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# Oral Administration of Live Exopolysaccharide-Producing *Pediococcus parvulus*, but Not Purified Exopolysaccharide, Suppressed *Enterobacteriaceae* without Affecting Bacterial Diversity in Ceca of Mice

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**Growing evidence indicates that the gut microbiota could have an important role in the development of diet- and lifestyle-induced diseases. It has been shown that modulation of the gut microbiota by means of probiotics and prebiotics could improve host health. An oat-based product fermented by the exopolysaccharide (EPS)-producing organism *Pediococcus parvulus* 2.6 has been reported to have a bifidogenic effect. To find out whether the effect could be attributed to the EPS or the bacterium, mice were fed a diet supplemented with 2% purified EPS or 10<sup>8</sup> CFU/g of live *P. parvulus* 2.6 for 6 weeks. Both supplementations altered the gut microbiota composition but in different directions. Purified EPS not only significantly lowered the microbial diversity ( $P < 0.001$ ) but decreased the bifidobacterial population ( $P = 0.01$ ). In contrast, the live EPS-producing bacterium *P. parvulus* 2.6 antagonized *Enterobacteriaceae* without disturbing the homeostasis of the cecal microbiota.**

As the worldwide burden of diet- and lifestyle-induced diseases, such as obesity, type 2 diabetes, and cardiovascular diseases, increases, research on functional foods sheds light on possibilities to improve host health. Under the concept of functional foods, prebiotics and/or probiotics are often used as means to regulate the gut microbiota, which is perceived as a key player in promoting the health of the host. The gut microbiota provides several thousands of extra genomes, more than the human genome itself (1), and could be viewed as an additional organ providing genetic and metabolic features (2). Probiotics are live microorganisms that provide a beneficial effect to the host when administered in sufficient amounts. Survival through the passage in the gastrointestinal tract and the ability to proliferate and colonize the digestive tract are prerequisites that need to be fulfilled by these organisms (3). Most commonly, certain strains of *Lactobacillus* and *Bifidobacterium* are used as probiotics, and some strains have been shown to affect the composition of the gut microbiota, primarily by increasing the gut load of lactobacilli (4–7) and bifidobacteria (8–10) in both animals and humans. Furthermore, the bacterial diversity of the human rectal mucosa may be increased after consumption of lactobacilli (11).

The gut microbiota composition can be affected by the availability of fermentable carbohydrates reaching the colon (12). Digestive fibers labeled as prebiotics are fibers that provide a selective stimulation of health-beneficial components of the indigenous microbiota. The bacterial taxa currently used to measure prebiotic effects are bifidobacteria and lactobacilli, but other genera can be of interest as soon as their health-beneficial influence has been proven. Most beneficial health effects associated with prebiotics have so far been derived from studies on inulin-type fructans and galacto-oligosaccharides; however, this does not mean that other substances can be excluded from having prebiotic effects (13). Well-known prebiotics have been reviewed by Patel and Goyal, and some new prebiotic options have been pointed out showing

the possibility of finding new prebiotics (14). One such option could be exopolysaccharides (EPS), which are polymeric carbohydrates produced and excreted by various microorganisms. EPS are used as stabilizers and viscosifiers in food due to their unique physical properties (15), and the physical and technological properties of EPS have been extensively studied. However, little is known about the effect of EPS on the gut microbiota *in vivo*. Attempts to evaluate the prebiotic capacity of some EPS have been made *in vitro* but with inconsistent results. Certain EPS produced by lactobacilli (16–18) and bifidobacteria (12, 19) modulate the intestinal flora positively; however, not all EPS seem to have the desired effect. EPS produced by *Lactococcus lactis* subsp. *cremoris* NIZO B40 could be fully recovered in rat feces (20), and the EPS from *Lactobacillus rhamnosus* RW-9595 M was not degraded but still decreased the lactobacillus count (21). The EPS [a 2-substituted-(1, 3)- $\beta$ -D-glucan] produced by *Pediococcus parvulus* 2.6, can be used as a substrate by different probiotic *Lactobacillus* strains (22).

Thus, EPS of different origin may act differently in the gut, and it is important to demonstrate prebiotic effects *in vivo*. It has been shown in humans that an oat-based product, fermented with the EPS-producing lactic acid bacterium *Pediococcus parvulus* 2.6 had a bifidogenic effect, whereas the fermented product without EPS failed to do so (23). This suggests that the EPS produced by *P. parvulus* 2.6 selectively stimulates bifidobacteria in the human gut;

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TABLE 1 Additions to the premix of the experimental diets

Ingredient	Amt added to premix for group:		
	Control	EPS	<i>P. parvulus</i> 2.6
MRS broth (%)	20	20	20
Cellulose (%)	4	2	4
EPS (%)	0	2	0
Live pediococci (CFU/g)	0	0	10 <sup>8</sup>

however, synergistic effects with the oats and live *P. parvulus* 2.6 cannot be ruled out. The survival ability of *Pediococcus parvulus* 2.6 in the digestive tract has previously been shown *in vivo* in mice (24). The aim of the present study was to clarify to what extent live *P. parvulus* 2.6 or purified EPS from *P. parvulus* 2.6 could affect the cecal microbiota of mice. Mice were fed an experimental diet containing 10<sup>8</sup> CFU *P. parvulus* 2.6 per g of feed (*P. parvulus* 2.6 group) or 2% EPS (EPS group). Changes in the microbiota were monitored by terminal restriction fragment length polymorphism (T-RFLP) analysis (total community) and quantitative PCR (qPCR) (certain taxa).

## MATERIALS AND METHODS

**Animals.** The experimental setup for the mouse trial is described elsewhere (24). Briefly, homozygous, female low-density lipoprotein receptor-deficient (LDLr<sup>-/-</sup>) mice (WEIBL.B6.129S7 Ldlr<sup>tm1Her/J</sup>; Charles River, Sulzfeld, Germany) were randomly divided into groups of 10 individuals upon arrival, excluding one group of 4 animals that were housed in their own cage. The mice were acclimated for 2 weeks being fed normal chow (R34 rodent chow; Lactamin, Vadstena, Sweden) and were housed in plastic cages (22°C, relative air humidity, 60%; 12-h light/dark cycle) with housing material allowing unlimited supply to food and water. After acclimatization, at the age of 9 to 10 weeks (body weight, 18.1 ± 1 g), the mice were fed a high-fat Western-type experimental diet for 6 weeks. The mouse care and use procedures followed national guidelines and were approved by the Malmö/Lund Regional Ethical Committee for Laboratory Animals.

**Diets.** High-fat, isocaloric Western diets were prepared daily by addition of MRS broth to a premix (Research Diets, Inc., New Brunswick, NJ) at a concentration of 20% (vol/wt). The composition and energy content of the diets may be found elsewhere (24). Microcrystalline cellulose (Avicel PH-101; FMC Biopolymer, Newark, NJ) was used as the fiber source in all diets. The experimental groups were divided based on different additions to the MRS broth (Table 1). Group A (control group) was administered pure MRS broth with no EPS or bacteria added. Purified EPS (10% protein) produced by *P. parvulus* 2.6 was prepared as described elsewhere (24), dissolved in MRS broth, and added to the diet of group B (EPS group) at a final concentration of 2% (wt/wt) EPS based on dry matter. The amount of cellulose was decreased proportionally to give the same fiber content as the control (4%). For group C (*P. parvulus* 2.6 group), the MRS broth was inoculated with 0.6% of EPS-producing *P. parvulus* 2.6 and incubated for 48 h at 30°C before addition to the diet. The food contained 10<sup>8</sup> CFU/g, as determined by plate count. The objective of using MRS broth in the diet was due to multiple reasons. First, the growth of *Pediococcus parvulus* 2.6 for the production of EPS was performed in MRS broth due to the simplicity of using this medium. Glucmannans from the MRS broth may be coprecipitated with the EPS, but this was taken into consideration by adding MRS to all diets. Second, the addition of live *Pediococcus parvulus* 2.6 could easily be done through the MRS broth. Third, previous studies point to the important link between state of solubilization and physiological response (25). The EPS could be fully hydrated by dissolving it in the MRS broth before addition to the diet.

**Collection of material.** After acclimatization, at week 0, the group of four mice were anesthetized by isoflurane and killed by cervical dislocation.

Cecum contents were collected and snap-frozen in liquid nitrogen before storage at -80°C. These animals were used to provide a baseline value. After 6 weeks on the experimental diets, the rest of the mice were killed and cecum contents were collected and frozen by the procedure described above.

**T-RFLP analysis.** To evaluate the structure of the microbiota community, terminal restriction fragment length polymorphism (T-RFLP) analysis was performed. Each obtained fragment corresponds to one group of bacteria, accurate down to the genus and sometimes species level (26). Total DNA was extracted from thawed cecal content with the EZ1 DNA tissue kit (Qiagen AB, Sollentuna, Sweden) on a BioRobot EZ1 workstation (Qiagen). DNA extraction did not succeed for one of the samples from the *P. parvulus* 2.6 group and was thus excluded from the data analysis. The 16S rRNA gene was amplified by using the forward primer ENV1 (5'-AGAGTTTGATITGGCTCAG-3'), which was fluorescently labeled with 6-carboxyfluorescein (FAM) dye at the 5' end, and the reverse primer ENV2 (5'-CGGITACCTTGTTACGACTT-3'). The PCR setup and conditions were as described elsewhere (27). The amplicons were purified with the MinElute PCR purification kit (Qiagen), and the DNA concentration was measured by Nanodrop ND-1000 (Saveen Werner, Limhamn, Sweden). Next, 200 ng of the purified DNA was digested with the endonuclease MspI (Fermentas Life Science, Burlington, Canada) in a total volume of 10 µl according to the manufacturer's instruction. The digested 16S rRNA gene fragments were analyzed on an ABI 3130xl genetic analyzer (Applied Biosystems, Foster City, CA) at DNA-lab (SUS, Malmö, Sweden). The resulting T-RFLP pattern was analyzed with the Genemapper software version 4.0 (Applied Biosystems), and the terminal restriction fragments (T-RFs) were resolved between 40 and 580 bp. For diversity calculation and principal component analysis (PCA), the relative area percentage was calculated for every T-RF by taking the area for each peak divided by the total peak area for that individual sample.

**Cloning and sequencing to identify certain T-RFs.** Two samples from the control and EPS-administered groups with a high abundance of representative T-RFs were used to build clone libraries. The 16S rRNA gene was amplified using primers ENV1 and ENV2 as described above. The purified amplicons were cloned by using the pGEM-T Easy vector system (Promega, Madison, WI) and used in the transformation of *E. coli* JM109 high-efficiency competent cells. The clones were sent to GATC Biotech (Konstanz, Germany) for sequencing with primer 519r (5'-GWATTACCGCGGCKGCTG-3'). The obtained sequences were checked for chimeric sequences using the DECIPHER's chimera detection tool at <http://decipher.cce.wisc.edu/FindChimeras.html>. Detected chimera sequences were removed from further analysis. For each clone, the ENV1 primer sequence was located using Bioedit Sequence Alignment Editor version 7.1.3.0 (<http://www.mbio.ncsu.edu/bioedit/bioedit.html>), and the resulting plasmid vector sequence was trimmed off together with the first 20 to ~40 nucleotides (nt) with low sequence quality. The edited sequences were aligned, and the closest matches were searched for with Ribosomal Database Project (RDP) release 10 (<http://rdp.cme.msu.edu/>), and the two clone libraries were compared with a confidence threshold of 80%.

**Putative identification of the T-RFs *in silico*.** The theoretical length of a T-RF was determined by calculating the distance between the ENV1 sequence (starting at *E. coli* position 8) and the first cutting site of enzyme MspI (5'-C<sup>^</sup>CGG-3' [where <sup>^</sup> indicates the cutting point]). Because of the sequencing primer 519r, the targeted sequence length between ENV1 and 519r will be 529 nucleotides (nt). However, due to the limitation of sequencing, the first 35 to 50 nt are usually not reliable due to low sequence quality. Thus, the longest T-RF (sequence between ENV1 and the MspI cutting site) will not exceed 495 nt, which limits the identification of T-RF longer than 490 nt. With the described experimental settings, a 5- to 7-nt difference was seen between theoretical and actual T-RF size by checking type strains and clones (unpublished data). To identify fragments of interest that were only present in one of the different experimental groups (T-RF118, T-RF293, and T-RF491), the "PROBE MATCH" function at

TABLE 2 Primers used in qPCR for bacterial quantification

Primer	Sequence (5'→3')	Target group	Amplicon size (bp)	Reference
Bif-F Bif-R	TCGCGTCYGGTGTGAAAG CCACATCCAGCRTCCAC	Bifidobacteria	243	41
Lact-16S-F Lact-16S-R	GGAATCTTCCACAATGGACG CGCTTACGCCAATAAATCCGG	<i>Lactobacillus</i>	217	42
Eco1457-F Eco1652-R	CATTGACGTTACCCGCAGAAGAAGC CTCTACGAGACTCAAGCTTGC	<i>Enterobacteriaceae</i>	195	43
AM1-F AM2-R	CAGCACGTGAAGGTGGGGAC CCTTGCGGTTGGCTTCAGAT	<i>Akkermansia</i>	327	44
g-Bfra-F g-Bfra-R	ATAGCCTTTCGAAAGRAAGAT CCAGTATCAACTGCAATTTTA	<i>Bacteroides fragilis</i> group	495	45
Tot-F Tot-R	GCAGGCCTAACACATGCAAGTC CTGTGCCTCCCGTAGGAGT	Total bacteria	292	46

RDP was used by setting the two probes as sequences of the ENV1 primer and MspI recognition site. Based on the BLAST result, sequences from closely matched bacterial groups were downloaded both from type strains and isolates, and the theoretical size of the T-RFs was calculated as described previously.

**SYBR green qPCR.** Recombinant plasmid standards were constructed by cloning the corresponding 16S rRNA gene fragments specific for bacterial species into the pGEM-T vector system (Promega). Briefly, genomic DNA was extracted from *Lactobacillus plantarum* CCUG 35035 (Culture Collection, University of Gothenburg, Sweden) and used as the template to amplify 16S rRNA fragments of *Lactobacillus* and total bacteria with the primers listed in Table 2. The amplicons were purified with a Wizard SV gel and PCR clean-up system (Promega) and used for plasmid standard construction. The same procedure was done to construct plasmid standards for bifidobacteria using *Bifidobacterium infantis* DSM15159 (*Bifidobacterium infantis* CURE 21; Probi AB, Lund, Sweden) as the template, *Escherichia coli* CCUG 29300 for *Enterobacteriaceae*, and *Bacteroides fragilis* ATCC 25285 for the *B. fragilis* group. An *Akkermansia* clone obtained from mouse cecum was used for targeting of the *Akkermansia muciniphila*-specific region on the 16S rRNA gene. The bacterial groups were amplified using the Rotor-Gene SYBR green PCR kit (Qiagen) in a real-time PCR cycler Rotor-Gene Q (Qiagen). The PCR profile was set as follows: activation at 95°C for 15 min followed by 40 cycles of (i) 95°C for 5 s and (ii) annealing and extension at 60°C for 10 to 25 s, depending on the amplicon size. Melt curve analysis was performed for each run to check the specificity of the primers. The PCR was prepared in a 20- $\mu$ l mixture consisting of 10  $\mu$ l of 2 $\times$  Rotor-Gene SYBR green PCR master mix, 0.5  $\mu$ M each primer (Table 2), and 2  $\mu$ l of template DNA. Samples that exceeded the detection limit were diluted accordingly and rerun. Triplicate reactions were performed for each sample, standard, and negative controls.

**Data analysis.** Data obtained from the T-RFLP analysis were used to calculate the number of taxa and different diversity indices using the free data analysis program PAST version 2.17b (available at <http://folk.uio.no/ohammer/past/>). The data matrix was composed of detected T-RFs and the relative area percentage of each T-RF. For normally distributed data, a one-way analysis of variance (ANOVA) test was performed with a Tukey's honestly significant difference (HSD) *post hoc* test if necessary. For the data that were not normally distributed, including diversity indices and qPCR data, the Kruskal-Wallis test was performed. If significance was found, the Nemenyi-Damico-Wolfe-Dunn test for pairwise comparisons using the packages "stats" and "coin" in the R program (version 2.15.1) was applied. Values of  $P < 0.05$  were considered statistically significant.

T-RFLP data were analyzed by principal component analysis (PCA) with SIMCA-P software version 12.0.1.0 (Umetrics, Umeå, Sweden).

## RESULTS

**Animals.** There was no difference in final body weights ( $23.6 \pm 0.4$  g) and food intakes ( $3.4 \pm 0.04$  g) between groups. All animals tolerated the study well.

**Effects of pure EPS and *P. parvulus* 2.6 on microbial diversity.** The T-RFLP profile revealed that the number of taxa (T-RFs) were detected at a significantly lower rate in the EPS ( $P < 0.01$ ) and *P. parvulus* 2.6 ( $P < 0.05$ ) groups than the control group. Even though no significant difference was found in the Buzas-Gibson's evenness ( $e^H/S$ ) and equitability with either of the supplements, a significant decrease in diversity in Simpson's index ( $P < 0.01$ ) and Shannon's index ( $P < 0.001$ ) accompanied by significantly higher Berger-Parker dominance ( $P < 0.05$ ) was seen in the EPS group compared to the control group (Table 3).

**Microbiota compositions shifted by different diets.** Principal component analysis (PCA) was applied to the T-RFLP data to give an overview of the cecal microbiota of the different experimental groups. The data were scaled with Pareto scaling to not amplify the impact of the T-RFs detected occasionally or with low abundance, and the first two principal components (PCs) were calculated. PC1 (19% of the variance) explained the difference between mice administered EPS and those administered the other three diets. The most important variables for the EPS group were T-RF118 and T-RF491. PC2 (14% of the variance) mainly explained the difference between the mice administered live *P. parvulus* 2.6 and the mice administered the control diet without supplementation. As many of the variables lie in the middle of the biplot, the overall explained variance is rather low. If the noninformative variables had been removed, the explained variance would have increased considerably; however, all variables were kept in the model to convey the results also for the noninformative variables. The three diets shaped the cecal microbiota in the mice in different ways that led to a separation between groups (Fig. 1). The control diet shifted the cecal microbiota toward the third quadrant as a result of the increase of certain bacterial groups such as T-RF293 and T-RF307. The supplementation of the live bacteria altered the ce-

TABLE 3 Number of taxa and diversity indices calculated from T-RFLP profile<sup>a</sup>

No. of taxa or index <sup>b</sup>	Control			EPS			<i>P. parvulus</i> 2.6		
	Mean	Median	Min–Max	Mean	Median	Min–Max	Mean	Median	Min–Max
No. of taxa (T-RFs) <sup>c</sup>	30.6 A	31	23–39	19.8 B	15.5	13–37	22.7 B	19	17–23
Simpson's index $(1 - D)^d$	0.92	0.93	0.87–0.95	0.86	0.88**	0.78–0.90	0.90	0.92	0.75–0.94
Shannon's index $(H)^d$	2.98	3.06	2.60–3.23	2.41	2.45***	1.96–2.89	2.73	2.70	2.11–3.10
Buzas-Gibson's evenness $(e^H/S)^c$	0.65	0.64	0.58–0.75	0.61	0.56	0.44–0.80	0.67	0.78	0.37–0.85
Equitability $(J)^c$	0.87	0.87	0.83–0.91	0.83	0.81	0.75–0.92	0.88	0.92	0.68–0.94
Berger-Parker dominance <sup>e</sup>	0.17 A	0.14	0.12–0.31	0.27 B	0.26	0.17–0.42	0.20 AB	0.16	0.12–0.47

<sup>a</sup> Mice in the control group ( $n = 10$ ) were administered control diet without supplementation, mice in the EPS group ( $n = 10$ ) were administered purified exopolysaccharide, and mice in the *P. parvulus* 2.6 group ( $n = 9$ ) were administered live *P. parvulus* 2.6. Min, minimum; Max, maximum.

<sup>b</sup> Calculations were done with the free data analysis program PAST version 2.17b (available at <http://folk.uio.no/ohammer/past/>).

<sup>c</sup> Data were analyzed with ANOVA. If significance was found, a Tukey's HSD *post hoc* test was used for pairwise comparison. Values marked with different letters were significantly different ( $P < 0.05$ ).

<sup>d</sup> Data were analyzed with the Kruskal-Wallis test. If significance was found, a Nemenyi-Damico-Wolfe-Dunn test was used for pairwise comparisons. \*\*,  $P < 0.01$  compared to control; \*\*\*,  $P < 0.001$  compared to control. The *P. parvulus* 2.6 group showed a trend of higher diversity by Simpson's index ( $P = 0.06$ ) and Shannon's index ( $P = 0.05$ ) than the EPS group.

cal microbiota mainly toward the second quadrant. The change was indicated, for instance, by proliferation of the taxon represented by T-RF284, which was only detected in the *P. parvulus* 2.6 group, and suppression of the taxon represented by T-RF159, which was only absent in this group. The theoretical T-RF size of *Pediococcus* is 586 nt, which is out of the detection limit of the method used in this work. The EPS group was separated from the other groups by PC1. T-RF118 and T-RF491 were detected with average abundances of 15% and 9%, respectively, in all mice from the EPS group but were absent in all mice from the control and *P. parvulus* 2.6 groups. T-RF265 and T-RF93 were present in higher numbers in the EPS group than the other groups. In addition, T-RF281, T-RF307, and T-RF536 were only detected occasionally in the EPS group and the *P. parvulus* 2.6 group. Furthermore, the T-RFLP data were interpreted using a partial least-square regres-

sion (PLS-R) followed by an uncertainty test using the Unscrambler X (Camo, Oslo, Norway). When comparing the PCA and the PLS-R models, both showed the same increase/decrease of abundance for the T-RFs, which was used as a validation of the system (results not shown).

**Putative identification of certain T-RFs.** With the aim of identifying the fragments representative of T-RF491, T-RF118, T-RF265, and T-RF93, one sample from the EPS group with high abundance of those T-RFs (15, 17, 8, and 27%, respectively) and one sample from the control group with a high abundance of T-RF293 (10%) were chosen to construct two clone libraries. Putative identification of the T-RFs was based on running T-RFLP on the sequenced clones followed by a database search at RDP. T-RF265 was recognized as *Akkermansia*-like and T-RF93 as the *Bacteroides fragilis* group, which have been putatively identified in previous work on mice (28, 29). A

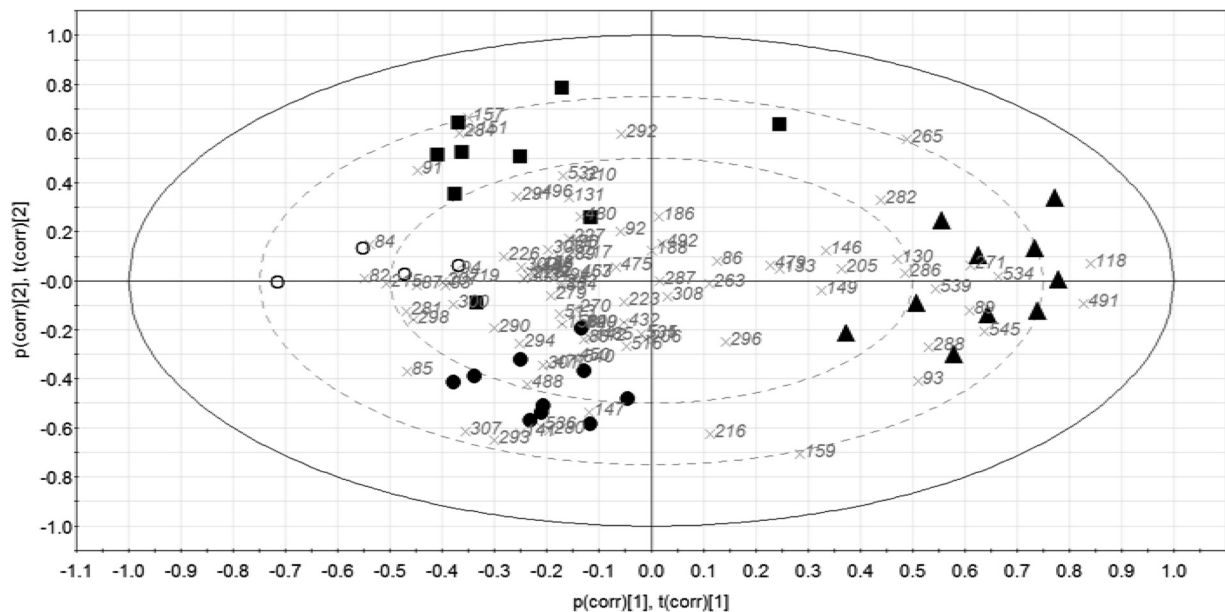


FIG 1 PCA loading biplot of T-RFLP data. ○, mice not administered any experimental diet, which served as baseline; ●, mice administered control diet (high fat) without supplementation; ▲, mice administered purified EPS from *Pediococcus parvulus* 2.6 as supplement to food; ■, mice administered EPS-producing live *Pediococcus parvulus* 2.6 as supplement to food. Numbers with the symbol "x" (e.g., "x91") denote T-RFs that represent different bacterial groups. PC1 and PC2 explained 19% and 14% of the data variance, respectively.

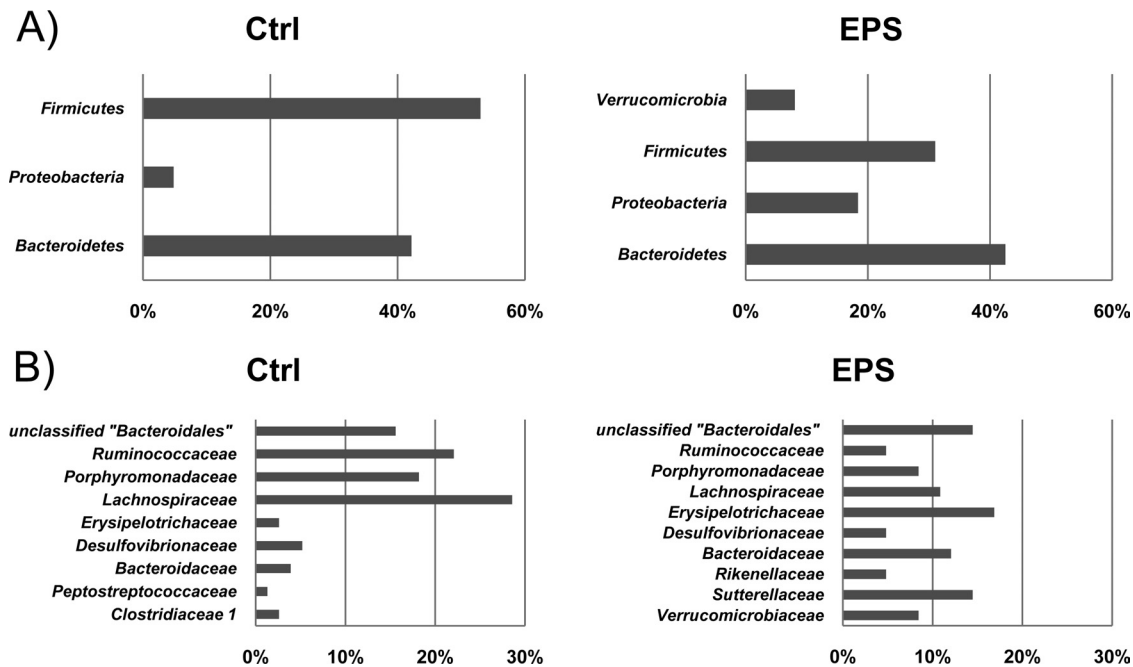


FIG 2 Bar charts of clone libraries of one mouse from the control group and one from the EPS group. (A) Phylum level; (B) family level. Eighty-three and 87 clones obtained from one mouse in the control group and the EPS group, respectively, were used for the phylum-level results, and 77 clones from the control libraries and 83 clones from the EPS libraries that can be matched to known family taxa in the RDP database were used to plot the family-level bar chart.

comparison between the two clone libraries (Fig. 2) revealed that *Akkermansia*-like bacteria (family *Verrucomicrobiaceae*; T-RF265) and *Parasutterella*-related bacteria (*Sutterellaceae*; T-RF491) were only detected in the EPS library. *Allobaculum*-related bacteria (*Erysipelotrichaceae*; T-RF118) were significantly more abundant in the EPS library than the control library ( $P < 0.01$ ). On the other hand, there were significantly fewer *Oscillibacter*-related (*Ruminococcaceae*; T-RF293) and *Lachnospiraceae*-related bacteria in the EPS library than in the control library.

**Bacterial population affected by different diets.** Quantification of the bacterial groups by SYBR green qPCR (Table 4) showed that there was no difference in total bacteria and the population of lactobacilli between groups. However, bifidobacteria were significantly decreased in the EPS group compared to the control group

( $P = 0.01$ ). On the other hand, *Akkermansia* was significantly increased in both the EPS ( $P < 0.001$ ) and *P. parvulus* 2.6 ( $P = 0.002$ ) groups compared to the control group. The *Bacteroides fragilis* group was significantly decreased in the *P. parvulus* 2.6 group compared to the EPS group ( $P = 0.02$ ) but not to the control group. Interestingly, *Enterobacteriaceae* were detected in all individuals of the control group, but the level was under the detection limit (200 copies/20  $\mu$ l PCR) in all mice of the *P. parvulus* 2.6 group and 3 out of 10 mice in the EPS group.

## DISCUSSION

The T-RFLP analysis showed that the purified EPS [2-substituted-(1,3)- $\beta$ -D-glucan] from *P. parvulus* 2.6 significantly decreased the diversity of the cecal microbiota measured with Simpson's index

TABLE 4 Quantification of bacterial groups by SYBR green qPCR<sup>a</sup>

Bacterial group	Log copy no./g					
	Control		EPS		<i>P. parvulus</i> 2.6	
	Median	25–75%	Median	25–75%	Median	25–75%
Bifidobacteria	8.4 A	8.1–8.6	7.4 B	6.9–7.8	8.0 AB	7.8–8.3
<i>Lactobacillus</i>	8.5	8.3–9.0	7.4	7.1–8.4	8.0	7.6–8.8
<i>Enterobacteriaceae</i>	6.6	6.5–6.8	6.5 <sup>b</sup>	5.9–6.8	ND <sup>c</sup>	NA <sup>d</sup>
<i>Akkermansia</i>	7.5 A	7.0–8.4	9.3 B	8.5–10.1	9.2 B	8.8–9.4
<i>Bacteroides fragilis</i> group	7.7 AB	7.4–8.3	8.3 B	8.1–8.7	7.6 A	7.4–7.7
Total bacteria	11.0	10.6–11.2	11.2	11.0–11.3	11.1	10.8–11.2

<sup>a</sup> Mice in the control group were administered diet without any supplementation, mice in the EPS group were administered purified exopolysaccharide as supplementation to the diet, and mice in the *P. parvulus* 2.6 group were administered EPS-producing live *Pediococcus parvulus* 2.6. The data are expressed as median values and 25th and 75th percentiles (25–75%). The Kruskal-Wallis test was used to compare the three groups, and if significance was found, the Nemenyi-Damico-Wolfe-Dunn test was used for pairwise comparison. Groups marked with different letters were significantly different ( $P < 0.05$ ).

<sup>b</sup> *Enterobacteriaceae* were not detected in 3 out of 10 mice in the EPS group.

<sup>c</sup> ND, *Enterobacteriaceae* was not detected in any mouse in the *P. parvulus* 2.6 group.

<sup>d</sup> NA, not available.

and Shannon's index. The decreased diversity is mainly caused by the decreased richness but not evenness (Table 4). The increased dominance in the EPS group indicates a strong selection pressure toward certain taxa; together with a reduced number of taxa, it seemed like the EPS group maintained evenness of the cecal microbiota. Even though *P. parvulus* 2.6 induced a decline in cecal bacterial richness compared to the control, there was no significant difference in dominance, implying that the selection pressure on the microbiota was not as strong as for purified EPS. This is also supported by the PCA (Fig. 1). On the other hand, no difference in total bacterial loads was observed by qPCR, reflecting similar degrees of evenness among the three groups. In addition, the qPCR results showed that the amount of bifidobacteria was significantly decreased in the EPS group compared to the other groups. Thus, the hypothesis that EPS from *P. parvulus* 2.6 should provide a bifidogenic effect (23) was refuted. It can be speculated that the bifidogenic effect previously seen with the ropy oat-based product instead was due to the phenomenon of cross-feeding (30). *P. parvulus* 2.6 might have been the primary degrader of some polymeric substances in the oat product, making new substrates available to the bifidobacteria and stimulating them to proliferate.

The clone libraries were built to putatively identify certain dominant T-RFs. Considering the large variation between individual microbiota of mice, the clone libraries built upon only two mice were not representative for the whole group, and thus the results are not comparable to the diversity statistics done on the T-RFLP data. However, the selection pressure of the EPS could be seen in the differences between the clone libraries. The comparison between clone libraries showed that at the phylum level, *Bacteroidetes* and *Firmicutes* were dominant in both the control and the EPS libraries. However, *Verrucomicrobia* was only detected in the EPS libraries. This is in agreement with the qPCR result, where the EPS and *P. parvulus* 2.6 significantly increased the load of *Akkermansia* (representative of *Verrucomicrobia*). *Akkermansia* is a Gram-negative bacterium with the ability to degrade mucin (31). It has previously been reported that *Akkermansia* was dramatically increased as higher concentrations of resistant starch were given to aged mice and found to be positively correlated with health markers (32). In a previous study, *Akkermansia* was found to be negatively correlated with risk factors for the development of type 2 diabetes (28). The increase in *Akkermansia* observed in the present study may have resulted from a selection toward *Akkermansia* by the EPS when used as the substrate.

The PCA of the T-RFLP data revealed that the composition of the microbiota was affected by the high-fat diet. In addition, both purified EPS and live *P. parvulus* 2.6 altered the microbiota in different directions. *Parasutterella*-related bacteria (T-RF491) were detected only in the EPS group, implying a strong selection pressure from the purified EPS. In a recent study, *Parasutterella* and *Lachnospiraceae* were detected in the cecal microbiota of rats in a group fed laminaran, a  $\beta$ -(1,3) glucan from brown algae, but not in a group fed sodium alginate or the control rats (33). The  $\beta$ -(1,3) bond may favor the proliferation of *Parasutterella*. *Allobaculum*-related bacteria (T-RF118) were found to be most abundant in the EPS group. Previously an increase in *Allobaculum* has been found in different experimental animal models, like an induced colitis rat model (34), rats that developed precancerous mucosal lesions (35), and mice that were susceptible to rheumatoid arthritis, where a negative correlation between bifidobacteria and *Allobaculum*-like bacteria was found (36). In contrast, enrichment of

*Allobaculum* has in some cases been reported to be positively linked to improvement in health (32, 37). Further studies are needed to clarify the role of the increased *Allobaculum*-like bacteria in response to EPS feeding. Previously the effects of *P. parvulus* 2.6 and its exopolysaccharide on plasma cholesterol and the inflammatory markers soluble vascular cell adhesion molecule 1 and serum amyloid A were investigated (24). Those parameters, however, could not be correlated with the microbiota changes found in the present investigation.

Due to the diverse chemical and structural composition of EPS, their biodegradabilities can vary (38), and presumably so can their physiological or physiopathological effects. The microbiota provide the digestive tract with a huge number of carbohydrate-active enzymes, and even though a core microbiome at a functional level is hypothesized (39), the responses to different carbohydrates would depend on the kind of enzymes that are present. This underlines the complexity of prebiotic evaluation where the choice of model and differences in microbiota between individuals may affect the outcome. EPS previously evaluated for prebiotic potential *in vitro* have different chemical compositions and structures compared to the EPS from *P. parvulus* 2.6. The chemical compositions and types of bonds between the monomers do not seem to give an explanation for the selectivities of the different EPS. It would be of interest to know the conformation in space of the polymers, but that information is lacking in most cases. The EPS that are reported to be nondegradable (20, 21) share a common denominator in large and/or many side groups that may prevent bacterial degradation of the EPS through sterical hindrance. This is somewhat strengthened by the results from Ruijsenaars et al. (38); however, the conformation in space has to be known before any conclusions may be drawn. The EPS from *P. parvulus* 2.6 is a  $\beta$ -glucan showing a helical conformation with extensive intramolecular hydrogen bonding between neighboring side-chain residues and between side-chain and backbone residues (40). Hypothetically, this compact molecule ought to be rather resistant to microbial degradation, but obviously it has a strong effect on the composition of the microbiota. EPS can affect the composition of the microbiota without being degraded (21), and it remains to be shown whether the EPS from *P. parvulus* 2.6 is partially or completely degraded by the microbiota or not. Russo et al. previously showed that the EPS from *P. parvulus* 2.6 could be used as the sole carbon source by some probiotic strains, indicating at least partial degradation (22).

In conclusion, purified EPS from *P. parvulus* 2.6 did not show any prebiotic effect in the mouse model. Instead, it suppressed bifidobacteria and lowered microbiota diversity. In contrast, the live EPS-producing bacterium *P. parvulus* 2.6 antagonized *Enterobacteriaceae* without disturbing the homeostasis of the microbiota.

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## REFERENCES

1. Ley R, Turnbaugh P, Klein S, Gordon J. 2006. Microbial ecology: human gut microbes associated with obesity. *Nature* 444:1022–1023.
2. Cani PD, Delzenne NM. 2009. The role of the gut microbiota in energy metabolism and metabolic disease. *Curr. Pharm. Des.* 15:1546–1558.
3. FAO/WHO. 2002. Guidelines for the evaluation of probiotics in food. Joint FAO/WHO Working Group on Drafting Guidelines for the Evaluation of Probiotics in Food, 30 April to May 1 2002, London, Ontario, Canada.
4. Xie N, Cui Y, Yin Y-N, Zhao X, Yang J-W, Wang Z-G, Fu N, Tang Y, Wang X-H, Liu X-W, Wang C-L, Lu F-G. 2011. Effects of two *Lactobacillus* strains on lipid metabolism and intestinal microflora in rats fed a high-cholesterol diet. *BMC Complement. Altern. Med.* 11:53. doi:10.1186/1472-6882-11-53.
5. Wang Y, Xu N, Xi A, Ahmed Z, Zhang B, Bai X. 2009. Effects of *Lactobacillus plantarum* MA2 isolated from Tibet kefir on lipid metabolism and intestinal microflora of rats fed on high-cholesterol diet. *Appl. Microbiol. Biotechnol.* 84:341–347.
6. Goossens D, Jonkers D, Russel M, Thijs A, van den Bogaard A, Stobberingh E, Stockbrügger R. 2005. Survival of the probiotic, *L. plantarum* 299v and its effects on the faecal bacterial flora, with and without gastric acid inhibition. *Dig. Liver Dis.* 37:44–50.
7. Nobaek S, Johansson ML, Molin G, Ahrné S, Jeppsson B. 2000. Alteration of intestinal microflora is associated with reduction in abdominal bloating and pain in patients with irritable bowel syndrome. *Am. J. Gastroenterol.* 95:1231–1238.
8. Mohan R, Koebnick C, Schildt J, Schmidt S, Mueller M, Possner M, Radke M, Blaut M. 2006. Effects of *Bifidobacterium lactis* Bb12 supplementation on intestinal microbiota of preterm infants: a double-blind, placebo-controlled, randomized study. *J. Clin. Microbiol.* 44:4025–4031.
9. Lahtinen SJ, Tammela L, Korpela J, Parhiala R, Ahokoski H, Mykkanen H, Salminen SJ. 2009. Probiotics modulate the *Bifidobacterium* microbiota of elderly nursing home residents. *Age (Dordr.)* 31:59–66.
10. Matsumoto K, Takada T, Shimizu K, Moriyama K, Kawakami K, Hirano K, Kajimoto O, Nomoto K. 2010. Effects of a probiotic fermented milk beverage containing *Lactobacillus casei* strain Shirota on defecation frequency, intestinal microbiota, and the intestinal environment of healthy individuals with soft stools. *J. Biosci. Bioeng.* 110:547–552.
11. Karlsson C, Ahrné S, Molin G, Berggren A, Palmquist I, Nordin Fredrikson G, Jeppsson B. 2010. Probiotic therapy to men with incipient arteriosclerosis initiates increased bacterial diversity in colon: a randomized controlled trial. *Atherosclerosis* 208:228–233.
12. Salazar N, Ruas-Madiedo P, Kolida S, Collins M, Rastall RA, Gibson GR, de los Reyes-Gavilan CG. 2009. Exopolysaccharides produced by *Bifidobacterium longum* IPLA E44 and *Bifidobacterium animalis subsp. lactis* IPLA R1 modify the composition and metabolic activity of human faecal microbiota in pH-controlled batch cultures. *Int. J. Food Microbiol.* 135:260–267.
13. Roberfroid MB, Gibson GR, Hoyles L, McCartney AL, Rastall RA, Rowland IR, Wolvers D, Watzl B, Szajewska H, Stahl B, Guarner F, Respondek F, Whelan K, Coxam V, Davicco M-J, Léotoing L, Wittrant Y, Delzenne NM, Cani PD, Neyrinck AM, Meheust A. 2010. Prebiotic effects: metabolic and health benefits. *Br. J. Nutr.* 104:S1–S63.
14. Patel S, Goyal A. 2012. The current trends and future perspective of prebiotics research: a review. *3 Biotech* 2:115–125.
15. Sutherland IW. 1998. Novel and established applications of microbial polysaccharides. *Trends Biotechnol.* 16:41–46.
16. Korakli M, Gänzle MG, Vogel RF. 2002. Metabolism by bifidobacteria and lactic acid bacteria of polysaccharides from wheat and rye, and exopolysaccharides produced by *Lactobacillus sanfranciscensis*. *J. Appl. Microbiol.* 92:958–965.
17. Dal Bello F, Walter J, Hertel C, Hammes WP. 2001. *In vitro* study of prebiotic properties of levan-type exopolysaccharides from lactobacilli and non-digestible carbohydrates using denaturing gradient gel electrophoresis. *Syst. Appl. Microbiol.* 24:232–237.
18. Hongpattarakere T, Chertong N, Wichienchot S, Kolida S, Rastall RA. 2012. *In vitro* prebiotic evaluation of exopolysaccharides produced by marine isolated lactic acid bacteria. *Carbohydr. Polym.* 87:846–852.
19. Salazar N, Gueimonde M, Hernández-Barranco AM, Ruas-Madiedo P, de los Reyes-Gavilán CG. 2008. Exopolysaccharides produced by intestinal *Bifidobacterium* strains act as fermentable substrates for human intestinal bacteria. *Appl. Environ. Microbiol.* 74:4737–4745.
20. Looijesteijn PJ, Trapet L, de Vries E, Abee T, Hugenholtz J. 2001. Physiological function of exopolysaccharides produced by *Lactococcus lactis*. *Int. J. Food Microbiol.* 64:71–80.
21. Cinquin C, Le Blay G, Fliss I, Lacroix C. 2006. Comparative effects of exopolysaccharides from lactic acid bacteria and fructo-oligosaccharides on infant gut microbiota tested in an *in vitro* colonic model with immobilized cells. *FEMS Microbiol. Ecol.* 57:226–238.
22. Russo P, López P, Capozzi V, de Palencia PF, Dueñas MT, Spano G, Fiocco D. 2012. Beta-glucans improve growth, viability and colonization of probiotic microorganisms. *Int. J. Mol. Sci.* 13:6026–6039.
23. Martensson O, Björklund M, Lambo AM, Duenas-Chasco M, Irastorza A, Holst O, Norin E, Welling G, Oste R, Onning G. 2005. Fermented, rosy, oat-based products reduce cholesterol levels and stimulate the bifidobacteria flora in humans. *Nutr. Res.* 25:429–442.
24. Lindström C, Holst O, Nilsson L, Oste R, Andersson KE. 2012. Effects of *Pediococcus parvulus* 2.6 and its exopolysaccharide on plasma cholesterol levels and inflammatory markers in mice. *AMB Express* 2:66. doi:10.1186/2191-0855-2-66.
25. Martensson O, Chasco MD, Irastorza A, Holst O, Rudling M, Norin E, Midtvedt T, Oste R. 2002. Effects of fermented, rosy, non-dairy, oat-based products on serum lipids and the faecal excretion of cholesterol and short chain fatty acids in germfree and conventional rats. *Nutr. Res.* 22:1461–1473.
26. Danovaro R, Luna GM, Dell’anno A, Pietrangeli B. 2006. Comparison of two fingerprinting techniques, terminal restriction fragment length polymorphism and automated ribosomal intergenic spacer analysis, for determination of bacterial diversity in aquatic environments. *Appl. Environ. Microbiol.* 72:5982–5989.
27. Karlsson C, Onnerfält J, Xu J, Molin G, Ahrné S, Thorngren-Jerneck K. 2012. The microbiota of the gut in preschool children with normal and excessive body weight. *Obesity* 20:2257–2261.
28. Axling U, Olsson C, Xu J, Fernandez C, Larsson S, Strom K, Ahrne S, Holm C, Molin G, Berger K. 2012. Green tea powder and *Lactobacillus plantarum* affect gut microbiota, lipid metabolism and inflammation in high-fat fed C57BL/6j mice. *Nutr. Metab.* 9:105. doi:10.1186/1743-7075-9-105.
29. Andersson KE, Axling U, Xu J, Swärd K, Ahrné S, Molin G, Hellstrand P. 2012. Diverse effects of oats on cholesterol metabolism in C57BL/6 mice correlate with expression of hepatic bile acid-producing enzymes. *Eur. J. Nutr.* doi:10.1007/s00394-012-0479-1.
30. Candela M, Maccaferri S, Turroni S, Carnevali P, Brigidi P. 2010. Functional intestinal microbiome, new frontiers in prebiotic design. *Int. J. Food Microbiol.* 140:93–101.
31. Derrien M, Vaughan EE, Plugge CM, de Vos WM. 2004. *Akkermansia muciniphila* gen. nov., sp. nov., a human intestinal mucin-degrading bacterium. *Int. J. Syst. Evol. Microbiol.* 54:1469–1476.
32. Tachon S, Zhou J, Keenan M, Martin R, Marco ML. 2013. The intestinal microbiota in aged mice is modulated by dietary resistant starch and correlated with improvements in host responses. *FEMS Microbiol. Ecol.* 83:299–309.
33. An C, Kuda T, Yazaki T, Takahashi H, Kimura B. 2013. FLX pyrosequencing analysis of the effects of the brown-algal fermentable polysaccharides alginate and laminaran on rat cecal microbiotas. *Appl. Environ. Microbiol.* 79:860–866.
34. Dicksved J, Schreiber O, Willing B, Petersson J, Rang S, Phillipson M, Holm L, Roos S. 2012. *Lactobacillus reuteri* maintains a functional mucosal barrier during DSS treatment despite mucus layer dysfunction. *PLoS One* 7:e46399. doi:10.1371/journal.pone.0046399.
35. Wei H, Dong L, Wang TT, Zhang MH, Hua WY, Zhang CH, Pang XY, Chen MJ, Su MM, Qiu YP, Zhou MM, Yang SL, Chen Z, Rantalainen M, Nicholson JK, Jia W, Wu DZ, Zhao LP. 2010. Structural shifts of gut microbiota as surrogate endpoints for monitoring host health changes induced by carcinogen exposure. *FEMS Microbiol. Ecol.* 73:577–586.
36. Gomez A, Luckey D, Yeoman CJ, Marietta EV, Miller MEB, Murray JA, White BA, Taneja V. 2012. Loss of sex and age driven differences in the gut microbiome characterize arthritis-susceptible \*0401 mice but not arthritis-resistant \*0402 mice. *PLoS One* 7:e36095. doi:10.1371/journal.pone.0036095.
37. Zhang X, Zhao YF, Zhang MH, Pang XY, Xu J, Kang CY, Li M, Zhang CH, Zhang ZG, Zhang YF, Li XY, Ning G, Zhao LP. 2012. Structural changes of gut microbiota during berberine-mediated prevention of obesity and insulin resistance in high-fat diet-fed rats. *PLoS One* 7:e42529. doi:10.1371/journal.pone.0042529.



38. Ruijsenaars HJ, Stingle F, Hartmans S. 2000. Biodegradability of food-associated extracellular polysaccharides. *Curr. Microbiol.* **40**: 194–199.
39. Turnbaugh PJ, Hamady M, Yatsunenko T, Cantarel BL, Duncan A, Ley RE, Sogin ML, Jones WJ, Roe BA, Affourtit JP, Egholm M, Henrissat B, Heath AC, Knight R, Gordon JI. 2009. A core gut microbiome in obese and lean twins. *Nature* **457**:480–484.
40. Lambo-Fodje AM, Leeman M, Wahlund KG, Nyman M, Oste R, Larsson H. 2007. Molar mass and rheological characterisation of an exopolysaccharide from *Pediococcus dammosus* 2.6. *Carbohydr. Polym.* **68**: 577–586.
41. Rinttila T, Kassinen A, Malinen E, Krogus L, Palva A. 2004. Development of an extensive set of 16S rDNA-targeted primers for quantification of pathogenic and indigenous bacteria in faecal samples by real-time PCR. *J. Appl. Microbiol.* **97**:1166–1177.
42. Abu Bakar F, Nordin N, Yoke TS. 2010. Methods for precise molecular detection of probiotic microflora: using adjusted molecular biology protocols, primer sets and PCR assays. *Biotechnology* **9**:25–32.
43. Bartosch S, Fite A, Macfarlane GT, McMurdo ME. 2004. Characterization of bacterial communities in feces from healthy elderly volunteers and hospitalized elderly patients by using real-time PCR and effects of antibiotic treatment on the fecal microbiota. *Appl. Environ. Microbiol.* **70**: 3575–3581.
44. Collado MC, Derrien M, Isolauri E, de Vos WM, Salminen S. 2007. Intestinal integrity and *Akkermansia muciniphila*, a mucin-degrading member of the intestinal microbiota present in infants, adults, and the elderly. *Appl. Environ. Microbiol.* **73**:7767–7770.
45. Matsuki T, Watanabe K, Fujimoto J, Miyamoto Y, Takada T, Matsumoto K, Oyaizu H, Tanaka R. 2002. Development of 16S rRNA-gene-targeted group-specific primers for the detection and identification of predominant bacteria in human feces. *Appl. Environ. Microbiol.* **68**: 5445–5451.
46. Castillo M, Martin-Orue SM, Manzanilla EG, Badiola I, Martin M, Gasa J. 2006. Quantification of total bacteria, enterobacteria and lactobacilli populations in pig digesta by real-time PCR. *Vet. Microbiol.* **114**:165–170.