Consumption of Breads Containing In Situ–Produced Arabinoylxyan Oligosaccharides Alters Gastrointestinal Effects in Healthy Volunteers1–3

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Abstract
Arabinoxylan oligosaccharides (AXOS) are studied as food compounds with prebiotic potential. Here, the impact of consumption of breads with in situ-produced AXOS on intestinal fermentation and overall gastrointestinal characteristics was evaluated in a completely randomized, double-blind, controlled, cross-over study. Twenty-seven healthy volunteers consumed 180 g of wheat/rye bread with or without in situ-produced AXOS (WR+ and WR−, respectively) daily for 3 wk. Consumption of WR+ corresponded to an AXOS intake of ~2.14 g/d. Refined wheat flour bread without AXOS (W−) was provided during the 3-wk run-in and wash-out periods. At the end of each treatment period, participants collected urine for 48 h as well as a feces sample. Additionally, all participants completed a questionnaire about stool characteristics and gastrointestinal symptoms during the last week of each period. Urinary phenol and p-cresol excretions were significantly lower after WR+ intake compared to WR−. Consumption of WR+ significantly increased fecal total SCFA concentrations compared to intake of W−. The effect of WR+ intake was most pronounced on butyrate, with levels 70% higher than after consumption of W− in the run-in and wash-out period. Consumption of WR+ tended to selectively increase the fecal levels of bifidobacteria (P = 0.06) relative to consumption of W−. Stool frequency increased significantly after intake of WR+ compared to WR−. In conclusion, consumption of breads with in situ-produced AXOS may favorably modulate intestinal fermentation and overall gastrointestinal properties in healthy humans. J. Nutr. 142: 470–477, 2012.

Introduction
AX11, which consists of a linear backbone of (1→4)-linked β-D-xylopyranose units with L-arabinofuranose as the main substituent, is one of the major dietary fiber fractions in cereals like wheat (1). However, AX content in grains varies largely within the layer of the kernel, because AX makes up 15–30% of the bran layers, whereas wheat endosperm contains only 1–3% AX (2). Typically, only a minor part of AX is WE-AX (3). WU-AX is poorly degraded in the colon and hence may, like other insoluble fibers, increase bowel mass and fasten transit of chyme through the gut (4). Conversely, WE-AX is readily fermented and its effect has been associated with a reduced intestinal pH, lower levels of proteolytic cataholites, and improved glucose and lipid metabolism (5,6). In general, a reduction of the pH in the large bowel, due to the formation of SCFA, the primary fermentation products of carbohydrates, is considered beneficial, because it may prevent the growth of some malignant bacteria (7). In addition, acidification of the large bowel may also impair the production of protein degradation products, of which some have shown to be carcinogenic in vitro (8).

In recent years, cereal AX have gained further interest, because their hydrolysis products, AXOS, have been proposed as food compounds with prebiotic potential (9). Prebiotics were first defined by Gibson et al. (10) as nondigestible food ingredients,
often oligosaccharides, that contribute to consumers’ well-being by selectively stimulating the growth and/or activity of beneficial bacteria in the gut. To date, an array of studies support the hypothesis that AXOS can be classified as food components with prebiotic potential (9). It was suggested that the selective bifidogenic effect of AXOS is closely related to their avDP, because levels of bifidobacteria increase with lower avDP of AXOS (6). Today, the term AXOS is typically reserved for hydrolyzed AX with an avDP between 5 and 50 (11). The positive impact of AXOS on intestinal fermentation was confirmed in clinical studies. Daily intake of 10.0 g of AXOS by healthy volunteers is well tolerated and promotes bifidobacterial growth in the colon while suppressing the excretion of urinary p-cresol, a potentially harmful catabolite of protein fermentation (12). Additionally, Cloetens et al. (8) indicated that consumption of AXOS at doses as low as 2.2 g is associated with a marked shift from urinary to fecal 13C excretion.

Currently, the production of AXOS is mainly performed at pilot scale by enzymatic hydrolysis of AX with wheat bran as a low-cost starting material (13). However, a method was recently established to produce AXOS in situ at high levels in fiber-rich breads through addition to the dough of a xylanase mixture comprising at least one thermostable xylanase (11,14). Given the status of bread as a staple food in the Western world with, e.g., an average consumption of 180 g/d in Western Europe (15), this technology would ensure the daily intake of AXOS at levels ≥2.0 g and with an avDP between 5 and 50 (11,14). Moreover, thermostable xylanase-mediated transformation of a substantial part of the AX population in fiber-enriched bread leads to the presence of different types of AX, in particular WU-AX and AXOS. A recent study on rats demonstrated that a combination of different types of AX synergistically enhanced putative markers associated with colonic health (16).

This study aimed to investigate whether intake of wheat/rye bread enriched in in situ-produced AXOS by healthy volunteers has an effect on carbohydrate and protein fermentation products and whether it alters the levels of microbiota in the feces, in particular fecal bifidobacteria, both hallmarks of prebiotic potential. In addition, the effect of intake of such bread on average stool frequency and consistency as well as on possible adverse gastrointestinal symptoms was assessed. The experimental setup equally allowed us to assess the impact of rye enriched bread compared to wheat bread on these end points.

**Methods**

**Participants**

A total of 27 volunteers [17 women and 10 men, median age 23 (IQR 23–29)] and BMI of 20.9 kg/m² (IQR 20.0–23.7) participated in the study. Inclusion criteria were: age between 18 and 46 y, good general health (determined by self-assessment), and regular eating habits (3 meals/d). Exclusion criteria were: history of abdominal surgery, intake of antibiotics <2 mo before the start of the study, and medical treatments influencing gut transit <2 wk before the start of the study. During the study, the volunteers were allowed to maintain their usual diets, except for the required intake of the supplied bread slices. At pre-assessment, the volunteers were informed about the pre- and probiotic concept and food substances containing pre- and/or probiotics.

The present study was conducted according to the guidelines laid down in the Declaration of Helsinki and all procedures involving human participants were approved by the Ethics Committee (Institutional Review Board) of the University of Leuven. Written informed consent was obtained from all participants.

**Materials**

Rye whole-grain bread (Type 1740) was provided by Panificio B.N. Rye bran was provided by A/S Bruggeman. Gluten was obtained from Syral. The xylanase-free bread improver mixture, providing 1.0 g of α-amylase (Bel’Ase A75), 15.0 g ascorbic acid, and 300 g di-acetyl tartaric acid ester of monoglyceride/100 kg total flour mixture, was from Puratos Group. The xylanase preparation with an activity of 2100 MU/L was from Puratos Group. Termamyl 120L, a thermostable amylase preparation, was from Novozymes.

All solvents, chemicals, and reagents were of at least analytical grade and purchased from Sigma-Aldrich unless otherwise specified.

**Production and characterization of the investigational products**

**Bread making.** W- was prepared by mixing 592.0 g flour with 11.8 g of salt, 11.8 g of baker’s yeast, 11.8 g of xylanase-free bread improver mixture, 17.8 g of gluten, and 355.0 mL water per kg total dough mix. WR- was prepared by mixing 430.0 g flour with 100.0 g rye whole meal, 43.0 g rye bran, 11.5 g of salt, 11.5 g of baker’s yeast, 11.5 g of xylanase-free bread improver mix, 17.2 g of gluten, and 375.0 mL of water per kg dough. WR′ was prepared as WR except that the baking absorption was reduced to 374 mL of water/kg total mix, and that the xylanase preparation (1.0 mL) was added.

After mixing, the doughs were divided into pieces of 820 g each, manually rounded, and allowed to rise at room temperature for 20 min, followed by mechanical molding, proofing, and proofing (90 min at 35°C and 95% relative humidity). Doughs were baked in a hot-air oven at 230°C for 35 min. The breads were sliced 2.5 h after baking, packaged in polypropylene bags in rations of 180 g/pack, and frozen at −20°C until consumption by the volunteers. All breads were prepared and packed at Puratos Group.

**Compositional analyses.** Bread slices from the middle of the loaf were dried (105°C, 12 h), followed by cooling to room temperature in an exsiccator for 1 h. The dried bread slices were homogenized using mortar and pestle to yield a dried bread powder.

Moisture and ash contents were analyzed according to American Association of Cereal Chemists. International methods 44–19 and 08–01, respectively (17). Protein contents were determined according to the Dumas combustion method as described earlier (6). Lipid content was determined by the Soxhlet method according to AOAC Official Method 954.16 (18). Fiber content was determined by AOAC Official Method 2001.03 (18).

For characterization of the AX population and starch content, aqueous extracts of breads were prepared by mixing dried and ground bread (2.00 g) with water (20 mL) and Termamyl 120L (120 μL) pretreated for 1 h at 90°C prior to use to destroy possible enzyme activities other than amylase activity. The suspension was incubated under constant shaking (37°C, 30 min) and subsequently centrifuged (3000 × g, 4°C, 15 min). The supernatant was stored at −20°C until further analysis. Total hydrolysable carbohydrate content of breads and total and reducing end saccharide contents of aqueous extracts thereof were measured by GC as described by Courtois et al. (19). The total AX content of bread was calculated using formula 1. The WE-AX/AXOS content and the avDP of WE-AX/AXOS was calculated using formulae 2 and 3, respectively. The starch content of bread was calculated using formula 4.

**Total AX (%) = (%arabinose in dried bread – 0.7× %galactose in bread extract) × 132/150 + [132 × (avDP – 1) + 150] / [150 × avDP] × %xylose in bread.**

**WE-AX/AXOS (%) = (%arabinose in bread extract – 0.7 × %galactose in bread extract) × 132/150 + [132 × (avDP – 1) + 150] / [150 × avDP] × %xylose in bread extract**

avDP of WE-AX/AXOS = (%arabinose in bread extract – 0.7 × %galactose in bread extract + %xylose in bread extract) / %reducing end xylose in bread extract

starch = 0.9 × (%glucose in dried bread).**

The formulae 1, 2, and 3 contain a correction for the galactose present in aqueous extracts under the form of wheat and rye water-extractable arabinoxylolan peptides with an arabinose:galactose ratio of 0.7 (20). The factors 132 and 150 in formulae 1 and 2 reflect the molecular mass of anhydropentose sugars and pentose sugars, respectively.
Study design
The total treatment period of the randomized, double-blind, controlled, cross-over trial was 12 wk divided over four consecutive periods of 3 wk each (Fig. 1). The run-in and/or wash-out periods prior to the WR+ and WR− treatment periods were denoted as preWR+ and pre WR− period, respectively. During these periods, W− was consumed by the participants. Throughout the study, 180 g fresh weight of the provided bread was consumed on a daily basis, which is close to the average daily bread intake in a number of representative Western European countries (15). The packages with frozen bread slices were supplied by members of the investigational staff to the volunteers once per week or more frequently, depending on the freezerer capacity at home of the volunteer.

From the morning of d 19 of each intake period, all participants collected urine for 48 h. A stool sample was collected on the evening of d 20 or morning of d 21 of each intake period. During the final week of each intake period, participants completed a questionnaire about defecation frequency, stool consistency [Bristol Stool Score (21)], and possible gastrointestinal symptoms. Upon delivery of samples, compliance with investigational product consumption was evaluated by participant interview. Participants were requested to record any noncompliance throughout the study, including the amount of bread not consumed per day.

Sample collection

Stool samples. Stool samples were stored immediately after defecation at 4°C and delivered at the collection point within 1 h. Upon delivery, 2.5 g of each stool sample was immediately fixed in paraformaldehyde (22) for microbial analysis by FISH. Fresh stool samples (~2.5 g) were freeze-dried and dry matter content was determined as an indicator for stool consistency. Aliquots of 2.0 g feces were stored at −20°C until further analysis of SCFA.

Urine samples. Urine was collected for 48 h in dedicated containers (5 L) to which 1.0 g neomycin was added to prevent bacterial growth (8). The volume of the urine fractions was measured and aliquots (20 mL) were stored at −20°C until further analysis.

Analytical procedures

SCFA analysis in feces. SCFA were extracted with diethyl ether and analyzed with GC as described by Van Craeyveld et al. (6). The total SCFA concentration was calculated as the sum of the concentrations of acetic, propionic, and butyric acids.

Phenol and p-cresol in urine. Total phenol and p-cresol content in urine samples was assayed by GC-MS as described by de Looor et al. (23). The GC was equipped with a RxiTM-5MS column (Restek, 30 m × 0.25 mm, 0.50-µm film thickness). Helium was used as carrier gas with constant flow of 1.5 mL/min. The oven 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 39 to 390 at two scans/s.

Microbiological analysis of feces. FISH was used to count the number of different bacterial groups in paraformaldehyde-fixed stool samples. Processing of fixed samples and FISH analysis were performed as described by Langendijk et al. (24). For total bacteria cell counts, 4′,6-diamidino-2-phenylindole was used. The probes used for group-specific analyses were Bif164 for Bifidobacterium species (24), Lac158 for Lactobacillus species (25), Ec1531 for enterobacteria (26), and an equimolar mixture of Chr150 and Clt135 for the Clostridium histolyticum/clostridium seburense group (22). Rod-shaped bacteria fluorescing with the Lac158 probe were scored as Lactobacillus rods. Fluorescent cells were counted by using Quantimet HR600 image analysis software (Leica). The detection limit of the log10 counts by the FISH procedure was 5.65 log10/g wet feces and values below the detection limit were set to one-half the detection limit.

Recording of stool characteristics and gastrointestinal symptoms. During the last week of each intake period, participants were requested to daily evaluate following symptoms: abdominal cramps, flatulence, and bloating. The severity of the symptoms was graded on a 4-step scale ranging from no (0) to severe (4) symptoms as described by van Munster et al. (27). Defecation frequency and consistency of the stool according to the Bristol Stool Form Scale (21) were recorded daily through appropriate questionnaires. The average stool frequency was calculated as the number of stools divided by the number of days of diary recording, whereas the average stool consistency corresponded to the sum of Bristol Stool Form Scales divided by the number of stools. Finally, the composite factor of stool frequency and consistency was defined as the sum of Bristol Stool Form Scales divided by the number of days of diary recording (28).

Statistics
A statistical analysis plan was made available prior to the review and statistical analysis of the study data by an external biostatistician, who had no links to either sponsor or institution of the principal investigator and who was not involved in the execution of the clinical trial.

The EE population was defined as all randomized participants who received control treatment (WR−) and at least one serving of investigatet treatment (WR+) and provided at least one postrandomization outcome data point during each of the two treatment phases. In the PP population, participants with major protocol violations were excluded. No imputation for missing data was performed.

Outlyingness of data was checked using box plots marking all data that were smaller than the 25th quartile – 1.5 × IQR or >75th quartile + 1.5 × IQR. All outlier data were checked for possible errors and biological significance. All the available data points were kept for further statistical analysis.

Nonparametric tests (Kruskall-Walliss) were used for the analysis of differences at baseline for age and length. All other outcome parameters, whether the outcomes were rank transformed or not, were analyzed using a parametric linear mixed model and inference on the treatment comparisons was done using tests for general parametric models.

To test for differences at baseline, the treatment sequence groups were compared with respect to age, gender, and BMI, the only relevant initial factors recorded at baseline in this study. Comparison of the

FIGURE 1 Schematic presentation of the study design. Breads (W−, WR−, and WR+) were consumed at a dose of 180 g/d during four consecutive periods (preWR+, WR+, preWR−, and WR− period) of 3 wk each. Participants (n = 27) were randomly divided into two groups. All volunteers collected urine (48 h) and feces samples at the end of each 3-wk intake period. The participants also completed a diary questionnaire about stool frequency, stool consistency, and gastrointestinal symptoms during the last week of each 3-wk intake period, W−, without in situ–produced arabinoxylan oligosaccharides; WR+, wheat/rye bread with in situ–produced arabinoxylan oligosaccharides; WR+, wheat/rye bread without in situ–produced arabinoxylan oligosaccharides.
groups for age and BMI was based on a 2-sample t test. In case the normality assumption of the 2-sample t test was rejected ($P < 0.05$), a Mann-Whitney U-test with Sidak’s correction for multiple comparisons was used to check baseline differences. Gender differences were tested using the chi-square test or, in case there were not enough data in each cell of the contingency table, the Fisher Exact test.

The treatment effect for the different outcome variables was analyzed using a linear mixed model (29). The fitted model included participant as a random effect and contained terms for treatment and treatment sequence. Because the spread of the inclusion period for all participants was only 1 wk, no term for inclusion period was used in the linear mixed models.

To test the validity of the linear mixed models, the normality of the residuals was checked with the Shapiro-Wilk test (30). In case this test rejected the null hypothesis of normality of the residuals at a Type I error rate of $\alpha = 0.01$, the dependent variable was rank-transformed and the linear mixed model was performed on the rank-transformed data (31). Ties occurring during the rank transformation were replaced with their mean rank. For each linear model, whether based on raw data (31). Ties occurring during the rank transformation were replaced and the linear mixed model was performed on the rank-transformed data. For these models, pairwise multiple comparisons were executed to compare the difference between the parameter at the treatment period and at the immediately preceding run-in of wash-out period and the difference between the parameters at the two treatment periods.

In case of models with interactions, the above-mentioned pairwise comparisons were made for each treatment sequence group. Next to that, the overall differences were also analyzed by aggregating over the interaction effects in the model.

The Bonferroni procedure was used to correct for all pairwise multiple comparisons and reported $P$ values were adjusted accordingly.

**Results**

**Participant characteristics** A total of 33 volunteers were screened and 28 were randomized to either one of the two groups differing in treatment sequence ($n = 13$ and 15, respectively) (Supplemental Fig. 1). One volunteer was asked to prematurely terminate the study due to noncompliance, because this volunteer frequently missed appointments. This volunteer did not receive the control treatment and was not included in the EE population. Hence, 27 volunteers were included in the EE population. Because no major protocol violations were reported for these 27 volunteers, the PP population was the same as the EE population. In the EE/PP population, treatment sequence group 1 consisted of 13 volunteers and treatment sequence group 2 consisted of 14 volunteers.

Sex, age, and BMI did not significantly differ at baseline between the two treatment sequence groups.

**Compositional analysis of applied bread types** WR⁺ and WR⁻ contained similar amounts of total fiber (Table 1). The low-molecular-weight fiber content of WR⁺ was higher than that of WR⁻. Xylanase treatment in WR⁺ resulted in an AXOS level corresponding to one-half of the total AX population, with an avDP of 18 (Table 1). The avDP of WR + was higher than the avDP of WR⁻, with an avDP of 174 and 212, respectively.

**Study compliance** The number of nonconsumed bread slices was low, with a mean of 1.4, 1.6, 1.0, and 1.2% during the preWR⁺, WR⁻, pre WR⁻, and WR⁻ periods, respectively. There were no differences observed between the treatments ($P > 0.10$).

**SCFA in feces** Fecal SCFA concentrations did not differ following the WR⁺ and WR⁻ periods (Table 2). The fecal total SCFA concentration was higher after the WR⁺ than during the preWR⁺ period ($P = 0.02$). Fecal concentrations of acetate and propionate in the WR⁺ period tended to be higher ($P < 0.1$) compared with the preWR⁺ period. Intake of WR⁺ resulted in higher ($P = 0.02$) fecal butyric acid levels than those observed at the end of the preWR⁺ period. Neither fecal acetate, fecal propionate, nor fecal butyrate levels differed significantly at the end of the WR⁻ and pre WR⁻ periods.

**Phenol and p-cresol in urine** Urinary phenol and p-cresol excretions were lower after the WR⁺ than after the WR⁻ period ($P \leq 0.05$) (Table 3). At the end of the WR⁻ period, urinary phenol excretion was higher ($P = 0.04$) and that of p-cresol tended to be higher ($P = 0.09$) than during the pre WR⁻ period. Urinary excretions of phenol and p-cresol did not differ between the preWR⁺ and WR⁺ periods.

**Microbiological analysis of feces** Total bacteria numbers were unaffected by administration of WR⁻ or WR⁺ (Supplemental Table 1). Fecal bifidobacteria counts, however, tended to be higher at the end of the WR⁺ period compared to the preWR⁺ period ($P = 0.06$).

Similar to total bacteria, levels of lactobacilli, rod-shaped lactobacilli, Enterobacteriaceae, and bacteria belonging to the C. bistolyticum/lituseburense group were not affected by the treatments (Supplemental Table 1).

**Stool characteristics and gastrointestinal symptoms** The stool frequency increased ($P = 0.02$) in the WR⁺ period relative to the WR⁻ period (Table 4). Stool consistency did not differ after the WR⁺ and WR⁻ periods. The composite factor for stool frequency/consistency was higher ($P = 0.001$) after 3-wk consumption of WR⁺ compared to WR⁻.

Abdominal cramping and bloating were virtually absent throughout the study and did not differ during wk 3 of the treatment periods (data not shown). Flatulence symptoms between the two treatment sequence groups.

**TABLE 1** Compositional analysis of the different bread types (WR⁻, WR⁺, and WR⁺) used in the study (W⁻, W⁺, and W⁺)

<table>
<thead>
<tr>
<th>Composition</th>
<th>W⁻</th>
<th>W⁺</th>
<th>W⁺</th>
</tr>
</thead>
<tbody>
<tr>
<td>Total AX</td>
<td>1.9</td>
<td>3.7</td>
<td>4.0</td>
</tr>
<tr>
<td>AX-AXOS</td>
<td>0.6</td>
<td>1.2</td>
<td>1.9</td>
</tr>
<tr>
<td>avDP of AX-AXOS</td>
<td>174</td>
<td>212</td>
<td>180</td>
</tr>
<tr>
<td>Fiber</td>
<td>5.7</td>
<td>10.1</td>
<td>9.2</td>
</tr>
<tr>
<td>HMW fiber</td>
<td>4.4</td>
<td>8.4</td>
<td>6.3</td>
</tr>
<tr>
<td>LMW fiber</td>
<td>1.3</td>
<td>1.7</td>
<td>2.9</td>
</tr>
<tr>
<td>Starch</td>
<td>76.8</td>
<td>70.2</td>
<td>72.7</td>
</tr>
<tr>
<td>Protein</td>
<td>17.4</td>
<td>18.1</td>
<td>17.1</td>
</tr>
<tr>
<td>Lipids</td>
<td>&lt;0.5</td>
<td>&lt;0.5</td>
<td>&lt;0.5</td>
</tr>
<tr>
<td>Ash</td>
<td>2.7</td>
<td>3.0</td>
<td>3.0</td>
</tr>
<tr>
<td>% of dry matter</td>
<td>36.0</td>
<td>37.0</td>
<td>37.4</td>
</tr>
</tbody>
</table>

1
data are mean, $n = 3$, avDP, average degree of polymerization; AX, arabinoxylan; AXOS, arabinoxylan oligosaccharides; HMW, high molecular weight; LMW, low molecular weight; WE-AX, water-extractable AX, W⁻, wheat flour bread without in situ-produced arabinoxylan oligosaccharides; WE-AX, water-extractable: AXOS; WR⁺, wheat/rye bread without in situ-produced arabinoxylan oligosaccharides; WR⁻, wheat/rye bread with in situ-produced arabinoxylan oligosaccharides.

Gastrointestinal effects of in situ–formed arabinoxylan oligosaccharides
increased from the pre WR− period (0.64, IQR 0.18–1.00) to the WR+ period (1.07, IQR 0.71–1.57) (P = 0.02).

Discussion

The present double-blind, cross-over study investigated the effects of consumption of xylanase-treated and untreated wheat/rye breads on putative markers of colonic health. The specific impact of xylanase treatment during wheat/rye bread making, resulting in the presence of wheat- and rye-derived AXOS, was assessed by comparing the gastrointestinal effects induced after 3-wk consumption of AXOS-rich wheat/rye bread with those observed after the intake of their xylanase-untreated counterparts (WR−). In addition, the potentially beneficial role of incorporation of dietary fiber as such, in bread, was investigated by comparing the effect of the consumption of WR+ with intake of control wheat bread without rye fiber (WR−). Finally, any combined effect of xylanase treatment and rye incorporation was analyzed by comparing the effect of WR+ consumption with the effect observed after intake of the control wheat bread (WR−).

Carbohydrate fermentation. Consumption of WR+ increased the fecal total SCFA concentration compared to the intake of WR− in the run-in and wash-out periods (P = 0.02). In general, it can be expected that partial replacement of wheat flour by fiber-rich whole-grain rye meal and rye bran enhances SCFA production, because these fiber-rich ingredients provide high levels of undigestible carbohydrates (32). However, fecal concentrations of SCFA remained unaffected after 3-wk intake of WR−. Therefore, the SCFA-boosting effect of WR+ must be ascribed mainly to the partial conversion of wheat and rye AX into AXOS, taking into account that AXOS is more fermentable in the colon than WU-AX and WE-AX.

Whereas intake of WR+ tended to promote the formation of acetate and propionate, the higher fecal SCFA concentrations at the end of the WR+ period were due to an intensive stimulation of butyrate production.

From a digestive health perspective, butyrate production is considered relevant, because it is generally acknowledged to play a key role in maintenance of homeostasis in the colon (33,34). Unlike the present results, most studies on the intake of fructans and galactooligosaccharides fail to observe an effect on fecal SCFA concentrations (35–37).

Interestingly, it was recently shown that a 1:1 combination of WU-AX and AXOS, as was obtained in WR+, synergistically promotes the production of butyrate in the cecum of rats (16). The stimulatory effect of AXOS on butyrate formation was explained by the fact that acetate, which is by far the primary metabolite of AXOS fermentation, is readily converted to butyrate by the many butyrogenic species present in the colon and their stimulation by the presence of WU-AX (38). Alternatively, it can be envisaged that AXOS stimulates xylanase production by butyrogenic bacterial species, allowing them to better ferment WE-AX and/or WR-AX.

Phenol and p-cresol excretion. Consumption of WR+ suppressed the urinary excretions of potentially detrimental phenol and p-cresol compared to the intake of WR−. The urinary excretion of phenols may give a good indication of colonic bacterial protein metabolism, because it has been established that under steady-state conditions, urinary excretion of phenolic compounds reflects their generation in the colon as bacterial catabolites of phenylalanine and tyrosine (39). In accordance

### Table 2

<table>
<thead>
<tr>
<th>Metabolite</th>
<th>preWR+ period (W− treatment)</th>
<th>WR+ period (W− treatment)</th>
<th>preWR− period (W+ treatment)</th>
<th>WR− period (W+ treatment)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Acetic acid</td>
<td>26.4 (19.5–39.5)</td>
<td>36.6 (24.0–46.7)</td>
<td>29.0 (18.0–38.4)</td>
<td>33.8 (26.6–40.3)</td>
</tr>
<tr>
<td>Propionic acid</td>
<td>7.9 (6.1–10.7)</td>
<td>11.1 (7.4–14.2)</td>
<td>8.5 (5.7–11.0)</td>
<td>9.8 (7.8–11.2)</td>
</tr>
<tr>
<td>Butyric acid</td>
<td>10.7 (6.0–19.8)</td>
<td>18.3 (12.4–28.0)*</td>
<td>11.4 (5.5–18.6)</td>
<td>13.7 (9.4–21.7)</td>
</tr>
<tr>
<td>Total SCFA</td>
<td>45.5 (32.3–70.0)</td>
<td>70.8 (46.8–84.0)*</td>
<td>48.9 (32.0–67.0)</td>
<td>58.5 (40.3–75.1)</td>
</tr>
</tbody>
</table>

*Data are median (IQR), n = 27. **Different from preWR− period, P < 0.05; *different from WR− period, P < 0.05. W−, wheat flour bread without in situ–produced arabinoxylan oligosaccharides; WR+, wheat/rye bread without in situ–produced arabinoxylan oligosaccharides; WR−, wheat/rye bread with in situ–produced arabinoxylan oligosaccharides.

### Table 3

<table>
<thead>
<tr>
<th>Metabolite</th>
<th>preWR+ period (W− treatment)</th>
<th>WR+ period (W− treatment)</th>
<th>preWR− period (W+ treatment)</th>
<th>WR− period (W+ treatment)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Phenol</td>
<td>44.5 (36.7–66.2)</td>
<td>39.5** (33.9–58.2)</td>
<td>43.0 (35.2–58.9)</td>
<td>50.0* (40.5–73.5)</td>
</tr>
<tr>
<td>p-Cresol</td>
<td>168 (93.3–304)</td>
<td>175** (88.8–288)</td>
<td>208 [114–288]</td>
<td>210 (150–365)</td>
</tr>
</tbody>
</table>

*Data are median (IQR), n = 27. **Different from preWR− period, P < 0.05; *different from WR− period, P < 0.05. W−, wheat flour bread without in situ–produced arabinoxylan oligosaccharides; WR+, wheat/rye bread without in situ–produced arabinoxylan oligosaccharides; WR−, wheat/rye bread with in situ–produced arabinoxylan oligosaccharides.
Microbiological growth. While there were no significant periods, consumption of WR+ did tend to increase fecal levels of bifidobacteria. In this regard, it was hypothesized that some bifidobacteria can take up small oligosaccharides but are unable to hydrolyze larger polymers, because they do not possess the required xylanase enzymes (9). Selective stimulation of colonic bifidobacteria may be related to reduced gut infections, inflammation, and lower prevalence of colon cancer initiation (42).

Stool and gastrointestinal symptoms. Consumption of WR+ resulted in a higher stool frequency and distinctly higher composite stool frequency/consistency factor compared to WR−. This possibly points to a faster stool transit upon intake of WR+ compared to WR− (43), thus suggesting that consumption of AXOS-enriched bread (WR+) is more beneficial for improving gastrointestinal transit than consumption of the corresponding bread in which the AX is not partially depolymerized. However, because consumption of WR+ and WR− had similar effects on stool frequency and consistency, it can be concluded that a clear effect of rye incorporation combined with xylanase treatment on stool characteristics was absent. In general, softer stools and a reduced gastrointestinal transit time upon fiber intake is often ascribed to its bulking effect and water-binding capacity (4). These physico-chemical effects are typically less pronounced in case of soluble fibers compared to insoluble fibers, because they are more prone to fermentation. Conversely, they may also contribute to softer stools by increasing bacterial biomass and host secretions, e.g., mucin.

Finally, consumption of WR+ had no effect on the assessed gastrointestinal symptoms, abdominal cramps, flatulence, and abdominal bloating, indicating that it is well tolerated by the volunteers (P > 0.1).

In conclusion, the intake of AXOS-rich wheat/rye bread at levels similar to the average daily bread intake noted in Western Europe may alter gastrointestinal fermentation characteristics relative to xylanase-untreated wheat bread and xylanase-untreated wheat/rye bread. Compared with consumption of wheat bread without AXOS, significantly higher total SCFA concentrations were observed in the feces of healthy participants after intake of AXOS-enriched wheat/rye bread. The increase in SCFA concentrations was largely attributed to an elevated production of butyrate, a SCFA of particular interest from a digestive health perspective. Conversely to xylanase-untreated wheat/rye breads, consumption of their xylanase-treated counterparts did not increase the concentrations of phenolic compounds in urine. Moreover, a trend (P =

TABLE 4 Defecation frequency, stool consistency, and composite stool frequency/consistency during wk 3 of the preWR+, WR+, preWR−, and WR− periods in which participants consumed 180 g fresh weight of W−, WR−, or WR+ daily

<table>
<thead>
<tr>
<th>Stool characteristics</th>
<th>preWR+ period (W+ treatment)</th>
<th>WR+ period (W+ treatment)</th>
<th>preWR− period (W− treatment)</th>
<th>WR− period (W− treatment)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Frequency, n/d</td>
<td>1.4 (0.9–1.6)</td>
<td>1.3* (1.1–1.7)</td>
<td>1.0 [0.9–1.5]</td>
<td>1.1 [1.0–1.4]</td>
</tr>
<tr>
<td>Consistency, score/stool</td>
<td>3.8 (2.8–4.4)</td>
<td>3.7 (3.1–4.2)</td>
<td>3.3 [2.5–4.1]</td>
<td>3.5 [2.7–4.1]</td>
</tr>
<tr>
<td>Composite frequency/consistency</td>
<td>3.8 (2.8 – 6.0)</td>
<td>5.1 (3.9–7.2)*</td>
<td>3.0 [2.3–5.3]</td>
<td>3.9 [2.4–4.9]</td>
</tr>
</tbody>
</table>

1 Data are median (IQR), n = 27. *Different from WR− period, P < 0.05. W− wheat flour bread without in situ–produced arabinoxylan oligosaccharides; WR+, wheat/rye bread with in situ–produced arabinoxylan oligosaccharides. 2 Stool frequency was calculated as the number of stools divided by the number of days of diary recording. 3 Stool consistency data are based on the Bristol stool scale (ranked from type 1, separate hard lumps, to type 7, watery no solid pieces, entirely liquid). Average stool consistency corresponded to the sum of Bristol Stool Form Scales divided by the number of stools. 4 Composite factor of stool frequency and consistency was defined as the sum of Bristol Stool Form Scales divided by the number of d of diary recording.
0.06) toward higher fecal bifidobacteria was observed after intake of AXOS-rich wheat/rye bread compared to consumption of xylanase-untreated wheat bread. Finally, this study showed that the consumption of AXOS-enriched wheat/rye bread is well-tolerated, because no adverse gastrointestinal symptoms were reported.

Acknowledgments
K.V., I.T., and W.F.B. conceived and designed the study; I.T. coordinated the experimental bread production and transport of the breads; K.V., C.M.C., G.W., J.A.D., L.C., and B.D. supervised or conducted research; W.F.B., I.F., O.L., and J.W. analyzed data or performed statistical analysis; K.V., W.F.B., L.C., and B.D. had full access to all of the data in the study and jointly take responsibility for its integrity; W.F.B., K.V., C.M.C., I.T., and B.D. wrote the paper; and B.D. and C.M.C. had primary responsibility for the final content. All authors read and approved the final manuscript.

Literature Cited