

In Situ Prebiotics for Weaning Piglets: *In Vitro* Production and Fermentation of Potato Galacto-Rhamnogalacturonan

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Postweaning diarrhea (PWD) in pigs is a leading cause of economic loss in pork production worldwide. The current practice of using antibiotics and zinc to treat PWD is unsustainable due to the potential of antibiotic resistance and ecological disturbance, and novel methods are required. In this study, an *in vitro* model was used to test the possibility of producing prebiotic fiber *in situ* in the gastrointestinal (GI) tract of the piglet and the prebiotic activity of the resulting fiber in the terminal ileum. Soluble fiber was successfully produced from potato pulp, an industrial waste product, with the minimal enzyme dose in a simulated upper GI tract model extracting 26.9% of the initial dry matter. The fiber was rich in galactose and galacturonic acid and was fermented at 2.5, 5, or 10 g/liter in a glucose-free medium inoculated with the gut contents of piglet terminal ileum. Fermentations of 5 g/liter inulin or 5 g/liter of a purified potato fiber were used as controls. The fibers showed high fermentability, evident by a dose-dependent drop in pH and an increase in the organic acid content, with lactate in particular being increased. Deep sequencing showed a significant increase in the numbers of *Lactobacillus* and *Veillonella* organisms and an insignificant increase in the numbers of *Clostridium* organisms as well as a decrease in the numbers of *Streptococcus* organisms. Multivariate analysis showed clustering of the treatment groups, with the group treated with purified potato fiber being clearly separated from the other groups, as the microbiota composition was 60% *Lactobacillus* and almost free of *Clostridium*. For animal studies, a dosage corresponding to the 5-g/liter treatment is suggested.

Postweaning diarrhea (PWD) is a serious condition frequently afflicting piglets after weaning (1–3) and is a leading cause of economic loss in industrial pork production (4, 5). The pathology and etiology of PWD are complex, but the causative agent is believed to be enterotoxigenic *Escherichia coli* (ETEC) (3, 6, 7). The infective action of ETEC is in turn facilitated by the immature gastrointestinal (GI) system in the piglet, the switch from immunoglobulin-rich maternal milk to solid foods with lower digestibility, increased emotional stress, and the lowered food intake (3, 4, 7).

A substantial amount of antibiotics and the heavy metal zinc are used for prevention and treatment of PWD, as they are presently the most cost-effective means of improving performance in swine production (8, 9). However, due to increasing problems with antibiotic resistance in bacteria of both veterinary and human importance, as well as environmental concerns about the use of heavy metals, alternative strategies for prevention of PWD are needed.

In humans, it is well established that prebiotic fibers, e.g., indigestible fibers fermented by the intestinal microbiota, such as polymers of fructose or galactose, can result in the increased growth of selected beneficial bacteria from the commensal microbiota (10). It is also known that various strains of *Lactobacillus* and *Bifidobacterium* act as suppressors or inhibitors of infectious bacteria (11), likely due to production of antimicrobial peptides and organic acids (OAs), as thoroughly reviewed by Liévin-Le Moal and Servin (12). It could then be hypothesized that prebiotic intervention would increase piglet resilience against infection by competitive inhibition of pathogenic bacteria or general improvement of health status. Studies in animals have, however, not been conclusive in establishing if the same effect is possible in weaning piglets (13–17), perhaps because of difficulties in observing an effect in a healthy animal. In contrast, studies using experimental

infections along with prebiotic intervention generally show a protective effect against PWD (18–20). *In vitro* fermentations, using inocula from pig intestines or pig feces, have, however, been fairly successful in showing increases in the numbers of *Lactobacillus* organisms and, to a much lesser extent, *Bifidobacterium* organisms and/or increases in the level of production of OAs resulting from bacterial fermentation (21–24).

Potato pulp is a high-volume waste product from the starch industry and is currently produced at $\sim 1 \times 10^6$ tons/year in Europe, where it is used in low-value applications, e.g., in animal feed, and is priced at ~ 10 €/ton (25). It has previously been shown that a novel, fermentable, and highly prebiotic fiber could be produced from potato pulp (26, 27) and has the potential for use as a beneficial feed supplement, resulting in a substantial increase in the value of potato pulp. These fibers are tightly bound to the pulp matrix and are insoluble and inaccessible to the gut bacteria in this state. The fibers consist of the rhamnogalacturonan-1 (RG-1) domain of pectin, e.g., an alternating backbone of rhamnose and galacturonic acid substituted with galactose and arabinose chains (28, 29). The pulp is very high in pectin containing this domain,

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and in 2011 the work of Thomassen et al. showed that a mixture of polygalacturonase (EC 3.2.1.15) and pectin lyase (EC 4.2.2.10) efficiently releases large amounts of RG-1 in an industrially relevant setting (26).

In the pig, peristalsis and body heat provide natural agitation and a constant temperature in the >2 h required for the feed to reach the terminal small intestine. In light of this, it was hypothesized that feeding of enzymes and substrate should allow the enzymatic degradation of highly complex molecules into prebiotic fibers *in situ*, thereby decreasing the issues of industrial fiber production, such as purification and transport. Release of these fibers from potato pulp by the gut microbiota is not possible, as the bacteria in the pig ileum do not possess genes for pectinolytic enzymes (30).

In this work, the feasibility of executing the enzymatic reaction in an *in vitro* piglet intestinal system, as well as the effect of these fibers on the microbiota composition in ileum samples obtained from 26-day-old piglets, was evaluated.

MATERIALS AND METHODS

Characterization of pectinolytic enzymes. Pectin lyase and polygalacturonase were produced by fermentations, as described by Silva et al. (31), using *Pichia pastoris* clones transformed with the pectin lyase gene AN2569.2 and the polygalacturonase gene AN4372.2, both from *Aspergillus niger*, as described by Bauer et al. (32). This method ensures the production of monocomponent enzymes by placing the gene under the control of a methanol promoter, allowing the enzyme to be expressed in large amounts by addition of methanol to the fermentation. Protein concentrations were determined by the bicinchoninic acid (BCA) assay with bovine serum albumin (BSA) as a standard, as described by the manufacturer (Thermo Fisher Scientific, Rockford, IL).

Pectin lyase activity was assayed by incubating pectin lyase enzyme with 1.5 g/liter citrus pectin substrate at a 0.25% (wt/wt) enzyme-to-substrate (E/S) ratio (Sigma-Aldrich, Steinheim, Germany) in McIlvaine buffer at pH 3 or pH 7 and 25°C in triplicate. The reaction was followed for 10 min in an Infinite200 microplate reader (Tecan, Salzburg, Austria) by recording the absorbance at 235 nm. Units of activity were defined as the number of μmol of unsaturated uronide released per minute using $5,500 \text{ M}^{-1} \text{ cm}^{-1}$ as the extinction coefficient (31).

Polygalacturonase was assayed by incubating polygalacturonase with 3 g/liter polygalacturonic acid (Sigma-Aldrich, Steinheim, Germany) at a 0.125% E/S ratio in McIlvaine buffer at pH 3 or 7 and 37°C in triplicate. The amount of reducing ends was quantified in the reducing ends assay described by Thomassen et al. (26). In brief, after 10 min of incubation, the enzymatic reaction was stopped by extracting 20 μl into a freshly made and preheated *p*-hydroxybenzoic acid hydrazide solution, and the mixture was then incubated at 70°C for 10 min. The amount of reducing ends was then quantified by determination of the absorbance at 410 nm. Units of activity were defined as the number of μmol of galacturonic acid released per minute, using free galacturonic acid as a standard.

Animals. Five suckling pigs were acquired from a commercial farm in Denmark at 26 days of age, 2 days before they would otherwise have been weaned. The animals were females of mixed breed, had been given a wheat-based creep feed since 7 days of age, and were healthy. Furthermore, they had not been given antibiotics.

The animals were euthanized at the National Veterinary Institute at the Technical University of Denmark by an overdose of pentobarbital and jugular vein puncture. All handling of animals was performed by trained personnel and veterinarians and fulfilled the regulations of the Danish Ministry of Justice.

Contents were collected from the stomach and middle jejunum, placed in sterile tubes, and snap-frozen in dry ice before storage at -80°C . Contents from the terminal ileum were collected, placed in sterile tubes, and mixed 1:1 with 50% (wt/wt) glycerol before they were snap-frozen in

dry ice and stored at -80°C . The use of frozen samples rather than fresh samples has formerly been verified (33, 34), and frozen samples have previously been used in similar experiments (35, 36).

Characterization of digestive enzymes. Stomach and jejunal contents were homogenized, followed by centrifugation at $4,000 \times g$ for 10 min at 4°C , and the pH in the supernatant was then measured using a pH strip (pH-fix; Macherey-Nagel, Germany). Dry matter was determined by drying of the samples at 105°C for 24 h in duplicate for each sample.

The pepsin in the stomach supernatant was assayed by incubating 50 μl supernatant in 250 μl 0.06 M HCl with 20 g/liter hemoglobin in triplicate for each sample. The reaction was run at 37°C and 900 rpm and terminated after 60 min with 500 μl of 5% (wt/vol) trichloroacetic acid. After 10 min of mixing, the samples were centrifuged for 2 min at $14,000 \times g$, and the amount of pepsin in 100 μl of the supernatant was measured by determination of the absorbance at 280 nm. Controls were made by adding the trichloroacetic acid before addition of the gastric sample. Units of activity were defined as the number of nmol of tyrosine released per minute using an extinction coefficient of $1,250 \text{ M}^{-1} \text{ cm}^{-1}$. The pepsin concentration was calculated by comparison to a standard curve of the concentrations of commercial pepsin (catalog number P7125, batch no. SLBB6557V; Sigma-Aldrich, Steinheim, Germany).

Small intestine proteases in the jejunal supernatant were assayed with an azurine-cross-linked (AZCL) casein assay (Megazyme, Bray, Ireland) according to the manufacturer's instructions, with some modifications: 50 μl of sample diluted 40-fold was added to 250 μl McIlvaine buffer (pH 7, 20 mM) containing 5 mg AZCL-casein in triplicate for each sample. The reaction was run at 37°C and 1,400 rpm and terminated after 5 min with 500 μl of 10 g/liter trisodium phosphate. After centrifugation for 5 min at $15,000 \times g$, the amount of protease in 100 μl of the supernatant was measured by determination of the absorbance at 590 nm in a microplate. Units of activity were defined as the change in 1 optical density unit at a 1-cm path length per minute. The corresponding concentration of pancreatin was determined by comparison to a standard curve of the concentrations of commercial pancreatin (catalog number P7545; batch no. SLBD0640V; Sigma-Aldrich, Steinheim, Germany).

Production of IVSF. A porcine gastrointestinal digestion process was simulated by sequential digestion under stomach conditions and then small intestinal conditions, producing *in vitro*-solubilized fiber (IVSF). The concentrations and conditions were based on the measurements obtained by characterization of the animal digesta. The potato pulp used in the study, FiberBind, was kindly provided by KMC (Brande, Denmark) and is the dried residue remaining when potato starch has been extracted.

Ten milliliters of purified water with 0.32 mg/ml pepsin (catalog number P7125; Sigma) was added to 300 mg potato pulp in a 50-ml Falcon tube and adjusted to pH 3 with HCl in four replicates. After they were preheated to 39°C , pectin lyase and polygalacturonase were added at E/S ratios of 0 to 0.05% each, and the mixture was incubated at 60 rpm on a rolling mixer situated in an oven at 39°C . After 60 min, 20 ml of preheated water containing 16 mg/ml pancreatin, 50 mM bicarbonate, and 2 mg/ml bile salts, which made the pH ~ 7 , was added. This mixture was further incubated at 60 min and 60 rpm. The tubes were placed on ice for 5 min to stop the reaction and were centrifuged at $4,000 \times g$ for 5 min at 4°C , after which they were filtered through qualitative filter paper (no. 417; VWR, Darmstadt, Germany). Ten milliliters of the filtrate was added to 23.3 ml isopropanol to precipitate the soluble carbohydrates, and the mixture was centrifuged at $4,000 \times g$ for 5 min. The supernatant was discarded, and the pellet was lyophilized and weighed.

Production of UFSF. Ultrafiltered soluble fiber (UFSF) mimicking the one produced by Thomassen et al. (26) was made by adding 10 g of potato pulp to 1,000 ml 0.1 M phosphate buffer, pH 6, and preheating the mixture to 60°C . Pectin lyase enzyme and polygalacturonase substrate were added at 1% each, and the reaction was run for 1 min at 300 rpm, after which the reaction was stopped by 10 min of boiling. The solution was then centrifuged at $5,000 \times g$ for 60 min, and the supernatant was ultrafiltered with a 100-kDa-molecular-mass-cutoff VivaFlow 200 filter (Viva-

science, Hannover, Germany) with the addition of 5 liters of water until 90 ml remained in the retentate. The carbohydrate in the retentate was then precipitated by addition of isopropanol to 70% (vol/vol), and the mixture was centrifuged at $4,000 \times g$ for 5 min. The pellet was then lyophilized.

Carbohydrate composition. Monosaccharide composition analysis was done as described by Ravn and Meyer (37). In brief, samples were hydrolyzed by trifluoroacetic acid (soluble carbohydrate) or sulfuric acid (potato pulp), followed by quantification on a high-pH anion-exchange chromatography (HPAEC)-pulsed amperometric detection (PAD) system using a Dionex CarboPac PA1 analytical column (2 mm by 250 mm) combined with a CarboPac PA1 precolumn (2 mm by 50 mm) and 0.25 to 500 mM NaOH (37). Rhamnose, arabinose, galactose, glucose, mannose, xylose, and galacturonic acid were included as standards.

Size determinations were done with high-performance size exclusion chromatography (HPSEC) analysis by dissolving carbohydrate samples at 3 g/liter in 0.1 M sodium acetate, pH 6, with 0.02% sodium azide and filtering with a 0.22- μ m-pore-size filter. Samples were then injected in a Shodex OHPak SB-806 HQ column (8.0 mm by 300 mm; Showa Denko KK, Kawasaki, Japan) and eluted with 0.1 M sodium acetate (pH 6). The injection volume was 50 μ l, and the flow rate was 0.5 ml/min at 30°C on a system consisting of a P680 high-pressure liquid chromatography (HPLC) pump, an ASI-100 automated sample injector, and a Shodex RI-101 refractive index detector (Showa Denko KK). Pullulan standards of 1.3, 10, 110, 400, and 800 kDa were used.

Protein determination. The concentration of soluble protein in the extracted fibers was estimated using a micro-BCA protein assay reagent kit (Pierce, Rockford, IL) per the manufacturer's instructions with BSA as a standard.

In vitro fermentations. A fiber product was produced in the GI tract reactor to simulate the *in situ* production of what would be expected to reach the terminal ileum in an animal if the animal were fed enzymes and potato pulp. This fiber was then fermented in small-scale fermentations by the method of Vignæs and colleagues (35, 36). Briefly, IVSF from the simulated gastrointestinal reactor was fermented for 24 h at a final concentration of 0, 2.5, 5, or 10 g/liter of fiber (the control, IVSF-2.5, IVSF-5, and IVSF-10 groups, respectively) in a glucose-free medium consisting of 2 g/liter peptone water, 1 g/liter yeast extract, 0.1 g/liter (1.71 mM) NaCl, 0.04 g/liter (0.23 mM) K_2HPO_4 , 0.04 g/liter (0.29 mM) KH_2PO_4 , 0.01 g/liter (0.04 mM) $MgSO_4 \cdot 7H_2O$, 0.01 g/liter (0.07 mM) $CaCl_2 \cdot 2H_2O$, 2 g/liter (23.81 mM) $NaHCO_3$, 0.5 g/liter bile salts, 0.5 g/liter L-cysteine hydrochloride, 0.005 g/liter hemin, 10 μ l/liter vitamin K1 (0.02 mM), 2 ml/liter Tween 80, and 0.05 % (wt/vol) resazurin. Inulin (INU; OrafitiHPX, Orafiti, Oreye, France) and UF5F, both of which were added at 5 g/liter (INU-5 and UF5F-5, respectively), were used as positive controls. The fibers were dissolved by 15 min of agitation in a boiling water bath, which provided the added advantage of sterilizing the fibers, which were contaminated with *Bacillus cereus* as well as other bacteria. The samples from the terminal ileum of piglets, which had been stored in 25% glycerol, were thawed on ice, pooled, and diluted 5-fold in 10 mM degassed phosphate-buffered saline. Digesta were pooled across piglets, as in earlier studies (23, 34, 38), in order to control biological variation (39). This mixture was diluted 10-fold in the fiber-containing medium, which had been degassed in an anaerobic cabinet overnight in Nunc 14-ml round-bottom tubes. The resulting 1% solution of ileal content was then fermented for 24 h in an anaerobic cabinet at 37.5°C. All treatments were performed in 10 replicates. A fermentation mixture that was harvested immediately after inoculation and that had no added fiber served as a baseline control, and fermentation of this mixture was also performed in 10 replicates.

pH and organic acid analysis. Following the fermentations, the tubes were centrifuged for 5 min at $5,500 \times g$ at 4°C, and the pH was measured with a pH meter. The sample was then resuspended, and the suspension was transferred to a 2-ml Eppendorf tube, which was spun at $13,000 \times g$ for 10 min. The supernatant was then filtered through a 0.22- μ m-pore-size filter and assayed for OAs using HPLC. A Shimadzu HPLC system

fitted with an RSpak KC-811 column and a refractive index detector and that used 12 mM H_2SO_4 at a flow rate of 0.6 ml/min at 63°C was used. The standards used were lactic acid, acetic acid, propionic acid, isobutyric acid, *n*-butyric acid, isovaleric acid, and *n*-valeric acid.

DNA extraction. Fermentation samples were spun at $13,000 \times g$ for 10 min, and the DNA contained in the cell pellets was purified with a Maxwell LEV blood DNA purification kit (Promega Corporation, Madison, WI, USA) as described by Ingerslev et al. (40).

16S rRNA gene PCR. A PCR targeting the V1/V2 regions of the bacterial 16S rRNA gene from the bacteria contained within the fermentations was performed. The PCR was performed using the universal primers V1-forward (5'-AGAGTTTGATCCTGGCTCAG-3') and V2-reverse (5'-CTGCTGCCTYCCGTA-3') (41) (Sigma-Aldrich, Broendby, Denmark). Both primers were tagged with a 6-nucleotide bar code at the 5' end, and each specific bar code was assigned to a specific DNA sample. The reaction was carried out in 50- μ l reaction mixtures containing 5 μ l of 5 \times Gold *Taq* buffer (Applied Biosystems, Branchburg, NJ, USA), 1 μ l of each primer (20 μ M), 2 μ l of 10 mM deoxynucleoside triphosphates, 4 μ l of 25 mM $MgCl_2$, 0.5 μ l of AmpliTaq Gold polymerase (Applied Biosystems), 34.5 μ l of nuclease-free H_2O , and 2 μ l of DNA template (10 ng/ μ l). Reaction times and cycling conditions were 94°C for 6 min; 30 cycles of 94°C for 45 s, 57°C for 45 s, and 72°C for 90 s; and 72°C for 10 min. The resulting PCR products were then analyzed on an Agilent 2100 bioanalyzer using an Agilent DNA 1000 kit (Agilent Technologies, Waldbronn, Germany) and further pooled in equimolar ratios (50 ng per bar-coded sample). The pooled DNA was then purified of primers and detergents using a Qiagen MinElute PCR purification kit (Qiagen GmbH, Hilden, Germany) according to the manufacturer's instructions.

Illumina MiSeq sequencing. The DNA was submitted to the National High-Throughput DNA Sequencing Centre at the University of Copenhagen, Copenhagen, Denmark, for sequencing on an Illumina MiSeq 250PE platform. The long reads obtained were analyzed using BION-meta software (more information about BION-meta software and acquisition of the software is available from the Danish Genome Institute, Aarhus, Denmark). In brief, demultiplexing was performed according to the primer and bar code sequences. Forward and reverse sequences were joined, with no gaps, a maximum mismatch percentage of 80, and a minimum overlap length of 20 bp being allowed. Next, the sequences at both ends were cleaned by removal of bases with a quality of less than 98%, which is equivalent to a Phred score of 17. Identical sequences were further dereplicated into consensus sequences. Consensus sequences of at least 260 nucleotides in length were mapped into a table according to the individual bar codes. Finally, the consensus sequences were taxonomically classified against the sequences in the Ribosomal Database Project II (RDP-II) SSU database (RDP II; <http://rdp.cme.msu.edu/index.jsp>) using a word length of 8 and a match minimum of 90%. The top 1% of the sequences obtained from the RDP-II database showing similarity were used for taxonomic classification of the consensus sequences. The resulting operational taxonomic units (OTUs) in each bar-coded sample were normalized in order to enable direct statistical comparisons of relative abundance in each sample.

Statistics. The dose-response relationship of enzyme dosage ($[E/S]$) versus dry matter release in the intestinal reactor was fitted to a modified Monod equation of the form percent release = $\phi_1 + \{\phi_2 \cdot [E/S]/(\phi_3 + [E/S])\}$, where ϕ_1 is the value of the control, ϕ_2 is the maximum value with the control value subtracted, and ϕ_3 is the enzyme concentration at which half of the maximum release is attained. Analysis of variance (ANOVA) was used to evaluate significant differences in dosage. pH and organic acid concentrations were analyzed with an ANOVA, followed by Tukey's test, with the fiber treatment being the main effect, using the lm- and HSD.test procedures in R. Two-way ANOVAs of the fiber composition and organic acid data were avoided due to uneven variances, and instead, when multiple one-way ANOVAs were performed, adjustments for multiple comparisons were made by use of the Sidak correction. Sequencing data were analyzed at a given taxonomic level by ANOVA of log-transformed data,

TABLE 1 Composition of fibers extracted from potato pulp by simulated *in vitro* digestion (IVSF) and by using an established purification process (UFSF)^a

Fiber type	Column composition (% [wt/wt] released dry matter)								
	Rha	Ara	Gal	Glc	Xyl	Man	GalA	Protein	NA
IVSF	3.4 ± 0.4*	6.5 ± 0.8	36.3 ± 3.6*	2.4 ± 0.4*	0 ± 0	0 ± 0*	20.3 ± 5.0*	13.2 ± 0.5*	17.9 ± 0
UFSF	2.4 ± 0.1	5.4 ± 0.3	45.6 ± 1.5	4.7 ± 0.4	0 ± 0	6.7 ± 0.2	9.3 ± 0.2	10.7 ± 0.4	15.2 ± 0

^a Total dry matter release was determined by weighing after freeze-drying, and the monosaccharide composition was determined by acid hydrolysis followed by HPAEC-PAD analysis. Protein was determined with a bicinchoninic acid assay. NA, nonaccountable (the fraction which could not be recovered by HPAEC-PAD or protein determination). Data for monosaccharides were calculated as dehydrated values ($n = 4$). *, significant difference ($P < 0.05$) within column by *t* test after correction for multiple comparisons.

followed by Tukey's *post hoc* test and correction for multiple comparisons by use of the Sidak correction (42) when multiple OTUs were compared. The microbial community was analyzed by subjecting species-level data to principal coordinates analysis (PCoA) with treatment groups as constraints and using the Bray-Curtis dissimilarity index with the capscale procedure in R, followed by analysis of similarities (anosim procedure) as well as k-means clustering (k-means procedure). Shannon indices were calculated by use of the diversity command. Principal coordinates regression, e.g., using the scores from the first axis of the PCoA as a predicting variable, was used to compare the overall bacterial composition with the presence of individual OAs.

RESULTS

Characterization of piglet digestive parameters. The dry matter content, the pH, and the pepsin levels in the stomach digesta were determined in order to use the values to produce a realistic *in vitro* GI system. There was substantial variation within the piglets, especially in the activity of pepsin, which was, on average, 43.3 units/ml digesta (pooled standard deviation [SD], 3.3 units/ml digesta) but ranged from 13.4 to 98.1 units/ml digesta. The average dry matter content was 21.42% (wt/vol) and ranged from 6.78 to 30.65% (wt/vol), whereas the pH averaged 4.16 and ranged from 4.1 to 4.6.

The average protease content in the ileum was 39.2 units/ml digesta (pooled SD, 1.32 units/ml digesta) and ranged from 7.0 to 78.0 units/ml. The dry matter content in the ileum was, on average, 13.4% (wt/vol) (range, 7.28 to 19.30), and the average pH was 6.6 (range, 6.0 to 7.0).

***In vitro* GI tract reactor.** The GI tract reactor was set up on the basis of the measurements from the piglet digesta with an additional overhead. The protease content in particular was deemed to be of importance, and therefore, protease was added to an amount equal to twice the highest measured concentration for both the stomach and the small intestine steps. The lowest measured pH in the stomach was 3.5, and therefore, the pH used in this step was 3. The pH in the small intestine was set to be neutral (pH 7).

The activities of the polygalacturonase and the pectin lyase under stomach conditions (pH 3) were 22.70 U/mg and 0.02 U/mg, respectively. Under small intestine conditions (pH 7), the activities were 18.79 U/mg and 2.38 U/mg, respectively.

Characterization of IVSF and UFSF. The release of water-soluble fibers from potato pulp showed a clear dose dependency as a function of enzyme dosage, which could be modeled by a nonlinear 3-parameter equation, which in turn suggested that the maximal release was 26.9% of the initial dry matter. The proportion of the initial dry matter released without addition of pectinolytic enzymes was $5.56\% \pm 0.1\%$, and since less than 20% of this fraction could be accounted for by HPAEC-PAD or as protein, it presumably consists of various proteins, lignin, and salts. The compositions of all other fractions released showed that they con-

tained large amounts of galactose, galacturonic acid, and, to a lesser degree, arabinose and rhamnose. Analysis of variance of the data revealed that there was a plateau in yield at a dosage consisting of an E/S ratio of 0.05%; for this reason, an E/S ratio of 0.03% was chosen as a cost-beneficial dosage with which to do future work with. At this dose, 24.6 mg dry matter is released from 100 mg potato pulp, corresponding to 9.5 mg galactose. The composition of the *in vitro*-released fiber, IVSF, and UFSF is shown in Table 1, and it is seen that IVSF contained more galacturonic acid and less galactose than UFSF, as well as slightly more protein. UFSF also contained a small amount of mannose, presumably from the pectinase formulation. HPSEC analysis of IVSF revealed a molecular size distribution with two distinct peaks, notably, a large fraction of ~900 kDa as well as a smaller fraction of ~10 kDa (Fig. 1). The HPSEC chromatogram of the UFSF showed that high-molecular-mass polysaccharides (i.e., polysaccharides with molecular masses of >800 kDa) were much more abundant than the low-molecular-mass polysaccharide fractions. Estimation of the amount of monosaccharide was done by comparing the area under the curve (AUC) of the monosaccharide peak (24.6 min) with the total AUC. The AUC of the monosaccharide peak constituted ~3.8% of the total AUC in UFSF, while this number was 10.6% in IVSF, suggesting that the monosaccharide content was 2.8-fold higher in IVSF.

***In vitro* fermentation characteristics.** IVSF, UFSF, and inulin were fermented in the terminal ileal content from piglets in an *in*

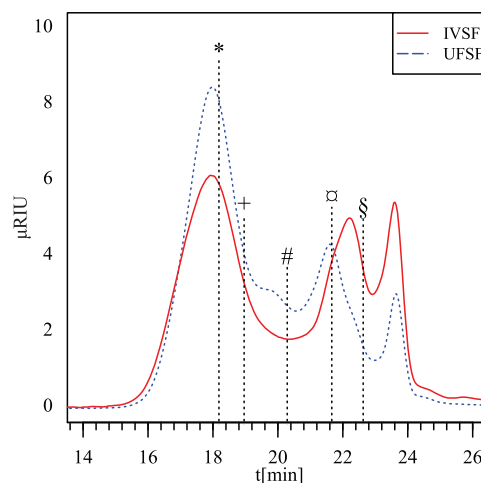


FIG 1 High-performance size exclusion chromatogram of the fibers extracted from potato pulp by simulated *in vitro* digestion (IVSF) and by using an established purification process (UFSF). *, 800 kDa; +, 400 kDa; #, 110 kDa; □, 12 kDa; \$, 1.3 kDa. μ RIU, refractive index unit; t, time.

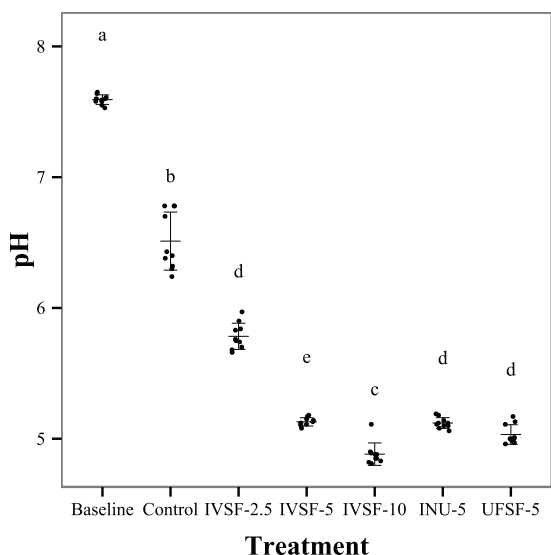


FIG 2 pH as a function of treatment after 24 h of *in vitro* fermentation of IVSF at 2.5 to 10 mg/ml, INU-5, or UFSF-5. Data are for 10 fermentations. Non-similar letters indicate significant differences.

in vitro reactor for 24 h. The fermentations resulted in a fiber dose-dependent decrease in pH ($P < 0.0001$, general linear model), as shown in Fig. 2. The pH in the INU-5 fermentation was the same as that in the IVSF-5 fermentation ($P = 1$, Tukey's *post hoc* test), although the pH was not as low as that in the IVSF-10 fermentation. The UFSF-5 treatment induced a pH similar to that in the INU-5 and IVSF-5 treatments but a pH higher than that in the IVSF-10 treatment.

Organic acids. All the fibers were highly fermentable. Fermentation treatment had a significant effect (ANOVA, $P < 0.001$) on all organic acids (Table 2). Total OA levels markedly increased with the concentration of IVSF, and INU-5 and UFSF-5 treatments resulted in OA levels between the OA levels from IVSF-2.5 and IVSF-5 treatments. The level of lactic acid was especially markedly increased by the experimental fiber (20 times from the baseline level) and was significantly higher than that in the INU-5 treatment group. Lactate was also the most affected OA in relative terms, constituting 3% of total OAs in the control treatment and 17% in the IVSF-10 treatment. The propionate level increased with dosage, although the levels plateaued beyond IVSF concentrations of 5 g/liter. Both butyrate and valerate levels increased from the baseline levels with all treatments, although the only

treatment that had results statistically significantly different from those for the other treatments was the IVSF-2.5 treatment. The acetate level increased with the IVSF concentration, as well as in the INU-5 and UFSF-5 treatments. As expected, a highly significant correlation ($r^2 = 0.94$, $P < 0.00001$) existed between the total OA concentration and pH.

Microbiota composition. After MiSeq sequencing of the 16S rRNA gene PCR products followed by pattern demultiplexing, sequence cleaning, unification, and chimera filtering by the BION-meta software, 1,279,205 sequences were available for taxonomic classification.

At the phylum level, the samples were generally heavily dominated by the phylum *Firmicutes*. The native microbiota, e.g., the microbiota in the baseline samples, consisted of more than 97% *Firmicutes*, with reads for members of the classes *Bacilli*, *Clostridia*, and *Erysipelotrichia* accounting for 47, 33, and 17% of the total reads, respectively. Fermentation for 24 h without added fiber resulted in increasing numbers of organisms of the class *Clostridia*, even in the no-fiber control group, whereas the presence of the *in vitro*-solubilized fibers was associated with a rise in the numbers of organisms of the class *Negativicutes*. Incubation with INU-5 resulted in a decrease in the numbers of organisms of the class *Clostridia* and an increase in the numbers of organisms of the class *Negativicutes*, whereas the UFSF-5 incubation eliminated organisms in the class *Clostridia* entirely, while it increased the numbers of organisms of the class *Bacilli* and the class *Negativicutes*.

At the genus level (Fig. 3), the fibers made *in vitro* caused a significant decrease in the numbers of organisms of the genus *Streptococcus* and a significant increase in the numbers of organisms of the genus *Lactobacillus*. The amount of *Clostridium* bacteria did not change significantly from that in the control group treated with IVSF, although it was decreased significantly in the INU-5 and UFSF-5 treatment groups. *Clostridium perfringens* was found in all groups, but its levels were not changed by the addition of IVSF and the numbers of *Clostridium perfringens* organisms were significantly depressed by the INU-5 and UFSF-5 treatments. No *Clostridium difficile* organisms were found in any of the groups. Only about 90 reads for *Clostridium* cluster XIVa of more than a million total reads were found. The numbers of organisms of the genus *Olsenella* were elevated only in the IVSF-2.5 and IVSF-5 treatment groups. The ratio of the number of organisms of the genus *Clostridium* to the number of organisms of the genus *Lactobacillus* was markedly heightened with the control treatment but was not different between the other treatments. Exclusion of data for the control group from the analysis to retain homosce-

TABLE 2 Concentrations of OAs as a function of treatment after 24 h of *in vitro* fermentation of IVSF at 2.5 to 10 mg/ml, INU-5, or UFSF-5

Group	Concn ^a (mmol/liter)					Total
	Lactate	Propionate	Acetate	Butyrate	Valerate	
Baseline	0.41 ± 0.04 ^D	0.89 ± 0.02 ^E	2.53 ± 0.05 ^E	0.11 ± 0.02 ^B	0 ± 0 ^A	3.95 ± 0.09 ^F
Control	0.84 ± 0.09 ^D	5.87 ± 0.18 ^D	19.73 ± 3.17 ^D	0.96 ± 0.50 ^{AB}	0.29 ± 0.25 ^A	27.69 ± 4.15 ^E
IVSF-2.5	0.95 ± 0.07 ^{CD}	8.09 ± 1.69 ^C	22.25 ± 1.92 ^{CD}	3.65 ± 2.85 ^{AB}	1.40 ± 1.21 ^A	36.34 ± 1.51 ^D
IVSF-5	4.23 ± 0.69 ^{BC}	11.79 ± 0.63 ^A	28.36 ± 1.34 ^{AB}	1.86 ± 0.77 ^{AB}	0.50 ± 0.38 ^A	46.73 ± 1.22 ^B
IVSF-10	9.11 ± 3.85 ^A	10.77 ± 1.51 ^{AB}	30.50 ± 5.09 ^A	4.49 ± 4.29 ^A	1.52 ± 1.92 ^A	56.39 ± 3.68 ^A
INU-5	2.21 ± 0.86 ^{CD}	10.30 ± 0.63 ^{AB}	26.08 ± 1.86 ^{BC}	2.72 ± 1.49 ^{AB}	0.94 ± 0.69 ^A	42.25 ± 1.96 ^C
UFSF-5	6.19 ± 3.60 ^{AB}	9.56 ± 0.59 ^{BC}	23.57 ± 1.75 ^{CD}	3.22 ± 2.23 ^{AB}	0.95 ± 0.89 ^A	43.49 ± 1.84 ^{BC}

^a Values are for 10 piglets for each group. Nonsimilar letters denote significant differences ($P < 0.05$) for each column by one-way ANOVA after correction for multiple comparisons.

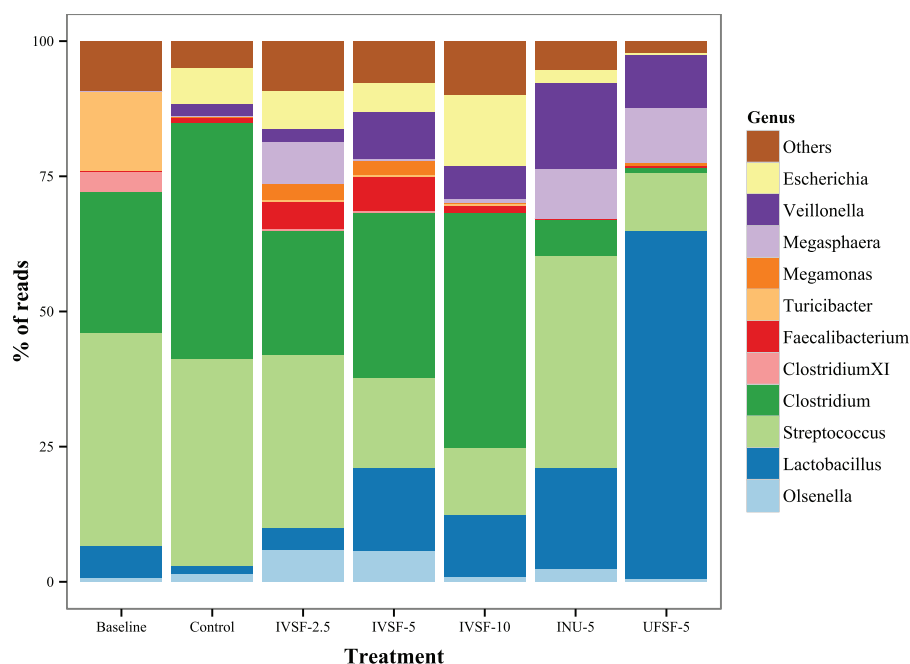


FIG 3 Genus-level composition of the microbial community after sequencing. DNA from 24-h *in vitro* fermentations of IVSF at 2.5 to 10 mg/ml, INU-5, or UFSF-5 was sequenced. Data are for 9 to 10 fermentations in each group.

lasticity suggested that the IVSF-10 group had a higher ratio of these two genera than the UFSF-5 group, mainly since in this study the amount of *Clostridium* bacteria was very low. The numbers of organisms of the genus *Megasphaera* were increased significantly by all fiber treatments except the IVSF-2.5 treatment. Members of the family *Enterobacteriaceae* were present in all baseline samples, and their levels increased from those at the baseline in all fermentations, including the control fermentation, although the numbers in the INU-5 and UFSF-5 groups were slightly lower than those in the control group. *Bifidobacteria* were consistently observed only in the INU-5 treatments and constituted only less than ~0.1% of the microbiota.

At the genus level, the Shannon diversity index after treatment was significantly lower than that at the baseline for the control and UFSF-5 groups; the IVSF-2.5, IVSF-5, IVSF-10, and INU-5 groups had significantly higher indices than the control and UFSF-5 groups. A constrained analysis of principal coordinates (CAP) on species-level data by use of the treatment groups as constraints resulted in 71.1% of the variation being explained on the first two dimensions, as seen in Fig. 4. On the first axis, *Lactobacillus* spp. as well as *Megasphaera* spp. had positive coefficients, whereas species of *Clostridium*, *Streptococcus hyointestinalis*, and *Erysipelotrichaceae turicibacter* had negative coefficients. On the second axis, *Streptococcus hyointestinalis*, *Lactobacillus delbrueckii*, and an unclassified *Turicibacter* species had positive coefficients, whereas various species of *Clostridium* and unclassified *Lactobacillus* species had negative coefficients. Clustering by use of the k-means algorithm showed distinct clusters, namely, one containing the UFSF-5 treatment, a second cluster containing the INU-5 treatment, a third cluster containing the IVSF-5 and IVSF-10 treatments, and a fourth cluster containing the control group. In contrast, the IVSF-2.5 group was not clearly clustered, and although the control samples were clearly separated on the first axis,

there was no separation on the second axis. The clustering was supported by analysis of similarities ($R = 0.811$, $P < 0.001$).

The scores obtained from the constrained analysis of principal coordinates were further used as an independent variable to test correlations of the scores with the pH and the OA composition using linear regression (Fig. 5). A significant negative association between the score on dimension 1 (CAP1) and the pH and acetic acid concentration was observed, whereas there was a positive association between the score on dimension 1 and the lactic acid, butyric acid, and valeric acid concentrations. This, in turn, suggests that a gut microbiota rich in species belonging to the genera *Lactobacillus* and *Megasphaera* and poor in organisms of the genus *Clostridium* is associated with a low pH and acetic acid concentration but high levels of butyric acid, valeric acid, and lactic acid.

DISCUSSION

In the present paper, the solubilization of potato galacto-rhamnogalacturonan 1 from potato pulp was attempted in a system simulating the piglet upper gastrointestinal tract. The operating parameters of the system were chosen from direct measurements on piglets. The pHs of the stomach and the small intestine were measured to be 4.1 to 4.6 and 6.0 to 7.0, respectively, which are in agreement with those reported in the literature (43). A pH of 3.0 was chosen as the value for the stomach, as this level of acidity is realistic in piglets that have eaten little or after an overnight fast (44, 45). Proteolytic activity, which can potentially degrade the pectinolytic enzymes, was added to mimic the measured values, as the values in the literature are not directly applicable due to differences in the methods and the definition of units of activity. The release of water-soluble fiber from the potato pulp required very little enzyme, with about 30 μg of each enzyme (E/S ratio, 0.03%) releasing ~21% of the dry matter from 300 mg potato pulp. With amounts of each enzyme above 90 μg , there was no further release.

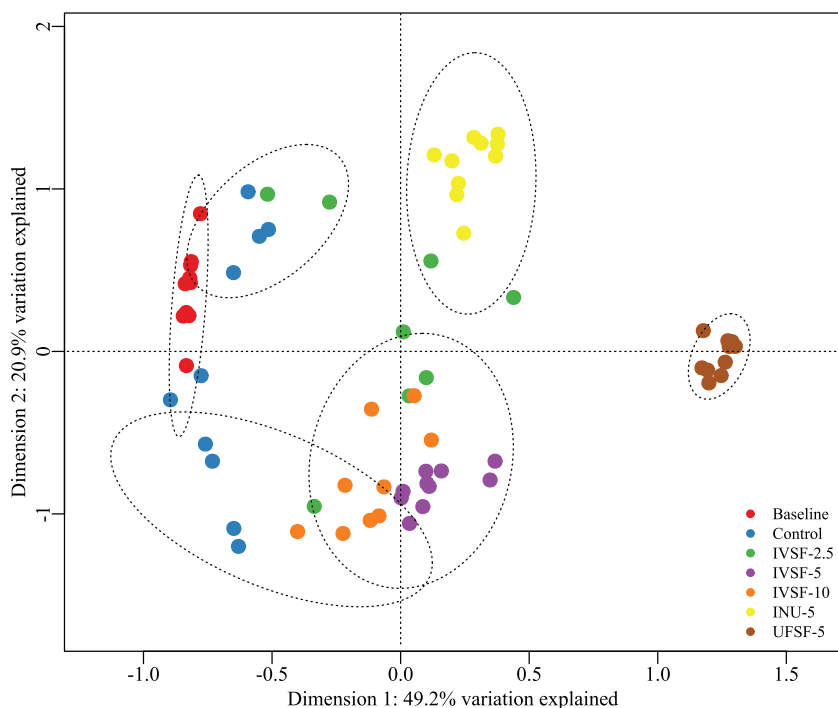


FIG 4 Constrained analysis of a principal coordinates plot on normalized bacterial reads at the species level using Bray–Curtis dissimilarities. The k-means method was used for clustering (clusters include a 95% confidence interval). DNA from 24-h *in vitro* fermentations of IVSF at 2.5 to 10 mg/ml, INU-5, or UFSF-5 was sequenced. Data are for 9 to 10 fermentations in each group.

The two monocomponent enzymes were not purified beyond ultrafiltration before use, so the use of even lower doses may be feasible, as the enzyme dose was determined from the total amount of protein after *Pichia* fermentation. Previous reaction schemes have involved an E/S ratio of 1%, which would correspond to 3 mg of each enzyme, an approximately 33 times higher enzyme dosage (26, 27), making *in situ* production an attractive possibility.

The monosaccharide composition of the released fraction suggests that this was indeed galacto-rhamnogalacturonan 1, corresponding to a backbone of rhamnose and galacturonic acid that was highly substituted with galactose chains as well as smaller amounts of arabinose, likely being flanked by domains of homogalacturonan (28, 46). UFSF was different from IVSF, in that the galactose content was higher in UFSF and the galacturonic acid content was lower. According to HPSEC analysis, both fibers encompassed two separate populations of the solubilized pectin, namely, high- and low-molecular-weight fractions. UFSF, which had a higher molecular weight than IVSF as well as a larger amount of galactose, can then be hypothesized to be richer in galactan-rich RG-1 fractions and to have smaller amounts of shorter polygalacturonan chains, which would have been filtered out in the ultrafiltration step, as in the UFSF product. The content of monosaccharide was also higher in IVSF than UFSF. The existence of a dual population in enzymatically solubilized potato pectin has previously been shown (26). An additional difference was the presence of mannose in the purified fiber, which presumably came from the enzyme preparation, e.g., as cell wall material from *Pichia pastoris*. Mannose has been detected (3 to 5%) in similarly produced fiber (26, 47) and appears to be >10 kDa in size.

The pH of the incubations followed a dose-dependent pattern,

dropping up to 2.5 pH points when incubation was with 10 mg/ml of IVSF for 24 h. Few *in vitro* fermentation studies have reported using porcine ileal microbiota, but the pH was reported to drop from 6.63 to ~6.2 and ~5.7, respectively, by fermenting 5 or 10 g/liter of predigested feed containing various oligosaccharides in pig feces (22). Another study used various human milk oligosaccharides (HMOs), inulin, and galacto-oligosaccharides at 1 mg/ml in sow-reared or formula-fed piglet colonic contents and reported pH drops from 6.5 to 5.3 after 12 h (23). Incubations of 10 g/liter inulin or transgalacto-oligosaccharides (TOSs) in the pig GI tract contents for 4 h showed a drop from pH 6.5 to 6.3 in distal small intestine content but a drop to pH 5.8 for colonic content (48). pH values reported from *in vivo* studies in the terminal ileum have been reported to range from 7.8 to 8.3 (47) and from 5.7 to 6.0 (49), suggesting that the low pH values observed in the *in vitro* fermentation studies may be lower than those that are physiologically relevant. The colonic pH has been reported to be 6.8, regardless of diet (50), suggesting that the clearance of fatty acids, which does not occur *in vitro* but is substantial in the colon (51), has a stabilizing effect on pH.

The fibers in this study showed excellent fermentability, evident by the increase in organic acid content (Table 2). The exact role of the individual fatty acids in piglet health is not entirely clear, but there is general consensus that butyric acid is both a fuel and a trophic factor for colonocytes, whereas propionate and acetate enter the circulation and participate in hepatic energy production (52–54). Interestingly, the control sample, with which 24 h of incubation without fiber was used, showed increases in acetate and propionate, probably reflecting the fermentation of residual material from digesta or the glycerol added at freezing. Of further interest are the relatively high levels of lactate and the

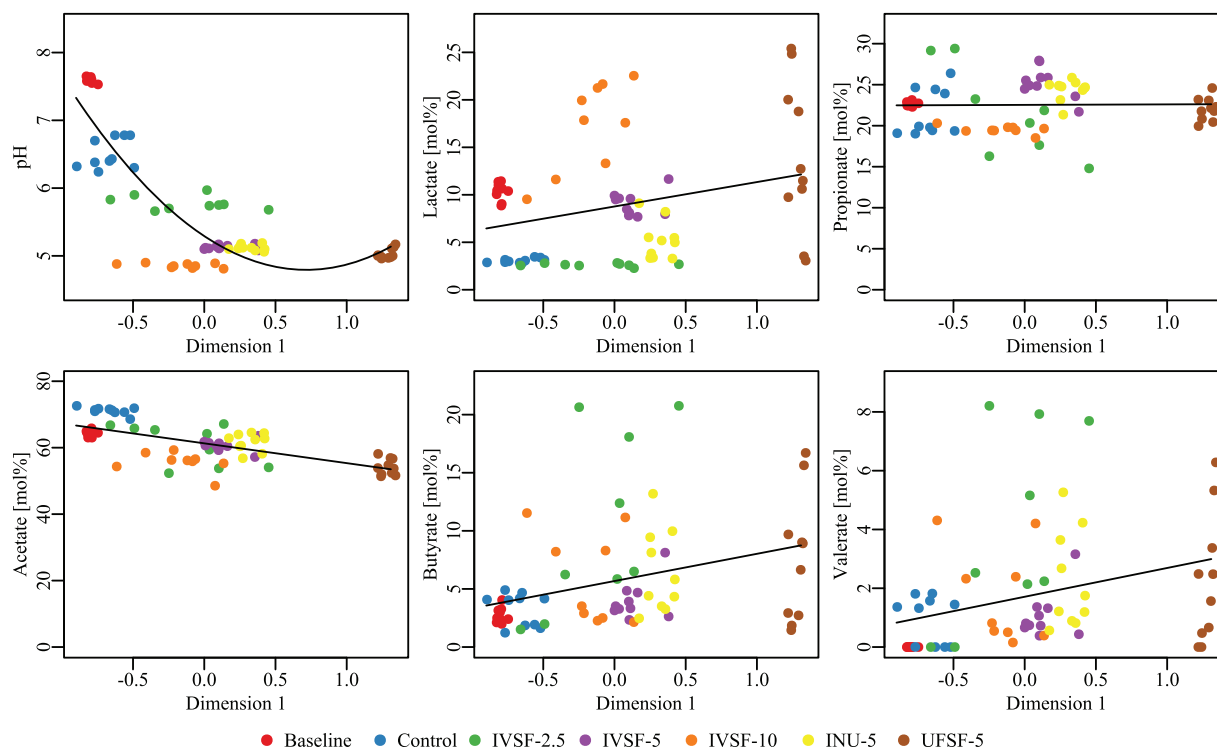


FIG 5 Associations between pH and fatty acids versus bacterial flora (CAP1). All associations apart from the association with propionate were significant ($P < 0.05$, general linear model). pH and OA levels were measured, and DNA from 24-h *in vitro* fermentations of IVSF at 2.5 to 10 mg/ml, INU-5, or UFSF-5 was sequenced. Data are for 9 to 10 fermentations in each group.

apparent dose-response relationship observed in the IVSF series and the UFSF-5 group, suggesting that lactate is a fermentation product of lactic acid bacteria. The high levels of lactic acid bacteria in these treatment groups support this notion (Fig. 3) as well as the correlations of CAP1 and the lactate concentration, as seen in Fig. 5. When fermenting inulin (55) or xylooligosaccharides (21), lactate is present after 11.5 h, and its level is markedly lowered after 30 h and is undetectable after 72 h, presumably since lactate is metabolized into other organic acids, such as propionate, through the acrylate pathway (54).

The microbiota was almost exclusively composed of the phylum *Firmicutes*, which is in contrast to the findings of other published pig metagenomic studies: in a study by Lamendella et al. (56), organisms of the *Firmicutes* and the *Bacteroidetes* phyla constituted 40 to 60% and 25 to 40% of the microbiota, respectively, depending on the sequencing platform and the classification database. These results were, however, for fecal samples from adult pigs. A more recent study using piglets weaned at 28 days and fed for 31 days showed 75% *Firmicutes*, 15% *Bacteroidetes*, and small amounts of organisms of the phylum *Proteobacteria* in fecal samples. In 2012, Looft et al., in an antibiotic feeding study with piglets weaned at 21 days and fed for 21 days, had results that contrasted with those of Lamendella et al. (56): 20% *Firmicutes*, 50% *Bacteroidetes*, and ~10% *Proteobacteria* (57). Another study from Looft and colleagues reported ~20 to 30% *Firmicutes* and 50 to 60% *Bacteroidetes* in animals weaned at 12 days and tested at 8 weeks (58). One explanation for the dominance of the *Firmicutes* in our study is the use of terminal small ileum samples rather than fecal samples, and that explanation is supported by a recent study examining the composition of the microbiota in several segments of

the porcine GI tract: here, the ileal microbiota was ~95% *Firmicutes*, whereas the cecal, colonic, and fecal flora was ~50% *Bacteroidetes* (30).

The composition of the microbiota in the baseline group, e.g., the microbial composition in the native piglet, was dominated by the genera *Streptococcus* (42% ± 6%), *Clostridium* (27% ± 7%), *Turicibacter* (15% ± 2%), and *Lactobacillus* (5% ± 0.5%). The populations of *Turicibacter*, a genus previously found in the ileum of piglets (59) but isolated only from a human patient with acute appendicitis (60), were close to entirely attenuated in all 24-h fermentations, suggesting that this genus requires host interaction or has specific growth requirements. Merely incubating the bacteria for 24 h resulted in a shift toward a microbiota consisting of equal amounts of *Clostridium* and *Streptococcus*. All the fiber treatments, apart from UFSF-5, resulted in a higher bacterial diversity, as evidenced by the Shannon index. The UFSF-5 group was almost exclusively composed of species of *Lactobacillus*, *Veillonella*, *Megasphaera*, and *Streptococcus*.

Addition of UFSF resulted in a significant increase in the numbers of organisms of the genus *Lactobacillus*, believed to be beneficial (10), along with a significant decrease in the numbers of organisms of the genus *Streptococcus* and a nonsignificant increase in the numbers of organisms of the genus *Clostridium*. Furthermore, the amount of *Clostridium* bacteria was substantially and significantly depressed with both the INU-5 and UFSF-5 treatments. A point of main interest is the difference between the microbial community composition in the IVSF-5 treatment group and that in the UFSF-5 treatment group, as these treatments were supposedly chemically identical but resulted in markedly different microbiota profiles. The UFSF-5 group did, however, have 25%

more galactan and half the galacturonic acid content, findings which suggest that the galactan chains of the molecule have a more pronounced effect on the genus *Lactobacillus*, whereas the galacturonic acid chains may be favored by bacteria belonging to the genus *Clostridium*. It has previously been shown that in human feces, pectin fractions with neutral sugars were efficient in increasing the amount of *Bifidobacterium* organisms, whereas the amount of *Clostridium* bacteria was increased by polygalacturonic acid, as enumerated by fluorescent *in situ* hybridization (FISH) (61). In contrast, another study using a pectin fraction with high levels of galacturonic acid also showed by means of FISH little growth of clostridia and increased levels of *Bifidobacterium* (62).

The *Clostridium* spp. observed in these fermentations appear to be a normal part of the porcine healthy gut microbiota, as they were present in high numbers in the baseline group and grew to high numbers in the control group, an observation consistent with the findings of other studies in healthy animals (56, 59, 63, 64). In the absence of genuine illness, the actual implications of *Clostridium* populations are difficult to discern, and it is also uncertain whether or not the eradication of *Clostridium*, as seen in the UFSF treatment group, is a positive effect. *Clostridium* cluster XIVa bacteria, previously found in the colon of piglets (23), were not found to any relevant extent in the samples in the present study, which could be because this study used the contents of the terminal ileum rather than the colon. Moreover, this cluster or organisms appears to preferably colonize the mucus layer (65), whereas this study used the luminal content. *Clostridium perfringens* appears to be a problem in the neonatal animal (66) but less of a problem in the weaning piglet, where *E. coli* is the main pathogenic agent (6, 7). In this study, the amount of *Enterobacteriaceae* in the groups treated with IVSF series was not different from that in the control group, although the amount in the INU-5 and UFSF-5 groups was decreased from that in the control group. The observation that the number of organisms of the genus *Escherichia* was higher in the IVSF-5 group than the UFSF-5 group can possibly be explained by the apparent 3-fold higher levels of monosaccharide in the IVSF-5 group. *Escherichia* is well adapted to the catabolism of monosaccharides, including galacturonic acid (67), and may have had an advantage in the early stages of the fermentation owing to the quick rate of replication of this genus.

Streptococcus spp. are also a normal component of the porcine gut microbiota, and in this study, their amount appeared to be diminished by both the fiber made *in vitro* and the fiber from UFSF-5. Since the abundance of *Lactobacillus* spp. was increased in the same groups, it is difficult to establish whether it is a direct effect of the fiber or occurs through the *Lactobacillus* organisms present. The consequence of this alteration is unknown.

Bifidobacterium, which is generally considered to be a health-promoting organism, is ubiquitous in the human gut microbiota, but it was not detected in notable numbers either in the baseline samples or after fermentation, and it is reported to comprise a negligible portion of the pig intestinal microbiota (23, 68, 69). The importance of *Bifidobacterium* in the pig remains to be illuminated.

The genus *Veillonella* grew well in all treatments but not at the baseline, likely owing to the fact that this genus metabolizes organic acids rather than carbohydrate (70). The amount of bacteria belonging to the genus *Megasphaera*, including the species *Megasphaera elsdenii*, suggested to protect the host against the pathogen *Brachyspira hyodysenteriae* (19), was also elevated by the fiber

treatment, in agreement with the fact that this genus feeds on organic acids (71).

With the use of PCoA, the entire microbiota could be visualized as well as clustered at the species level. It was seen that there was marked clustering of microbiota within each treatment group but that the microbiotas from the IVSF-5 and IVSF-10 groups were closer to one another than to the microbiota from any other group. In particular, the microbiota of the UFSF-5 group clustered far from the microbiota of the IVSF-5 group, but the compositions of these two groups should be chemically similar. This could be explained by small but possibly key differences in the monosaccharide content (3.8% in UFSF versus 10.6% in IVSF). The scores that were obtained, which provide an aggregate measure of the bacterial composition, could, furthermore, be correlated with the levels of organic acids and pH, showing patterns in the production of fatty acids and microbiota, namely, that a microbiota low in acetic acid but high in butyric acid, valeric acid, and lactic acid is associated with the genera *Lactobacillus* and *Megasphaera* and but not with the genus *Clostridium*.

For use in animal trials, it would appear that a treatment corresponding to IVSF-5 should be used, since the treatment with a higher dose of fiber, IVSF-10, tended to produce levels of *Clostridium* higher than those produced by the control treatment. UFSF appeared to decrease bacterial diversity, and it is unclear if having 60% *Lactobacillus* has a positive impact on the host.

In conclusion, it has been established that targeted enzymatic catalysis can be used to extract the RG-1 domain of low-value potato pulp in a simulated *in vitro* digestion. Furthermore, the fiber that was produced was highly fermentable and capable of changing the microbial community when fermented in the contents of the terminal ileum of piglets, notably, by increasing the *Lactobacillus* counts. Whether the interanimal variation is too large to verify a consistent response *in vivo* remains to be determined. For use in animal studies, use of a fiber dosage corresponding to that in IVSF-5 may be a valid option.

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