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Different Human Gut Models Reveal the Distinct Fermentation Patterns of Arabinoxylan versus Inulin

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Supporting Information

ABSTRACT: Different in vitro models have been developed to assess how food compounds affect the human gut microbiota. Using two such models (SHIME(R) and TIM-2), we compared how long-chain arabinoxylan (LC-AX), a wheat-derived potentially prebiotic fiber, and inulin (IN), a well-established prebiotic compound, modulate SCFA production and bifidobacteria composition. While both the SHIME and TIM-2 differ in experimental design, they both demonstrated that LC-AX and IN specifically increased the health-promoting metabolites propionate and butyrate, respectively. Furthermore, LC-AX stimulated *Bifidobacterium longum*, while IN stimulated other bifidobacteria including *Bifidobacterium adolescentis*. The SHIME experiment also revealed that effects of LC-AX were more persistent during the 2-week wash-out period. These results confirm a recent in vivo study, during which humanized rats were treated with the same LC-AX/IN. In conclusion, results from different human gut models suggest that, besides IN, LC-AX are promising prebiotic candidates with high specificity toward *Bifidobacterium longum* and a selective propionate increase.

KEYWORDS: *bowel, intestine, microflora, fructan, bacteria*

INTRODUCTION

Intestinal micro-organisms closely interact with the host and have an important potential to influence host health.[1−4] A strategy to modulate the human gut microbiome in order to improve health includes the use of prebiotics, defined as "indigestible compounds that selectively stimulate the growth and/or activity of one or a limited number of microbial genus(era)/species in the gut that confer(s) health benefits to the host".[5] While lactobacilli and bifidobacteria have been considered as beneficial microbes for many decades, specific genera of the Firmicutes (e.g., *Faecalibacterium*, *Eubacterium*, and *Roseburia*) are recently gaining interest, given their ability to produce butyrate. Prebiotic compounds have been shown to improve intestinal functioning, increase mineral absorption, modulate energy metabolism, regulate the immune system, and reduce the risk of intestinal infections, type II diabetes, intestinal inflammation, and colon cancer.[6]

Fructan-type carbohydrates, such as inulin (IN) and fructo-oligosaccharides (FOS), have been studied frequently and are considered as a "gold standard" in this field.[7−11] However, a limitation of existing prebiotics is their rapid proximal fermentation.[11,12] Since many diseases originate in the distal colon,[13] a more distal fermentation of prebiotics would be desirable.[12] In this context, arabinoxylans (AX) are an interesting novel class of potential prebiotic compounds.[14,15] Because their structure consists of a xylose backbone substituted with arabinose monomers and potentially also ferulic acid,[16] a spectrum of enzymes is needed for its degradation:[15] xylosidases (release xylose from nonreducing ends), xylanases (cleave xylose backbone), arabinofuranosidases (detach arabinose), and feruloyl esterases (release ferulic acid). Therefore, AX may be more gradually fermented along the colon. AX structure depends on its origin [17] and can be further modified to shorter fractions.[18] While most of its effects remain unknown, early studies indicate that AX structure affects fermentation patterns[19,20] and immune modulation, with highest activity for long-chain AX.[21]

To investigate prebiotic effects on the intestinal microbiota, different in vitro gut models have been used ranging from simple batch cultures without stirring and pH control [22] to complex models involving pH controlled single [23] and multiple-component continuous culture systems such as the dynamic TNO in vitro model of the colon (TIM-2) [24] and the Simulator of the Human Intestinal Microbial Ecosystem (SHIME).[25] Although both models have been validated,[24,26,27] they use specific standard protocols to focus on the simulation of certain digestive parameters (Figure 1). Therefore, the consistency of results obtained with different models may require further research.

To address this issue, we compared potentially prebiotic long-chain arabinoxylans (LC-AX) with the well-studied prebiotic inulin (IN) in two in vitro models of the human gut, the SHIME and the TIM-2. All experiments and microbial activity and community analysis were performed according to the standard lab practices as developed in the respective laboratories (LabMET and TNO). Results were compared with those obtained in a similar in vivo study with humanized rats [11] (= germfree rats inoculated with human faeces) treated with the same IN and LC-AX during similar incubation times as the SHIME study, while also being inoculated with a faecal sample of the same donor as during this SHIME study. The aims were

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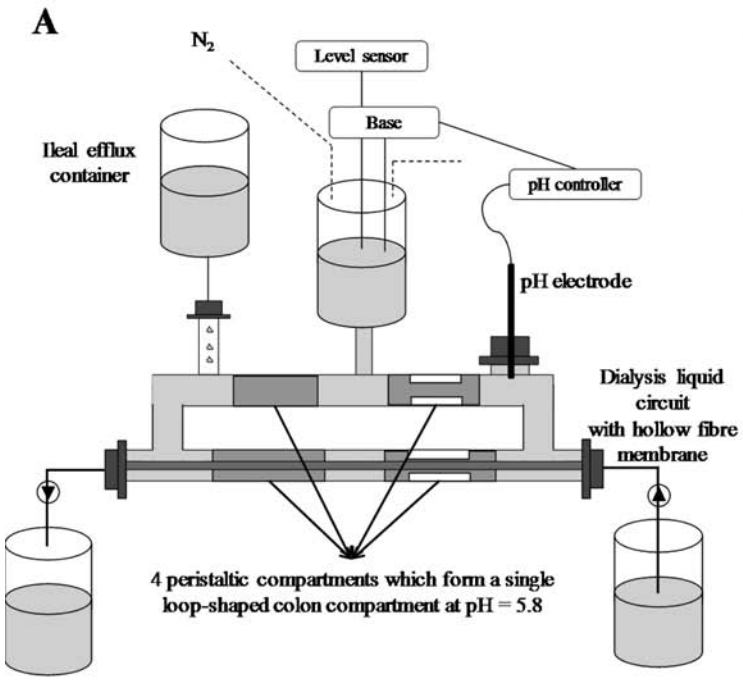

	TIM-2 (A)	SHIME (B)
Stabilization period	16 h	14 d
Control period	/	14 d
Treatment period	3 d	21 d
Washout period	/	14 d
Inoculum	✓ Mix of 10 subjects ✓ Frozen	✓ 1 subject ✓ Fresh
Test compound	Additional energy source	Replaces part of the starch
Dosage test compound/d	5 g/d	1.3 g/d
Concentration test compound in reactor	5 g/ 120mL = 41.7 g/L	1.3 g/ 500mL = 2,6 g/L
Mucins in nutritional medium	No	Yes
Dialysis	Yes	No
Mixing	Peristaltic movements	Stirring

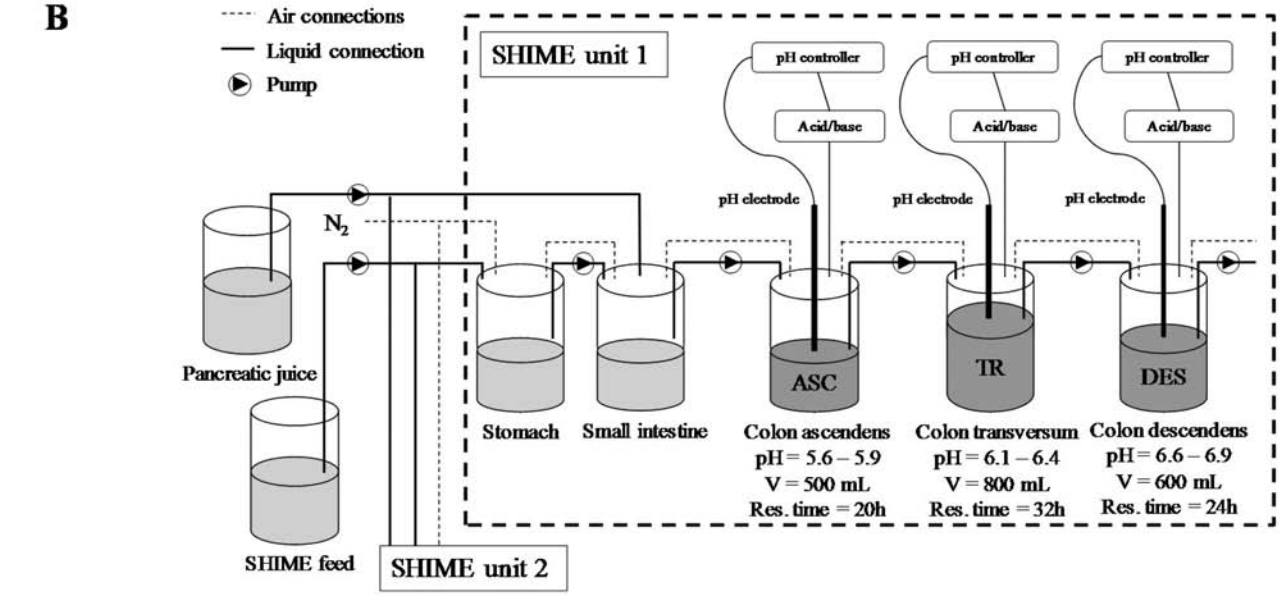

Figure 1. (A) Schematic representation of the TIM-2 and (B) the Twin-SHIME.

to evaluate the consistency of LC-AX and IN to stimulate (i) the production of specific SCFA, and (ii) specific *Bifidobacterium* species. Further, during the long-term study with the SHIME, we additionally assessed (iii) in which colon region both carbohydrates are fermented, and (iv) how reversible the intestinal microbiota was modified upon such a long-term treatment.

■ MATERIALS AND METHODS

Chemicals and Carbohydrates. The medium components for the TIM-2 experiment were provided by Tritium Microbiology (Veldhoven, The Netherlands). Other chemicals were obtained from Sigma (Bornem, Belgium) unless stated otherwise. Cosucra (Warcoing, Belgium) provided IN (Fibruline instant) with a purity of 92% and a degree of polymerization between 3 and 60 with an average of 10. Bioactor (Gent, Belgium) provided a concentrate of water-extractable LC-AX with a purity of 60%, a degree of substitution of 0.7, a high degree of polymerization (>60), and a high average molecular weight of approximately 20 kDa. The remaining fraction consists of proteins, fat, and other carbohydrates (e.g., arabinogalactan and β-glucan).

Description of in Vitro Models for the Human Gastro-Intestinal Tract. Two continuous in vitro models for the human intestinal tract were compared: the TIM-2 (TNO, The Netherlands) and the SHIME(R) (Ghent University-Prodigest, Belgium) (Figure 1). Both studies were performed according to their respective standard procedures.

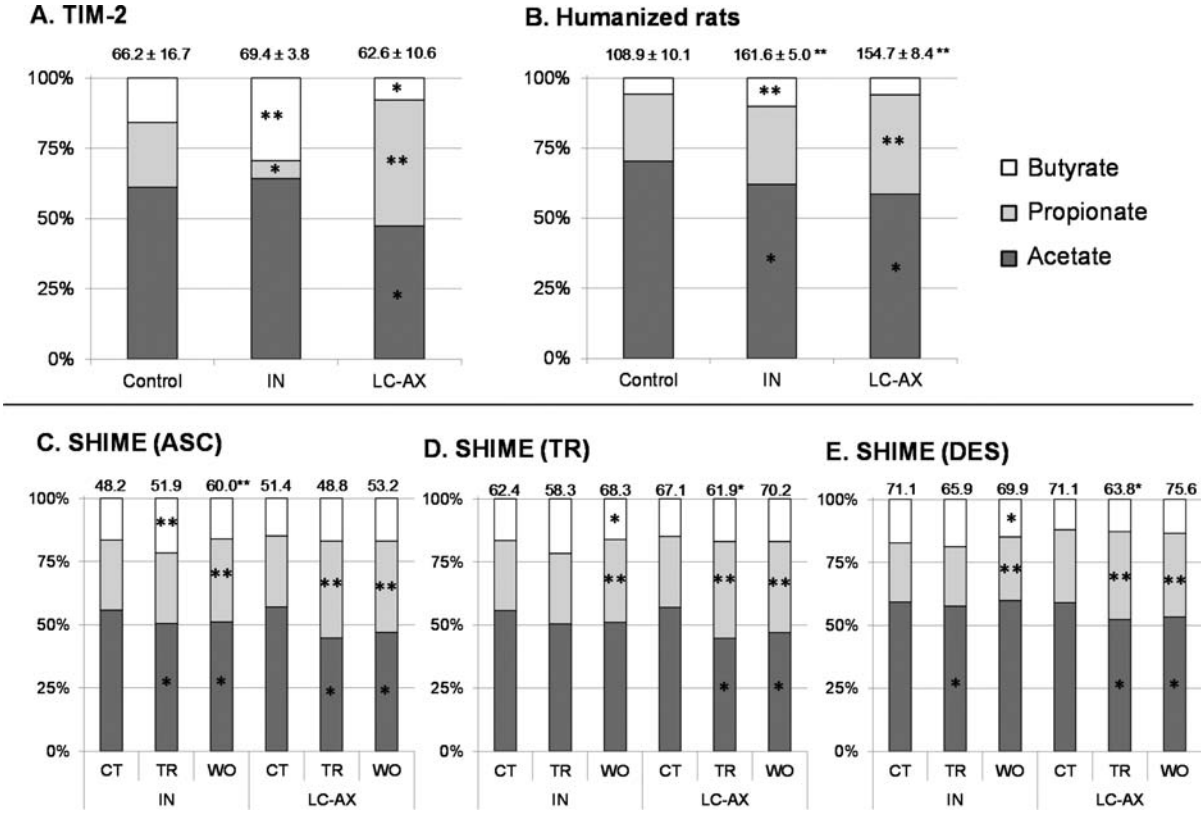

Figure 2. SCFA ratios upon IN/LC-AX treatment in two in vitro (TIM-2 and SHIME) and one in vivo (humanized rats) human gut model. Absolute SCFA levels are indicated above each bar (mmol in A; mM in B–E) (A) Average sum of the SCFA levels in the luminal content and dialysate of the TIM-2 model between several time points after IN/LC-AX treatment (0–24, 24–48, and 48–72 h; $n = 3$). Further, the figure contains SCFA levels in the ascending (C), transverse (D), and descending colon (E) of the SHIME during the control (CT; 2 weeks, $n = 6$), IN/LC-AX treatment (TR; 3 weeks, $n = 9$) and washout (WO; 2 weeks, $n = 6$), and the average cecal SCFA levels upon a 3-week treatment with IN/LC-AX ($n = 8$), as adapted from Van den Abbeele et al. (2011) [11] (B) Significant decreases compared to the control are indicated by *, while significant increases are indicated by ** ($p \leq 0.05$).

The TIM-2 simulates the proximal colon in a loop-shaped system (pH = 5.8), consisting of four glass units with a flexible wall inside allowing peristaltic movements.[9,24,28] The system is at 37 °C, kept anaerobic by flushing with N_2, and a dialysis liquid is pumped through hollow fibers in the lumen of the reactor. A standardized TIM-2 inoculum was used, which was created by pooling stools from 10 healthy adults (34.6 ± 3.7 y) in a fed-batch fermentor.[28] After snap-freezing in liquid N_2, it was stored at −80 °C. At the start of each experiment, 30 mL inoculum and 80 mL of two times diluted Standard Ileal Efflux Medium (SIEM) were combined. SIEM was slightly modified compared to its description by Gibson et al. [29] (g/L): pectin (9.4), xylan (9.4), arabinogalactan (9.4), amylopectin (9.4), casein (47.0), starch (78.4), Tween 80 (34.0), bactopepton (47.0), and ox-bile (0.8). The dialysis liquid contained (g/L) $K_2HPO_4 \cdot 3H_2O$ (2.5), NaCl (4.5), $FeSO_4 \cdot 7H_2O$ (0.005), $MgSO_4 \cdot 7H_2O$ (0.5), $CaCl_2 \cdot 2H_2O$ (0.45), bile (0.05), cysteine.HCl (0.4), and 1 mL vitamin mixture containing (mg/L) menadione (1.0), D-biotin (2.0), vitamin B12 (0.5), pantothenate (10.0), nicotinamide (5.0), *p*-aminobenzoic acid (5.0), and thiamine (4.0). The microbes were allowed to adapt to the in vitro conditions during a 16 h stabilization period, followed by a 2 h starvation and three-day treatment period. The feeding rate was 2.5 mL/h and during the first 8 h of each day, 5 g IN or 5 g LC-AX in sugar-depleted SIEM was added in the treatments, while sugar-depleted SIEM as such was added in the control. The remaining 16 h of the day, standard SIEM was given. After 24 and 48 h, 25 mL was removed from the lumen to simulate passage to the distal colon. Samples for metabolic analysis (SCFA) were taken from the lumen and the dialysate at the start (0 h) and after 24, 48, and 72 h of treatment. Samples for microbial community analysis with the Intestinal-Chip (I-Chip) were collected at 0 h and 72 h.

The SHIME (registered name from Ghent University and ProDigest) consists of five double-jacketed vessels, simulating the stomach, small intestine, and three colon regions (ascending at pH = 5.6–5.9; transverse at pH = 6.15–6.4; descending at pH = 6.6–6.9).[25,27] The vessels are held at 37 °C, stirred, and kept anaerobic by flushing the headspace with N_2. All colon regions were inoculated with a faecal sample of one healthy volunteer (23 y, m). Three times per day, 140 mL nutritional medium and 60 mL pancreatic juice was added to the stomach and small intestinal compartment, respectively. The nutritional medium contained (g/L) arabinogalactan (1.0), pectin (2.0), xylan (1.0), starch (4.0), glucose (0.4), yeast extract (3.0), peptone (1.0), mucin (4.0), and cystein (0.5). The pancreatic juice contained (g/L) $NaHCO_3$ (12.5), bile salts (6.0) (Difco, Bierbeek, Belgium) and pancreatin (0.9). A Twin-SHIME setup, consisting of two parallel SHIME-units, was used to attain similar microbiota in both units at the start of the treatment.[27] An initial two-week stabilization allowed faecal microbes to adapt to the in vitro conditions and to evolve to a colon region-specific microbiota.[27] Unlike the TIM-2 model, steady state conditions prevail at the end of this stabilization period. This was followed by a control period (day 1–14), under the same conditions as the stabilization period and a treatment period (day 15–35), during which the nutritional medium had a lower starch content (4.0 → 1.0), while supplemented with 3.0 g IN/L and 3.0 g LC-AX/L for the first and second SHIME-unit, respectively. Finally, both units were fed the initial nutritional medium during a wash-out period (day 36–49). During the control, treatment and wash-out period, samples were taken for metabolic activity (SCFA, $n = 3$/wk) and community composition analysis (plate counts, $n = 3$/wk; DGGE, $n = 1$/wk).

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Microbial Community Analysis: I-Chip, Plate Counts and DGGE. The Intestinal-Chip (I-chip) is a phylogenetic microarray, which is standardly applied to monitor microbial shifts in the TIM-2. The I-Chip was executed as described by Ladirat et al.[30] Briefly, DNA was isolated using a commercial kit (Agowa, Germany). Subsequently, DNA was labeled and hybridized to arrays. After washing, the arrays were scanned and analyzed. Imagene 5.6 software (BioDiscovery, Marina del Rey, CA) was used for data analysis. Signals were quantified by calculating the mean of all pixel values of each spot and calculating the local background around each spot. For each spot, a signal to background ratio was calculated. For further analysis, spots which had a minimal number of observations more than four times above its local background were selected. When multiple values are listed for a given species, multiple probes were designed for different variable regions. Due to differences in accessibility of variable regions in the 16S rRNA molecule during hybridization, these probes hybridize to a different degree so that results of different probes of a given bacterial species may differ. However, rather than averaging effects of different probes into a single value per species, individual values are presented.

Plate counts were performed to assess microbial changes in the SHIME: brain–heart infusion agar (facultative anaerobes and anaerobes, Oxoid, Hampshire, U.K.), raffinose *Bifidobacterium* agar (bifidobacteria),[31] LAMVAB agar (lactobacilli),[32] tryptose sulfite cycloserin agar (clostridia, Merck, NJ), *Enterococcus* agar (enterococci, Difco, Lawrence), mannitol salt agar (staphylococci, Oxoid) and McConkey agar (coliforms, Oxoid). Samples were serially diluted in saline solution (8.5 g/L NaCl), after which plates were inoculated and incubated at 37 °C (43 °C for coliforms). Anaerobic incubations were performed in jars with a gas atmosphere (84% N_2, 8% CO_2 and 8% H_2) regulated by the Anoxomat 8000 system (Mart, Sint-Genesius-Rode, Belgium).

Denaturing Gradient Gel Electrophoresis (DGGE) was applied to monitor shifts within the SHIME microbiota. After DNA extraction,[33] DGGE was applied on PCR products for the total microbiota,[34] bifidobacteria, and lactobacilli. For the latter two groups, a PCR with group-specific primers[35,36] was followed by a nested PCR with general bacterial primers.[34] Gels had a denaturating gradient from 45% to 60% and were run using an Ingeny PhorU apparatus (Ingeny International, Goes, The Netherlands). Bands of interest were cut from the gel and sequenced (Agowa, Berlin, Germany). Further analysis was carried out using BioNumerics version 5.10 (Applied Maths, Sint-Martens-Latem, Belgium). Pearson correlation and UPGMA clustering algorithm were used to calculate dendrograms, taking into account both band position and band density.

Metabolic Activity Analysis: Short-Chain Fatty Acids (SCFA). SCFA analysis of TIM samples was performed as reported by Van Nuenen et al.,[9] while SHIME samples were analyzed according to De Weirdt et al.[37] Because the TIM-2 simulates absorption, a significant amount of the SCFA which are produced in the simulated colon arrive in the dialysate. Therefore, in contrast to the SCFA results of the SHIME which are expressed as concentrations (mM), SCFA results of the TIM-2 are expressed as the sum of the daily produced amount in both the intestinal content and the dialysate (mmol). Finally, as there was a linear increase in the SCFA production in the TIM-2 during the 3-day incubation period (Figure S1 of the Supporting Information, SI), TIM-2 results are based on average produced amounts per day.

Statistics. All data were analyzed using SPSS 16 software (SPSS Inc., Chicago, U.S.). Normality and homogeneity of variances were studied with a Kolmogorov–Smirnov and Levene test, respectively. If so, an Anova with (post hoc) Bonferroni test was performed to investigate intergroup differences, while otherwise a Kruskal–Wallis with Mann–Whitney test was applied. Differences were significant if $p \leq 0.05$.

RESULTS

TIM-2: Metabolic Activity in Terms of SCFA. The microbial activity in the TIM-2 was monitored in terms of absolute (Figure S2A of the SI) and proportional SCFA production (Figure 2A). During the 72 h treatment period, both LC-AX and IN resulted in a similar total SCFA production of around 70 mmol per day. In contrast to LC-AX which resulted in a high propionate (31.2 mmol/d) and low butyrate production (5.3 mmol/d), IN resulted in a low propionate (4.2 mmol/d) but high butyrate production (19.4 mmol/d). When converting these values to proportional values, it was confirmed that LC-AX specifically stimulated propionate, while IN rather increased butyrate levels.

TIM-2: Quantification of the Microbiota Using the I-Chip. The I-Chip was used to compare shifts in the microbial community by LC-AX and IN within TIM-2 compared to a control experiment (Table 1). All initial samples (0 h) clustered

Table 1. Ratio of Bacterial Groups at the Final Time Point of Incubation (72 h) That Were at Least 4-Fold Different during a Treatment with LC-AX or IN Compared to a Control Experiment with the TIM-2 Model, Based on the I-Chip Analysis[a]

	bacterial species/group	IN	LC-AX
Actinobacteria	Bifidobacteriaceae	**10.0**	**4.2**
	Bifidobacterium catenulatum	**5.0–14.9**	2.4
	Bifidobacterium dentium	1.0	**0.2**
	Bifidobacterium longum	0.4–3.0	**44.1–394.9**
	Bifidobacterium adolescentis/ Bifidobacterium angulatum	**10.3**	1.5
	* *Bifidobacterium adolescentis*	**7.7–39.6**	0.9–1.1
	* *Bifidobacterium angulatum*	**6.1–16.1**	1.2
	Bifidobacterium bifidum	**4.3–34.0**	1.6–1.7
	Bifidobacterium group 1	**11.5**	1.0
	Bifidobacterium group 2	**28.7**	2.5
Bacteroidetes	Bacteroidales	1.0	**34.7**
	Bacteroides group	**0.2**	**0.2**
	* *Bacteroides ovatus*	**0.1**	0.1
	* *Bacteroides thetaiotaomicron*	**0.1**	0.3
	* *Bacteroides vulgatus*	**0.2**	1.0
	Prevotella bivia	**0.1**	**0.1**
Firmicutes	*Clostridium leptum*	1.1	**0.2**
	Clostridium sporogenes	1.0	**4.9**
	Clostridium sporogenes, C. botulinum	1.6	**0.2**
	Clostridium group 2	**4.1**	**4.5**
	Ruminococcus bromii	**0.1**	**18.1**
	Ruminococcus obeum	0.5	**0.1**
	Eubacterium group 1	9.5	2.9
	Lactobacillus sakei	**4.4**	1.6
	Lactococcus lactis subsp. *cremoris*	0.4	**0.1**
	Streptococcus group 4	**0.1**	1.3
	Carnobacterium group 1	3.0	**9.7**
Proteobacteria	*Enterobacter cloacae/ Serratia marcesens Salmonella typhi*	**7.1**	**0.2**
	Klebsiella group 1	0.4	**0.2**
	Salmonella typhi/ Klebsiella pneumonae	**9.4**	0.3
	β-Proteobacteria	**0.2**	1.1

[a]When two values are listed for a given bacterial species, probes were designed for different variable regions of the 16S rRNA gene.

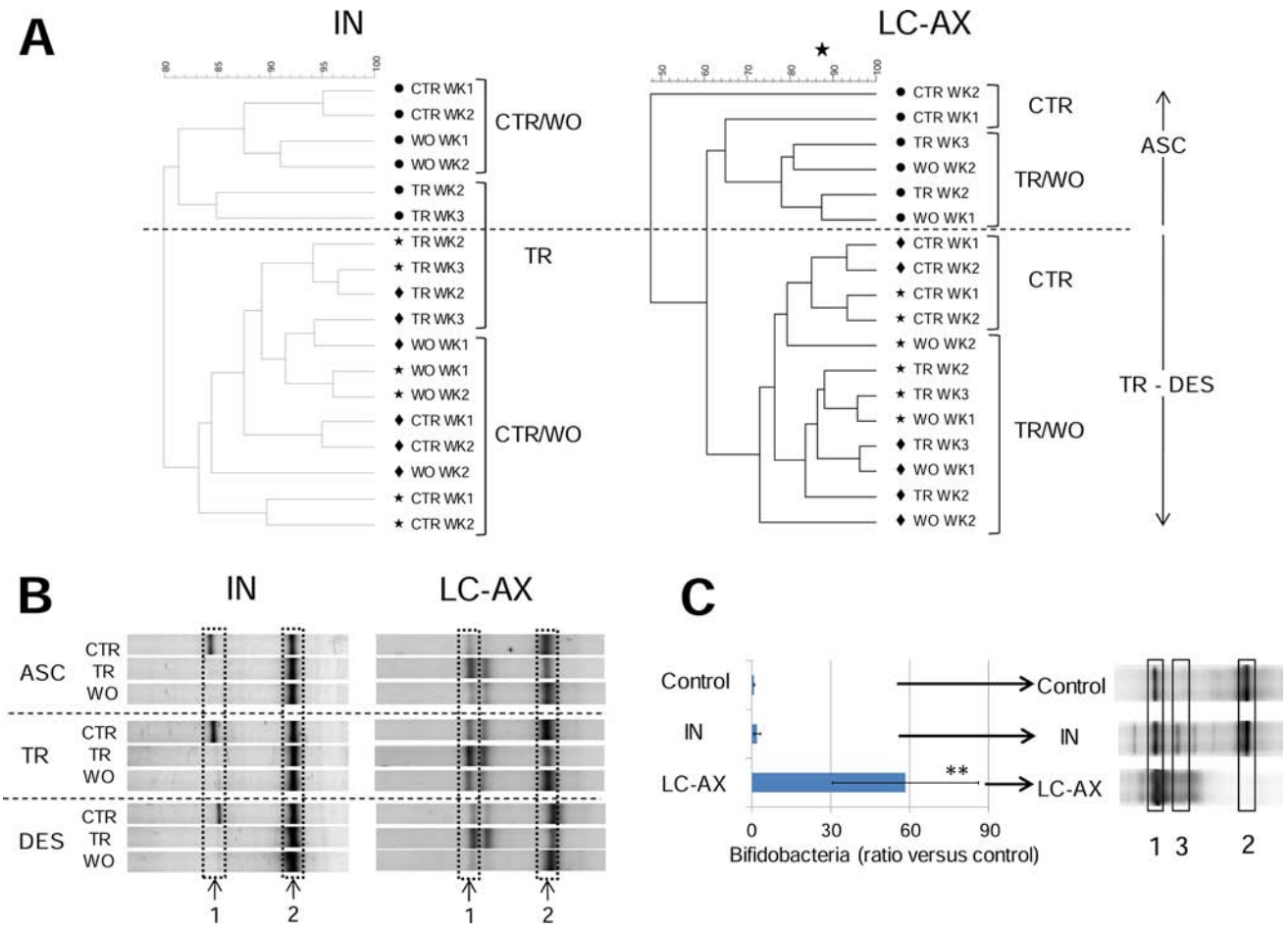

Figure 3. (A) Clustering tree of the DGGE-profiles in the ascending (●), transverse (★), and descending (♦) colon of a SHIME during the control (CO; 2 wks), treatment with IN or LC-AX (TR; 3 wks) and wash-out (WO; 2 wks), and (B) corresponding group-specific DGGE profiles for bifidobacteria (1 = *B. longum*; 2 = *B. adolescentis*). (C) Absolute cecal bifidobacteria levels as determined with the HITChip and expressed as a ratio compared to the control. Significant increases compared are indicated by ** ($p \leq 0.05$). Also representative *Bifidobacterium*-specific DGGE profiles in the cecum of control and IN/LC-AX treated humanized rats (3 = *B. bifidum*) are shown, as adapted from Van den Abbeele et al. (2011).[11]

together (data not shown). Both LC-AX and IN increased bifidobacteria, but whereas LC-AX specifically increased *B. longum* (~factor 400), IN increased a spectrum of *Bifidobacterium* species including *B. catenulatum, adolescentis, angulatum*, and *bifidum* (~factor 10). While members of the Bacteroidetes generally decreased during both treatments, members of the Firmicutes responded species-dependently. Regarding the latter phylum, a distinct difference between LC-AX and IN was the stimulation of *Ruminococcus bromii* by LC-AX, while IN strongly decreased the abundance of this species. Further, LC-AX generally lowered abundances of opportunistic pathogens belonging to the Proteobacteria, while IN increased several Proteobacteria species.

SHIME: Metabolic Activity in Terms of SCFA. As opposed to the TIM-2 model, where test compounds are added as an additional energy source, test compounds in the SHIME model are administered as a replacement of part of the starch (3.0 g/L). As the SHIME microbiota has reached steady-state conditions upon the 2-week stabilization period, this allows to observe subtle changes caused by qualitative changes in the diet. These subtle changes included that upon LC-AX treatment, acetate levels decreased in all colon regions of the SHIME (Figure S2C−E of the SI). This went along with significantly higher propionate concentrations in the ascending and transverse colon, while butyrate levels remained unaffected. After treatment, acetate levels returned to baseline, while propionate levels remained high and butyrate levels increased. This also followed from the proportional SCFA shifts (Figure 2C−E): decreased acetate ratios and increased propionate ratios in all three colon regions, both during treatment and washout.

Upon IN treatment, butyrate levels increased in the ascending colon and absolute acetate levels decreased only in the descending colon (Figure S2C−E of the SI). Propionate concentrations remained unaffected. This was also reflected by lower acetate and higher butyrate ratios in the ascending colon during treatment (Figure 2C−E). After the treatment, acetate and propionate levels increased, while butyrate decreased, again reflected by the proportional changes.

SHIME: Microbial Community Analysis Using DGGE and Plate Counts. DGGE was applied to compare the total bacterial composition, as well as the qualitative changes within the *Lactobacillus* and, especially *Bifidobacterium* communities of the LC-AX and IN-treated SHIME-units. When accounting for all three types of DGGE profiles, a clear separation between samples of the ascending colon was found as opposed to samples of the transverse and descending colon, both for LC-AX and IN (Figure 3A). Upon treatment, LC-AX and IN induced a distinct change of the microbial composition within both clusters. While for IN, samples of the washout period

Table 2. Average (± SD) Abundances (log CFU/mL) of Bacterial Groups in the Ascending, Transverse and Descending Colon of the SHIME during the Control (CT; 2 weeks, $n = 6$), Treatment with IN or LC-AX (TR; 3 weeks, $n = 9$) and Wash-Out Period (WO; 2 weeks, $n = 6$)[a]

		IN			LC-AX		
		ascending	transverse	descending	ascending	transverse	descending
facultative anaerobes	CT	7.48 ± 0.02	7.52 ± 0.13	7.37 ± 0.27	7.52 ± 0.26	7.32 ± 0.16	7.42 ± 0.38
	TR	7.58 ± 0.10	7.01 ± 0.24	6.84 ± 0.59	7.77 ± 0.43	7.60 ± 0.31	7.45 ± 0.58
	WO	8.02 ± 0.30	7.10 ± 0.19	6.99 ± 0.04	7.52 ± 0.17	7.31 ± 0.29	7.16 ± 0.02
anaerobes	CT	8.03 ± 0.79	7.88 ± 0.55	7.81 ± 0.56	7.98 ± 0.77	7.82 ± 0.83	7.78 ± 0.65
	TR	7.74 ± 0.28	7.37 ± 0.27	7.04 ± 0.46	8.02 ± 0.56	7.44 ± 0.44	7.63 ± 0.43
	WO	7.84 ± 0.13	7.60 ± 0.11	7.34 ± 0.27	7.59 ± 0.16	7.21 ± 0.19	7.50 ± 0.19
Bifidobacteria	CT	5.89 ± 0.15	5.87 ± 0.13	5.69 ± 0.03	5.99 ± 0.06	5.78 ± 0.02	5.51 ± 0.14
	TR	**6.67 ± 0.21**	**6.52 ± 0.29**	6.04 ± 0.42	**6.82 ± 0.14**	**6.35 ± 0.26**	**6.36 ± 0.30**
	WO	6.23 ± 0.21	5.95 ± 0.03	5.78 ± 0.09	**6.70 ± 0.06**	**6.15 ± 0.04**	**6.34 ± 0.04**
Clostridia	CT	7.42 ± 0.02	6.82 ± 0.63	7.30 ± 0.02	7.36 ± 0.05	7.14 ± 0.12	7.29 ± 0.21
	TR	7.42 ± 0.09	6.98 ± 0.24	**6.65 ± 0.47**	7.37 ± 0.06	7.14 ± 0.34	7.12 ± 0.30
	WO	7.64 ± 0.07	7.03 ± 0.06	6.78 ± 0.10	7.37 ± 0.07	6.79 ± 0.05	6.86 ± 0.04
Lactobacilli	CT	4.06 ± 0.19	3.70 ± 0.06	3.32 ± 0.03	3.54 ± 0.09	3.33 ± 0.26	3.38 ± 0.42
	TR	**5.75 ± 0.30**	**5.55 ± 0.31**	**5.13 ± 0.54**	**5.57 ± 0.37**	**5.70 ± 0.62**	**5.22 ± 0.21**
	WO	5.37 ± 0.48	**5.29 ± 0.30**	**4.70 ± 0.40**	**5.49 ± 0.46**	**4.77 ± 0.39**	**4.81 ± 0.16**
Enterococci	CT	6.08 ± 0.13	6.20 ± 0.18	6.20 ± 0.27	5.69 ± 0.39	5.76 ± 0.13	5.83 ± 0.04
	TR	**5.38 ± 0.16**	**5.18 ± 0.11**	5.04 ± 0.06	5.48 ± 0.51	5.33 ± 0.33	**5.11 ± 0.12**
	WO	5.34 ± 0.20	5.20 ± 0.28	4.84 ± 0.27	5.01 ± 0.05	4.86 ± 0.22	**4.91 ± 0.16**
coliforms	CT	7.28 ± 0.14	7.18 ± 0.09	7.16 ± 0.13	7.27 ± 0.16	7.21 ± 0.16	7.07 ± 0.16
	TR	7.39 ± 0.16	6.94 ± 0.19	6.75 ± 0.47	7.34 ± 0.12	7.22 ± 0.30	7.19 ± 0.26
	WO	7.38 ± 0.14	6.75 ± 0.38	6.95 ± 0.09	7.27 ± 0.04	7.01 ± 0.11	6.97 ± 0.04
Staphylococci	CT	6.35 ± 0.09	6.27 ± 0.11	5.88 ± 0.33	6.14 ± 0.76	5.98 ± 0.68	6.14 ± 0.52
	TR	6.52 ± 0.28	6.11 ± 0.32	5.69 ± 0.66	6.61 ± 0.68	6.30 ± 0.66	6.41 ± 0.44
	WO	6.56 ± 0.09	5.85 ± 0.07	5.38 ± 0.29	6.22 ± 0.46	6.19 ± 0.18	5.98 ± 0.04

[a]A significant increase or decrease compared to the control period is indicated in bold ($p \leq 0.05$).

clustered together with those of the control period, samples of the washout period of LC-AX still clustered together with those of the treatment period. Further, whereas IN induced a shift toward *B. adolescentis*, LC-AX enhanced the presence of *B. longum* (Figure 3B). *L. plantarum* was the dominant *Lactobacillus* species in both SHIME-units (data not shown).

Plate counts were performed to assess the effect of IN and LC-AX on several bacterial groups in the SHIME (Table 2). Both LC-AX and IN selectively increased the levels of bifidobacteria and lactobacilli but these increases were only maintained during the washout period with LC-AX. Further, IN lowered the abundance of enterococci in the ascending and transverse colon, and those of clostridia in the descending colon. LC-AX decreased the abundance of only one bacterial group, i.e., the enterococci in the descending colon.

DISCUSSION

In this study, we compared the fermentation of water-extractable LC-AX with the well-established prebiotic IN using two distinct in vitro models that are frequently used to evaluate modulation of the human intestinal microbiota [38] (TIM-2 and SHIME). These models were operated according to their respective standard procedures. This includes that while the TIM-2 was inoculated by a pooled frozen faecal sample allowed to stabilize over 16 h, the SHIME was inoculated by a single, fresh faecal sample, which is allowed to adapt to the in vitro conditions over 2 weeks (Figure 1). Further, the TIM-2 simulates peristaltic movements and absorption of metabolites in a single colon compartment, while the SHIME simulates the colonic microbiota in three consecutive colon regions, which allows specifying the location of a treatment effect. Other important differences are that the TIM-2 is limited to short-term studies (3d) so that high concentrations of a test compound are used, while the SHIME considers long-term studies (7 weeks), so that lower concentrations are used (factor 16) in order to observe more gradual changes over longer time periods. This also allows us to evaluate the persistence of the treatment during a washout period (2 weeks). Despite these differences in setup and despite the use of specific in-house methods for SCFA and microbial community analysis (I-chip vs DGGE), both models revealed a compound-specific modulation in vitro microbiota by LC-AX and IN in terms of SCFA production and stimulation of specific *Bifidobacterium* species. Moreover, it was particularly interesting to compare the obtained in vitro results with a recent in vivo study with humanized rats (= germfree rats inoculated with human faeces) treated with the same IN and LC-AX, while being inoculated with a faecal sample of the same donor as during the current SHIME study.

Regardless of what in vitro model was used, LC-AX specifically stimulated *B. longum* (Table 1 and Figure 3B). In contrast, upon administration to the SHIME, IN correlated with *B. adolescentis*, while in the TIM-2, IN correlated with a broad spectrum of *Bifidobacterium* species. This corresponded with the humanized rat study where HITChip analysis on the cecal microbiota revealed that LC-AX specifically targeted the bifidobacteria (Figure 3C).[11] Furthermore, in this humanized rat study, LC-AX also specifically stimulated *B. longum*, both in the intestinal lumen and mucus layer, while IN did not modify the bifidobacteria community compared to the control (*B. longum*, *B. adolescentis*, and *B. bifidum*) (Figure 3C). *B. longum* has been shown an excellent AX-degrader in vitro[39,40] and

possesses endoxylanases to degrade AX.[41] Also in a recent study where IN was administered to 12 human volunteers, *B. adolescentis* was specifically upregulated by IN.[42] This specific species has been shown to degrade IN through for instance β-fructofuranosidases.[43] The structurally different LC-AX and IN thus targeted specific bifidobacteria species. Especially the strong and specific stimulation of *B. longum* with LC-AX stresses the potential of this compound which should be further evaluated in relation to its potential beneficial effects on host health.

Another consistent finding between both in vitro models was that LC-AX selectively increased the production of propionate (Figure 2A,C−E), which again corresponded with the humanized rat study (Figure 2B). Increased propionate levels upon AX treatment have already been described in vitro,[10,20] as well as in vivo.[44] This health-promoting metabolite has been shown to lower cholesterol absorption.[45] Moreover, it can be absorbed from the intestine and, via bloodstream, reach the liver,[46] where it has been associated with reduced cholesterol synthesis and improved insulin sensitivity in humans and rats.[47,48] Propionate has also been shown to increase leptin levels and reduce inflammation in adipose tissue ex vivo.[49,50] The increase of propionate with LC-AX, as observed in the current study, may thus partly explain following beneficial effects of LC-AX that were recently established. Clinical trials demonstrated a beneficial effect of LC-AX on glucose and insulin response, both in healthy [51] and in prediabetic human volunteers.[52] Moreover, an in vivo study with diet-induced obese mice revealed that LC-AX counteracts the adverse effect of a high-fat diet as it significantly lowered the inflammatory tone, total cholesterol, adiposity, and body weight gain.[53] These beneficial effects were attributed to an improved gut barrier function due to the increase of bifidobacteria with LC-AX. However, as propionate levels were not determined, propionate may also have attributed to the reported beneficial effects. Future in vivo studies should thus try to establish this connection between propionate production and health benefits of LC-AX.

Regardless of the in vitro model used, IN selectively increased the production of butyrate (Figure 2A/C), which was also the case in the humanized rat study (Figure 2B). Higher butyrate levels after IN supplementation have been observed during both in vitro[8,10,20] and in vivo studies.[54,55] Butyrate acts as an important energy source for colonocytes [56] and may protect against colon cancer development.[3] Moreover, a depletion in butyrate or butyrate producing microbes has been related to human patients suffering from inflammatory bowel diseases such as Crohn's disease.[2,4] IN thus selectively increased this beneficial metabolite with potentially important health benefits for the human host.

The SHIME model was additionally used to evaluate some other aspects of IN and LC-AX fermentation. First, the main colon region of butyrate stimulation by IN was the ascending colon, while LC-AX induced higher propionate levels along the different colon regions (Figure 2C−E). Previous studies have shown that short-chain AX, arabinoxylo-oligosaccharides (AXOS), are specifically degraded in the transverse colon due to inhibition of the involved enzymes in the ascending colon.[10] Such inhibition phenomena might also apply to the LC-AX, causing a more gradual degradation of LC-AX along different colon regions. Furthermore, a more distal fermentation might be caused by the high molecular weight of LC-AX, as was for instance seen for Polydextrose.[57,58] Extending the sugar fermentation toward distal colon regions has been an important goal of functional foods in order to deal with colonic diseases which often localize in this area.[13] While the proximal colon is generally the site of sugar fermentation and beneficial SCFA production, the distal colon is characterized by proteolytic activity resulting in higher concentrations of more hazardous compounds and a higher pH.[59] Because the main site of IN fermentation is the ascending colon and that of LC-AX extends to the consecutive colon regions, IN and LC-AX might also be complementary in achieving health-promoting effects along the entire colon.

The SHIME model was also used to evaluate the persistence of the prebiotic effects after the treatment was stopped. On the basis of comparison of the washout and control period, it was found that the effects of LC-AX on the in vitro microbiota were more permanent than those of IN. This was the case for the production of propionate (Figure 2 C−E) and the stimulation of lactobacilli and bifidobacteria (Table 2). Moreover, the overall community composition changes as assessed with DGGE were more profound and more permanent for LC-AX. This more permanent effect may be of interest in prolonging a prebiotic effect.

Until now, only few studies investigated whether wheat-derived LC-AX could have beneficial effects on host health by modulating the intestinal microbiota. Using two distinct in vitro models for the human gut, we show that a specific concentrate of water-extractable LC-AX and the well-established prebiotic IN may be complementary as they both induced specific fermentation patterns within the intestinal microbiota with specific potential health benefits. While LC-AX specifically increased *B. longum* and propionate production, IN increased *B. adolescentis* (among other bifidobacteria) and butyrate levels. Future research should establish how widespread these specific microbial responses to LC-AX and IN are among different human subjects. This can be achieved by in vivo trials or by large-scale in vitro studies during which faecal samples of multiple individuals are tested. Finally, even if a direct extrapolation of the present study to humans is not straightforward, our results suggest that besides IN, LC-AX seem to fulfill the requirements to be considered a promising prebiotic compound and that they might confer beneficial health effects through gut microbiome modulation, potentially in a more specific and potent manner as compared to IN.

■ ASSOCIATED CONTENT

Ⓢ Supporting Information

Figure S1 demonstrates the linear increase of the SCFA levels in the TIM-2 model. Figure S2 contains the absolute SCFA values of TIM-2, SHIME and humanized rat samples, of which the proportional values are already depicted in Figure 2. This information is available free of charge via the Internet at http://pubs.acs.org.

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Notes

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■ ABBREVIATIONS USED

DGGE,Denaturing Gradient Gel Electrophoresis; IN,Inulin; I-chip,Intestinal-Chip; LC-AX,Long-chain arabinoxylans; SCFA,-Short-Chain Fatty Acids; SHIME,Simulator of the Human Intestinal Microbial Ecosystem; SIEM,Standard Ileal Efflux Medium; TIM-2,TNO in vitro model of the colon

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