0·00	0·20	0·70	NS
Flatulence	0·35	0·49	1·10	0·79	0·003	0·40	0·67	0·40	0·60	NS
Cramps	0·30	0·57	0·50	0·51	NS	0·25	0·55	0·20	0·52	NS
Bloating	0·30	0·47	0·70	0·80	NS	0·15	0·37	0·35	0·49	NS
Total score	1·85	0·07	3·65	0·34	NS	1·40	0·12	1·90	0·09	NS

* Wilcoxon test.

were expressed as mean values and standard deviations. The use of mean values and standard deviations was more useful for these scores since the medians of the different periods were mostly equal (0 or 1). The means are more appropriate to show small differences.

SPSS software (SPSS 15.0 for Windows, SPSS 1989–2003; SPSS Inc., Chicago, IL, USA) was used for statistical analysis. Because of the limited number of subjects, non-parametric tests (Wilcoxon signed ranks test) were used to compare values before and after substrate intake or to compare the effect of AXOS with placebo. Correlation analyses were performed calculating the Spearmen correlation coefficient (*r*). *P* values of or lower than 0·05 were considered statistically significant.

Results

Haematological and clinical chemistry parameters

Table 3 shows the effects of AXOS and placebo on blood parameters, including haematological and clinical chemistry parameters. Most of these parameters were not affected by either treatment. Small, yet significant, changes in the levels of packed cell volume, erythrocyte count, lactate dehydrogenase, total protein and K were found after intake of AXOS. After placebo intake, the levels of mean corpuscular Hb concentration, platelet count, lactate dehydrogenase and albumin were significantly different from the levels before the intake of placebo. All values remained within the normal ranges of the respective parameters.

Blood lipids

At baseline, total cholesterol, TAG, HDL-cholesterol and LDL-cholesterol were 1670 (IQR 1530–1840), 540 (IQR 470–790), 660 (IQR 550–800) and 860 (IQR 700–1020) mg/l, respectively. These parameters did not change significantly after the intake of AXOS or placebo (data not shown).

Gastrointestinal symptoms

Table 4 presents the influence of AXOS and placebo on gastrointestinal symptoms. After intake of AXOS, the total score of gastrointestinal symptoms tended to be increased, although not in a statistically significant way. When compared with the score before AXOS intake, only the flatulence score was significantly increased ($P=0·003$). However, the average flatulence score as well as other symptom scores after AXOS intake were graded as mild. Administration of placebo did not induce statistically significant changes in any of the gastrointestinal symptoms and total score.

Faecal parameters

At baseline, faecal wet weight and % DM were 66·7 (IQR 42·6–102·3) g/d and 25·4 (IQR 24·3–28·1) %, respectively. At baseline, ^{3}H recovery was 58·4 (IQR 44·5–68·3) %. All these faecal parameters remained unchanged after either AXOS or placebo intake (data not shown).

Urinary p*-cresol and phenol excretion*

The completeness of each urine collection was estimated from the calculation of the observed to expected creatinine ratio as proposed by Knuiman *et al.* [16]. Out of 100 urine collections, eleven were classified as incomplete as their ratio was lower than 0·7, and were not taken into account for statistical analysis of *p*-cresol and phenol excretion.

In general, *p*-cresol levels were five to ten times higher than the phenol levels (Table 5). *p*-Cresol content was significantly decreased after intake of AXOS for 2 weeks ($P=0·011$). Neither AXOS nor placebo intake influenced urinary phenol excretion.

Faecal microbiota

Table 6 shows the effects of AXOS and placebo on the levels of bifidobacteria, *Bifidobacterium adolescentis*, lactobacilli and total bacteria. The bifidobacteria levels were significantly increased after 2 and 3 weeks' intake of AXOS relative to bifidobacteria levels before AXOS intake ($P=0·028$ and $P=0·012$, respectively). With placebo, there was also a significant increase in bifidobacteria after 3 weeks ($P=0·008$), but not after 2 weeks. The magnitude of the median increase after 2 weeks' AXOS intake was significantly larger than after placebo intake ($P=0·025$). After 3 weeks, the

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Table 5. Influence of arabinoxylan-oligosaccharides (AXOS) or placebo on urinary *p*-cresol and phenol excretion (Medians and interquartile ranges (IQR))

	Before AXOS		After 2 weeks AXOS		After 3 weeks AXOS		*P**	Before placebo		After 2 weeks placebo		After 3 weeks placebo		*P**
	Median	IQR	Median	IQR	Median	IQR		Median	IQR	Median	IQR	Median	IQR	
p-Cresol (mg/24 h)														
n 10	32·07	17·56–67·55	14·89	11·82–29·03			0·011	32·88	24·94–53·39	27·33	21·26–48·97			NS
n 20	32·07	21·00–47·18			27·18	16·94–46·69	NS	28·22	20·55–47·01			25·24	15·98–44·00	NS
Phenol (mg/24 h)														
n 10	2·88	2·04–6·65	3·17	2·41–5·02			NS	4·68	2·41–5·82	3·67	3·00–14·56			NS
n 20	5·15	2·88–7·19			3·72	2·73–7·06	NS	4·68	2·61–6·04			4·14	2·82–10·24	NS

*Wilcoxon test.

effect of AXOS (+0·81 log bifidobacteria/g dry faecal weight) was not significantly more pronounced than that of the placebo (+0·41 log bifidobacteria/g dry faecal weight).

A significant inverse correlation was observed between the levels of bifidobacteria before AXOS intake and the change (crude increase) in bifidobacteria levels after the 3-week intake period (Fig. 2(a)). No significant correlation was seen between bifidobacteria levels before placebo and the effect of placebo intake on the numbers of bifidobacteria (Fig. 2(b)).

In one female subject, *Bifidobacterium adolescentis* could not be detected in any of the measurements, whereas three subjects had relatively low levels of *Bifidobacterium adolescentis* at all time points. After 3 weeks of AXOS intake, but not after 2 weeks, the levels of *Bifidobacterium adolescentis* significantly increased ($P=0·013$).

No changes in the levels of lactobacilli were seen after 2 weeks of AXOS intake, whereas a borderline significant decrease ($P=0·048$) was found after 3 weeks. With placebo, lactobacilli levels did not differ.

The levels of total bacteria, *Roseburia–Eubacterium rectale* and enterobacteria remained unchanged after the administration of either AXOS or placebo.

Discussion

AX and AXOS are intrinsic components of the human diet and, as such, already ingested by the consumption of cereal-based food products such as bread and beer[26]. Bread and beer are the main dietary sources of AXOS. During malting and beer brewing, cereal AX are exposed to either or both endogenous and exogenous xylanase activity, thus resulting in the generation of AXOS. Courtin *et al.* (2009) found that beers contain on average about 1·0 g AXOS per litre, with an avDP varying between 19 and 40, depending on the type of beer[26]. Based on the data on beer consumption, as provided in the Concise European Food Consumption database[27], we calculated that the mean consumption of AXOS through beer in countries such as Czechia or Iceland with relatively high beer consumption reaches up to 0·85 g/consumer per d, with 95th percentile consumption up to 3·0 g/consumer per d. In breads AXOS can be generated during kneading, leavening and/or baking through the action of endogenous and exogenous xylanases[28,29]. Van Haesendonck *et al.* determined the avDP and average degree of arabinose substitution for the water-extractable AX contained in different types of bread[30]. The avDP varied from 100 to 300 according to the type of bread. Rye bread within the German and East European tradition was found to contain 2·64 g AXOS per 100 g DM, while refined wheat bread made with exogenously added xylanases according to current commercial practices comprised 1·32 g AXOS per 100 g DM. Considering the mean bread consumption as provided by Euromonitor International (2006 data) we calculated that the mean AXOS consumption through refined wheat bread and rye bread reaches up to 4·1 and 8·2 g/consumer per d, respectively, in countries with relatively high bread consumption such as Turkey or Iran. Recent evidence indicates that AXOS exert beneficial nutritional effects in animals, and it therefore seemed worthwhile to further investigate their gastrointestinal effects in human subjects.

Table 6. Influence of arabinoxylan-oligosaccharides (AXOS) or placebo on the microbiota expressed as log bacteria/g dry faecal weight
(Medians and interquartile ranges (IQR))

	Before AXOS		After 2 weeks AXOS		After 3 weeks AXOS			Before placebo		After 2 weeks placebo		After 3 weeks placebo		
	Median	IQR	Median	IQR	Median	IQR	*P**	Median	IQR	Median	IQR	Median	IQR	*P**
Bifidobacteria														
n 10	7·91	7·39–8·47	8·52	7·91–9·17			0·028	7·88	7·16–8·23	8·08	7·38–8·72			NS
n 20	7·69	7·34–8·20			8·23	7·73–8·99	0·012	7·65	7·17–8·16			8·16	7·81–8·35	0·008
Bifidobacterium adolescentis														
n 10	7·02	4·04–7·59	6·98	4·23–8·45			NS	6·84	3·66–7·37	6·84	3·96–7·57			NS
n 20	6·94	5·98–7·24			7·56	6·09–7·81	0·013	6·80	6·28–7·33			7·24	5·33–7·50	NS
Lactobacilli														
n 10	6·32	5·34–6·72	6·03	5·36–7·08			NS	6·21	5·19–6·70	6·11	5·00–6·89			NS
n 20	6·06	5·54–6·58			5·99	5·51–6·90	0·048	5·96	5·38–6·71			6·36	5·37–6·76	NS
Total bacteria														
n 10	9·69	9·15–10·2	9·60	9·42–9·97			NS	9·67	9·36–10·06	9·52	9·42–9·95			NS
n 20	9·41	9·17–9·98			9·77	9·22–10·11	NS	9·55	9·17–9·91			9·73	9·21–10·13	NS
Roseburia–Eubacterium rectale														
n 10	8·46	7·58–8·87	8·00	7·17–8·56			NS	7·82	7·40–8·70	8·15	7·58–8·75			NS
n 20	8·17	7·76–8·85			8·07	7·84–8·95	NS	8·15	7·51–8·51			8·42	8·16–8·63	NS
Enterobacteria														
n 10	5·40	2·94–6·84	5·44	4·56–6·20			NS	5·57	5·00–7·01	6·16	4·40–6·90			NS
n 20	5·88	3·06–7·51			6·20	4·09–7·23	NS	5·46	4·67–6·80			5·38	4·11–6·87	NS

* Wilcoxon test.

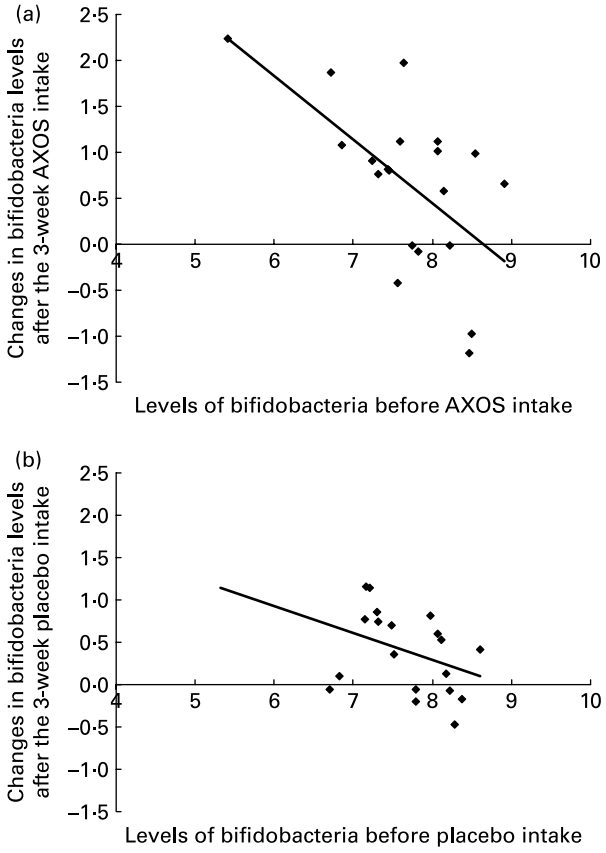

Fig. 2. Correlation (Spearman) between the bifidobacteria levels before and the change in bifidobacteria levels after arabinoxylan-oligosaccharide (AXOS) (a) and placebo (b) intake. Results are expressed as log bifidobacteria/g dry faecal weight. For AXOS, $r - 0{\cdot}51$ ($P = 0{\cdot}022$); for placebo, $r - 0{\cdot}45$ (NS).

In the present study, 10·0 g AXOS were daily administered to healthy subjects for 3 weeks. This dose was relatively high, since our group previously demonstrated the bifidogenic effect of AXOS with a low avDP in healthy subjects upon a dose of only 2·2 g[14]. Here, a higher dose was selected to assess the tolerance of AXOS. We found small, yet significant, differences for packed cell volume, erythrocyte count, lactate dehydrogenase, total protein content and K levels following intake of AXOS and for mean corpuscular Hb concentration, platelet count, lactate dehydrogenase and albumin following intake of placebo. However, these outcomes were not considered toxicologically relevant since they were slight and within the normal range.

Intake of FOS has been shown to increase absorption of minerals, in particular Ca, in young healthy individuals[31,32]. It has also been suggested that prebiotics stimulate the gastrointestinal production of vitamins. Indeed, Thoma *et al.* demonstrated that plasma folate concentrations are significantly increased in rats after administration of FOS[33]. The underlying mechanisms for the stimulation of vitamins by prebiotics have not yet been elucidated. Recently, an *in vitro* study showed that *Bifidobacterium adolescentis* MB 239 significantly increased folate biosynthesis[34]. In the present study, we found an increased level of *Bifidobacterium adolescentis* after 3 weeks of AXOS intake, whereas no influence was seen on folate concentrations. Plasma levels of vitamin A and minerals also remained unchanged.

The administration of a 10 % FOS-containing diet to normolipidaemic rats resulted in significantly decreased plasma TAG, phospholipids and cholesterol levels[35]. In hamsters, a significantly reduced plasma total cholesterol and TAG concentration was observed after the administration of inulin for 5 weeks[36]. In rodents, it is clear that dietary inulin-type fructans have beneficial effects on TAG and cholesterol. In contrast, data on the lipid-lowering effects of prebiotics in healthy human subjects are more conflicting[37–40]. For instance, the intake of 2·7 g xylo-oligosaccharides significantly lowered serum cholesterol and TAG in healthy normolipidaemic subjects[41], whereas the intake of 3·8 g xylo-oligosaccharides had no effect on blood lipids in elderly individuals[42]. No significant differences in blood lipid levels were observed in the present study.

Mild to moderate flatulence was observed in several other intervention studies with FOS and inulin and is probably due to increased gas production during colonic fermentation[43–45]. Also in the present study, flatulence was the most frequently reported adverse gastrointestinal symptom during the study and significantly increased after AXOS intake. The mean score of flatulence after AXOS intake was 1·10, indicating that this symptom was still mild.

Proteolytic fermentation results in the production of potentially toxic compounds such as phenolics. Phenolic compounds are unique bacterial metabolites of tyrosine, and are not produced by human enzymes. They are rapidly absorbed from the colonic lumen, detoxified in the mucosa and the liver (by sulfate or glucuronide conjugation) and finally excreted in urine[46]. More than 90 % of the urinary phenolic compounds consist of *p*-cresol, with the principal remainders being phenol and 4-ethylphenol[47]. Phenols have been implicated in the pathogenesis of bladder and bowel cancers[48]. The marked decrease in urinary *p*-cresol excretion after 2 weeks of AXOS intake (reduction by more than 50 %) indicates reduced colonic protein degradation. The effects on the phenolic metabolism after 2 weeks of AXOS intake were clearly more pronounced than after 3 weeks. The main difference is that AXOS were ingested during the days of sample collection after 2 weeks, whereas the intake of AXOS was ceased the day before sample collection, performed at 3 weeks. This probably indicates that the effects on proteolytic metabolism occur concomitantly with AXOS intake, and that, after a period of AXOS intake the effects are only weakly sustained despite changes in the bacterial composition of the colon. The observed effects on proteolytic fermentation are in agreement with our previous studies in which we found a beneficial modulation of the colonic ammonia metabolism (another protein fermentation metabolite) after AXOS intake[49].

In the present study, faecal SCFA and faecal pH were not measured. We assume that AXOS are mainly fermented in the proximal colon, with production of SCFA resulting in a decreased colonic pH. However, since SCFA are rapidly absorbed through the mucosa[50,51], only little impact of AXOS on SCFA concentration and pH in faeces was expected. Indeed, in our previous intervention studies with AXOS, faecal pH was not influenced. Nevertheless, absence of changes in faecal SCFA or pH does not rule out an increased SCFA production in the proximal colon.

The significantly increased levels of bifidobacteria after 2 or 3 weeks AXOS intake confirm the prebiotic activity

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of AXOS. The present results showed that the stimulation of bifidobacteria is most pronounced in subjects with the lowest bifidobacteria at baseline. This observation suggests that prebiotics might be of particular benefit for specific population groups such as elderly individuals, since bifidobacteria are known to decrease with old age[52]. Several *in vitro* studies have shown that not all *Bifidobacterium* species can degrade AXOS. In an *in vitro* fermentation model, AXOS are completely degraded by *Bifidobacterium adolescentis* and *Bacteroides vulgatus*, partially degraded by *Bifidobacterium longum* and *Bacteroides ovatus*, and not degraded by *Bifidobacterium breve* and *Bifidobacterium infantis*[53]. Another *in vitro* study also showed that AXOS are selectively utilised by *Bifidobacterium adolescentis* and *Bifidobacterium longum*[3]. Therefore, we also measured the effect of AXOS on *Bifidobacterium adolescentis* counts. Our *in vivo* results confirm the *in vitro* observations, since the intake of AXOS by the healthy human subjects stimulates *Bifidobacterium adolescentis*. Although most studies report that dietary intervention with prebiotics for 7–14 d is sufficient to induce changes in microbial composition, a significant increase in *Bifidobacterium adolescentis* was found only after 3 weeks but not after 2 weeks[43]. A possible explanation might be the fact that *Bifidobacterium adolescentis* was measured after 2 weeks only in a subgroup of ten subjects.

No correlations were found between the effect of bifidobacteria and the effect on urinary *p*-cresol or phenol excretion, suggesting that changes in bifidobacteria are not related to changes in protein fermentation.

The numbers of *Roseburia* species and *Eubacterium rectale* were quantified because of their butyric acid-producing capacity[54]. It has been shown that bifidobacteria do not produce butyric acid and that a mechanism of cross-feeding with acetate- or lactate-converting bacteria is involved in colonic butyrate production[55,56]. Finally, we selected enterobacteria as an example of an undesirable bacterial group for which stimulation by AXOS intake is not wanted. However, administration of AXOS did not influence the numbers of these two bacterial groups. Therefore, a more extensive analysis of the microbiota, for example, using a fingerprinting technique, is warranted to better understand the impact of AXOS administration on the bacterial composition and colonic metabolism.

In conclusion, the intake of 10·0 g AXOS reduced the proteolytic fermentation and increased the levels of faecal bifidobacteria. No toxicologically significant differences in blood parameters were observed after AXOS intake, whereas flatulence score was graded as mild. These results indicate that AXOS is a well-tolerated non-digestible carbohydrate with prebiotic activity.

Acknowledgements

The present study was supported by the Research Foundation Flanders (FWO) grant no. G.0607.07 and by the framework of research project IOFKP/06/012 financed by the Research Fund Katholieke Universiteit Leuven.

L. C., W. F. B. and K. V. designed the study; W. F. B., C. M. C. and J. A. D. prepared the AXOS; L. C. was responsible for data acquisition and analysis; L. C., Y. D. and F. O. were responsible for microbiological analysis; L. C., W. F. B. and K. V. contributed to data interpretation. All authors read and approved the final manuscript.

All authors have stated that there are no conflicts of interest.

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