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In situ prebiotics for weaning piglets: In vitro production and fermentation of potato galacto-rhamnogalacturonan

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1 Abstract

Post weaning 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 gastro-intestinal tract (GI-tract) of the piglet and the prebiotic activity of the resulting fiber in the terminal ileum. Soluble fiber were successfully produced from potato pulp, an industrial waste product, with a minimal enzyme dose in a simulated upper GI-model extracting 26.9 % of initial dry matter. The fiber was rich in galactose and galacturonic acid and was fermented at 2.5, 5 or 10 g/L in a glucose-free media inoculated with the gut contents of piglet terminal ileum. Fermentations of 5 g/L inulin or 5 g/L of a purified potato fiber were used as controls. The fibers showed high fermentability, evident by a dose-dependent drop in pH and increase in organic acids, with lactate in particularly being increased. Deep sequencing showed a significant increase in Lactobacillus and Veillonella and an insignificant increase in Clostridium as well as a decrease in Streptococcus. Multivariate analysis showed clustering of the treatment groups, with the 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/L treatment is suggested.
2 Introduction

Post weaning 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 is 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 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 as well as the heavy metal zinc are used for prevention and treatment of PWD, as it is 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 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 (OA) as thoroughly reviewed by Liévin-Le Moal & Servin (12). It could then be hypothesized that prebiotic intervention would increase piglet infection resilience 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). \textit{In vitro} fermentations, using inocula from pig intestines or pig feces have, however, been fairly successful in showing increases in \textit{Lactobacillus} and, to a much lesser extent, \textit{Bifidobacterium}, and/or increases in production of organic acids (OA) resulting from bacterial fermentation (21–24).

Potato pulp is a high volume waste product from the starch industry, currently produced at \( \sim 1 \times 10^6 \) tonnes/year in Europe where it is used in low-value applications, e.g. as in animal feed priced at \( \sim 10 \) €/ton (25). It has previously been shown that a novel, fermentable and highly prebiotic fiber could be produced from potato pulp (26, 27), having 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 and the work of Thomassen et al. (2011) has shown that a mixture of polygalacturonase (3.2.1.15) and pectin lyase (4.2.2.10) efficiently released large amounts of RG-1 in an industrially relevant setting (26).

In a pig, peristalsis and body heat provides natural agitation and constant temperature in the \( > 2 \) hours required for the feed to reach the terminal small intestine. In light of this, it was hypothesized that feeding enzymes and substrate should allow the enzymatic degradation of highly complex molecules into prebiotic fibers \textit{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 does not possess genes for pectinolytic enzymes (30).

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

### 3 Materials and methods

#### 3.1 Characterization of pectinolytic enzymes

Pectin lyase and polygalacturonase were produced by fermentations as described in de 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 in Bauer et al. (32). This method ensures production of mono-component enzymes by placing the gene under 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 assay with bovine serum albumin as a standard as described by the manufacturer (Thermo Fisher Scientific, Rockford, IL).

Pectin lyase activity was assayed by incubating 0.25 % enzyme-to-substrate [w/w] (%E/S) pectin lyase with 1.5 g/L citrus pectin (Sigma–Aldrich, Steinheim, Germany) in McIlvaine buffer at pH 3 or pH 7 at 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 were defined as μmol of unsaturated uronide released per minute using 5500M⁻¹cm⁻¹ as the extinction coefficient (31).
Polygalacturonase was assayed by incubating 0.125 % E/S with 3 g/L polygalacturonic acid (Sigma–Aldrich, Steinheim, Germany) in McIlvaine buffer at pH 3 or 7 at 37 °C in triplicate. The amount of reducing ends was quantified in the reducing ends assay as described in 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 PAHBAH solution, which was then incubated at 70 °C for 10 min. The amount of reducing ends was then quantified by the absorbance at 410 nm. Units are defined as the released µmoles of galacturonic acid per minute, using free galacturonic acid as a standard.

3.2 Animals

Five suckling pigs were acquired from a commercial farm in Denmark at 26 days of age, two 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, had furthermore not been given antibiotics and were healthy. The animals were euthanized at the National Veterinary Institute at DTU, Denmark, by an overdose of pentobarbital and jugular puncture. All handling of animals was performed by trained personnel and veterinarians and fulfilled the regulations from the Danish Ministry of Justice.

Content from the stomach and middle jejunum was collected in sterile tubes and were snap frozen in dry ice before storage at -80 °C. Contents from terminal ileum were collected in sterile tubes and mixed 1:1 with 50 % w/w glycerol before snap freezing in dry ice and stored at -80 °C. The use of frozen compared to fresh samples has formerly been verified (33, 34) and frozen samples have previously been used in similar experiments (35, 36).
3.3 Characterization of digestive enzymes

Stomach and jejunal contents were homogenized followed by centrifugation at 4000g 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 at 105 °C for 24 hours in duplicate for each sample.

Pepsin in the stomach supernatant was assayed by incubating 50 µL supernatant in 250 µL 0.06 M HCl with 20 g/L hemoglobin in triplicate for each sample. The reaction was run at 37°C at 900 RPM and terminated after 60 min with 500 µL of 5 % w/v trichloracetic acid. After 10 min of mixing, the samples were centrifuged for 2 min at 14000g and 100 µL of the supernatant was measured at 280 nm. Controls were made by adding the trichloracetic acid before the gastric sample. Units were defined as nmol tyrosine released per minute using an extinction coefficient of 1250 M⁻¹cm⁻¹. Pepsin concentration was calculated by comparing the activity to a standard curve of commercial pepsin [P7125, #SLBB6557V] (Sigma-Aldrich, Steinhein, Germany).

Small intestine proteases in the jejunal supernatant were assayed with an AZCL-casein assay (Megazyme, Bray, Ireland) according to the manufacturer’s instructions with some modifications: 50 µL of 40-fold diluted sample 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 at 1400RPM and terminated after 5 min with 500 µL of 10 g/L trisodium phosphate. After centrifugation for 5 min at 15000g, 100 µL of the supernatant was measured at 590 nm in a microplate. Units were defined by a change in 1 OD at 1 cm path length per minute. The corresponding concentration of pancreatin was compared to a standard curve of commercial pancreatin [P7545, #SLBD0640V] (Sigma-Aldrich, Steinhein, Germany).
### 3.4 Production of *in vitro* solubilized fiber

A porcine gastrointestinal digestion process was simulated by sequential digestion in stomach conditions and then small intestinal conditions, producing *in vitro* solubilized fiber (IVSF). The concentrations and conditions were based on the measurements from 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.

10 mL of purified water with 0.32 mg/mL pepsin (Sigma, P7125) was added to 300 mg FiberBind in a 50 mL falcon tube and adjusted to pH 3 with HCl in four replicates. After preheating to 39 ºC, pectin lyase and polygalacturonase was added corresponding to 0-0.05 % E/S each and the mixture was incubated at 60RPM on a rolling mixer situated in an oven at 39 ºC. After 60 min, 20 mL of preheated water containing 16 mg/mL pancreatin, 50 mM bicarbonate and 2 mg/mL bile salts were added, making the pH ~7. This was further incubated at 60 min at 60RPM. The tubes were placed on ice for 5 min to stop the reaction and were centrifuged at 4000g for 5 min at 4 ºC, after which they were filtered through a qualitative filter paper no. 417 (VWR, Darmstadt, Germany). 10 mL of the filtrate was added to 23.3 mL isopropanol to precipitate the soluble carbohydrates and the mixture was centrifuged at 4000g for 5 min. The supernatant was discarded and the pellet was lyophilized and weighed.

### 3.5 Production of ultrafiltered soluble fiber

Ultrafiltered soluble fiber (UFSF), mimicking the one produced in Thomassen et al. (26) was made by adding 10 g of FiberBind to 1000 mL 0.1M phosphate buffer, pH 6, and preheating to 60 ºC. 1 % E/S of each polygalacturonase and pectin lyase was added and the reaction was run for 1 min at 300RPM, after which the reaction was stopped by 10 min of boiling. The solution
was then centrifuged at 5000g for 60 min and the supernatant was ultrafiltered with a 100 kDa VivaFlow 200 filter (Vivascience, Hannover, Germany) with the addition of 5 L of water until 90 mL remained in the retentate. The carbohydrate in the retentate was then precipitated by addition of isopropanol to 70 %v/v and the mixture was centrifuged at 4000g for 5 min. The pellet was then lyophilized.

### 3.6 Carbohydrate composition

Monosaccharide composition analysis was done as described in Ravn et al. (37). In brief, samples were hydrolyzed by trifluoroacetic acid (soluble carbohydrate) or sulfuric acid (FiberBind), followed by quantification on a HPAEC-PAD system using a Dionex CarboPac PA1 analytical column (2 mm × 250 mm) combined with a CarboPac PA1 precolumn (2 mm × 50 mm) and 0.25-500 mM NaOH (37). Rhamnose, arabinose, galactose, glucose, mannose, xylose and galacturonic acid were included as standards.

Size determinations were done by dissolving carbohydrate samples at 3 g/L in 0.1 M sodium acetate, pH 6, with 0.02 % sodium azide and filtering with a 0.22 µm filter. Samples were then injected in Shodex OHpak SB-806 HQ (8.0 mm × 300 mm) column (Showa Denko KK, Kawasaki, Japan), and eluted with 0.1 M sodium acetate (pH 6). The injection volume was 50 µL and the flow rate was 0.5 mL/min at 30 °C on a system consisting of a P680 HPLC pump and an ASI-100 automated sample injector using a refractive index detector Shodex RI-101 (Showa Denko KK). Pullulan standards of 1.3, 10, 110, 400 and 800 kDa were used.

### 3.7 Protein determination

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The concentration of soluble protein in the extracted fibers was estimated using Micro BCA Protein Assay Reagent kit (Pierce, Rockford, IL) as per the manufacturer’s instructions with BSA as a standard.

3.8 In vitro fermentations

A fiber product was produced in the GI-reactor to simulate an in situ production of what would be expected to reach the terminal ileum in an animal if fed with enzymes and FiberBind. This fiber was then fermented in small scale fermentations by the method of Vigsnaes et al. (35, 36). Briefly, in vitro solubilized fiber (IVSF) from the simulated gastrointestinal reactor was fermented for 24 hours at a final concentration of 0, 2.5, 5 or 10 g/L (groups control, IVSF-2.5, IVSF-5 and IVSF-10, respectively) to a glucose-free media consisting of 2 g/l peptone water, 1 g/l yeast extract, 0.1 g/l NaCl (1.71 mM), 0.04 g/l (0.23 mM) K₂HPO₄, 0.04 g/l (0.29 mM) KH₂PO₄, 0.01 g/l (0.04 mM) MgSO₄∙7H₂O, 0.01 g/l (0.07 mM) CaCl₂∙2H₂O, 2 g/l (23.81 mM) NaHCO₃, 0.5 g/l bile salts, 0.5 g/l L-cysteine hydrochloride, 0.005 g/l hemin, 10 μl/l vitamin K₁ (0.02 mM), 2 ml/l Tween 80 and 0.05‰ (w/v) resazurin. Inulin (INU) (Orafti®HPX, Orafti, Oreye, France), as well as an ultra-filtered soluble fiber (UFSF), both added at 5 g/L (INU-5 and UFSF-5, respectively), were used as positive controls. The fibers were dissolved with 15 min of agitation in a boiling water bath, having 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, stored in 25 % glycerol, were thawed on ice, pooled and diluted 5-fold in 10 mM de-gassed PBS. Digesta were pooled across piglets as in earlier studies (34, 38, 39), in order to control biological variation (40). This mixture was diluted 10-fold in the fiber-containing media, which had been de-gassed in an anaerobic cabinet overnight in...
Nunc 14 mL round bottom tubes. The resulting 1% solution of ileal content was then fermented for 24 hours in an anaerobic cabinet at 37.5 °C. All treatments were performed in 10 replicates. A fermentation harvested immediately after inoculation, and with no added fiber, served as a baseline control, also in 10 replicates.

3.9 pH and organic acid analysis

Following the fermentations, tubes were centrifuged for 5 min at 5500g at 4 °C and pH was measured with a pH-meter. The sample was then re-suspended and transferred to a 2 mL Eppendorf tube, which was spun at 13,000g for 10 min. The supernatant was then filtered with a 0.22 µm filter and assayed for OA using high-pressure liquid chromatography (HPLC). The system was a Shimadzu HPLC fitted with an RSpak KC-811 column, using 12 mM H₂SO₄ with a flow rate of 0.6 mL/min at 63 °C and a refractive index detector. The standards used were lactic acid, acetic acid, propionic acid, iso-butyric acid, n-butyric acid, iso-valeric acid and n-valeric acid.

3.10 DNA extraction

Fermentation samples were spun at 13,000g for 10 min, and the DNA contained in the cell pellets was purified with the Maxwell® LEV Blood DNA Purification Kit (Promega Corporation, Madison, WI, USA) as described in Ingerslev et al. (41).

3.11 16S rRNA gene PCR

PCR was performed targeting the V1/V2 regions of the bacterial 16S rRNA gene from the bacteria contained within the fermentations. The PCR was performed using the universal
primers V1-forward (5'-AGAGTTTGATCCTGGCTCAG-3') and V2-reverse (5'-CTGCTGCCTYCCGTA-3') (42) (Sigma-Aldrich, Broendby, Denmark). Both primers were 5'-barcode tagged (6-nt) and each specific barcode were assigned a specific DNA sample. The reaction was carried out in 50 µl reactions containing 5 µl of 5 x Goldtaq buffer (Applied Biosystems, Branchburg, NJ, USA), 1 µl of each primer (20 µM), 2 µl of 10 mM dNTP, 4 µl of 25 mM MgCl₂, 0.5 µl of AmpliTaq Gold® polymerase (Applied Biosystems), 34.5 µl of nuclease-free H₂O and 2 µl of DNA template (10 ng/µL). Reaction times and cycling conditions were 94°C for 6 min, 30 cycles of 94 °C for 45 s, 57 °C for 45 s, 72 °C for 90 s and 72 °C for 10 min. The resulting PCR products were then analyzed on an Agilent 2100 Bioanalyzer using the Agilent DNA 1000 kit (Agilent Technologies, Waldbronn, Germany) and further pooled in equimolar ratios [50 ng per barcoded sample]. The pooled DNA was then purified for primers and detergents using a Qiagen MiniElute PCR purification kit (Qiagen GmbH, Hilden, Germany) according to the manufacturer’s instructions.

3.12 Illumina MiSeq sequencing

The DNA was submitted to the National High-throughput DNA Sequencing Centre at University of Copenhagen, Denmark, for sequencing on an Illumina MiSeq™ 250PE platform. The obtained long reads were analyzed using the BION-meta software (for more information about BION-meta and for acquisition of software, see http://box.com/bion). In brief, de-multiplexing was performed according to the primer- and barcode sequences. Forward and reverse sequences were joined, allowing no gaps, a maximum mismatch percentage of 80, and a minimum overlap length of 20 bp. Next, the sequences were cleaned at both ends by removal of bases of a quality less than 98 %, which is equivalent to a Phred score of 17. Identical
sequences were further de-replicated into consensus sequences. Consensus sequences of at least 256 nucleotides in length were mapped into a table according to the individual barcodes. Finally, the consensus sequences were taxonomically classified against the Ribosomal Database Project II (RDP II; http://rdp.cme.msu.edu/index.jsp) using a word length of 8 and a match minimum of 90%. The top one percent of the obtained similarities from the RDP-II database was used for taxonomical classification of the consensus sequences. The resulting operational taxonomic units (OTUs) in each barcoded sample were normalized in order to enable direct statistical comparisons of relative abundance in each sample.

### 3.13 Statistics

The dose-response relationship of enzyme dosage versus dry matter release in the intestinal reactor was fitted to a modified Monod equation of the form $%\text{release} = \phi_1 + \phi_2[E]/(\phi_3 + [E])$. Analysis of variance was used to evaluate significant differences in dosage, pH and organic acid concentrations were analysed with an ANOVA followed by Tukey's test with the fiber treatment as the main effect using the lm- and HSD.test-procedures in R. Two-way ANOVAs in the fiber composition and organic acid data were avoided due to uneven variances, and instead adjustment for multiple comparisons when doing multiple one-way ANOVAs were made with the Sidak correction. Sequencing data was analysed on a given taxonomic level by ANOVA on log-transformed data followed by Tukey's post-hoc test, followed by correction for multiple comparisons by the Sidak correction (43) when comparing multiple OTUs. The microbial community was analysed by subjecting species-level data to principal coordinates analysis (PCoA) with treatment groups as constraints and using the Bray-Curtis dissimilarity index using the capscale-procedure in R, followed by analysis of similarities (anosim-procedure) as
well as k-means clustering (kmeans-procedure). Shannon-indices were calculated by 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 overall bacterial composition with individual OAs.

4 Results

4.1 Characterization of piglet digestive parameters

The dry matter content, the pH and the pepsin levels in the stomach digesta was determined in order to use the values to produce a realistic \textit{in vitro} GI-system. There was substantial variation within the piglets, especially in the activity of pepsin, which on average was 43.3 units/mL digesta (pooled SD 3.3), but ranged from 13.4 to 98.1 units/mL digesta. Dry matter was 21.42 %w/v, ranging between 6.78 and 30.65 %w/v, whereas pH averaged 4.16 and ranged from 4.1 to 4.6.

The protease content in the ileum was 39.2 units/mL digesta (pooled SD 1.32) and ranged from 7.0 to 78.0 units/mL. Dry matter content in the ileum was, on average, 13.4 %w/v (range 7.28-19.30) and the pH was 6.6 (range 6.0-7.0).

4.2 \textit{In vitro} GI reactor

The 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 equal twice the highest measured concentration for both the
stomach and small intestine step. The lowest measured pH in the stomach was 3.5 and therefore
the pH used in this step was 3. pH in the small intestine was set to be neutral (pH 7).

The activity of the polygalacturonase and the pectin lyase at stomach conditions (pH 3) were
22.70 U/mg and 0.02 U/mg, respectively. In small intestine conditions (pH 7), the activity was
18.79 U/mg and 2.38 U/mg, respectively

4.3 Characterisation 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 modelled by a non-linear 3-parametric equation,
which in turn suggested that the maximal release was 26.9 % of the initial dry matter. 5.56±0.1%
% of initial dry matter was released without addition of pectinolytic enzymes 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 composition of all other fractions released
contained high amounts of galactose, galacturonic acid and to a smaller degree, arabinose and
rhamnose. Analysis of variance on the data revealed that there was a plateau in yield at dosage
0.05 % E/S, for which reason 0.03 % E/S was chosen as a cost-beneficial dosage to do future
work with. At this dose, 24.6 mg dry matter is released from 100 mg FiberBind, corresponding
to 9.5 mg galactose. The composition of *in vitro* released fiber, IVSF, and UFSF is shown in
Table 1, and it is seen that IVSF contain more galacturonic acid and less galactose than UFSF
as well as slightly more protein. UFSF also contain 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 at
∼10 kDa (Figure 1). The HPSEC chromatogram of the UFSF showed that large molecular
weight polysaccharides >800 kDa were much more abundant than the smaller fractions. Estimation of the amount of monosaccharide was done by comparing 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 the UFSF, while this number was 10.6% in the IVSF, suggesting that the monosaccharide content was 2.8 fold higher in the IVSF.

### 4.4 In vitro fermentation characteristics

IVSF, UFSF and inulin were fermented in an in vitro reactor for 24 hours in the terminal ileal content from piglets. The fermentations resulted in a fiber dose dependent decrease in pH (p<0.0001, general linear model) as shown in Figure 2. Fermentation INU-5 group resulted in the same pH as the fiber at IVSF-5 (p=1, tukeys post-hoc test), although the pH was not as low as in the IVSF-10 fermentation. The UFSF-5 treatment induced a similar pH as the INU-5 and IVSF-5 groups, but a higher pH than the IVSF-10 group.

### 4.5 Organic acids

The fibers were all highly fermentable. Fermentation treatment had a significant effect on all organic acids (Table 2) (ANOVA, p<0.001). Total OA markedly increased with concentration of IVSF, and INU and UFSF-5 resulted in OA levels in-between IVSF-2.5 and IVSF-5 levels. Especially lactic acid was markedly increased by the experimental fiber, 20x from baseline and significantly higher than INU-5. Lactate was also the most affected OA relatively speaking, constituting 3% of total OA at control and 17% in IVSF-10. Propionate increased with dosage, although levels plateaued beyond IVSF-5. Both butyrate and valerate increased from baseline.
with all treatments, although the only treatment statistically different from others were the IVSF-2.5 treatments. Acetate increased with IVSF concentrations, as well as with INU-5 and UFSF-5. As expected, a highly significant correlation \( r^2 = 0.94, p<0.00001 \) existed between total OA and pH.

### 4.6 Microbiota composition

After MiSeq sequencing of the 16S rRNA PCR products followed by pattern de-multiplexing, sequence cleaning, uniqification and chimera filtering by the BION software, 1,279,205 sequences were available for taxonomical classification.

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

On the genus level (see Figure 3), the *in vitro* made fibers caused a significant decrease in *Streptococcus* and a significant increase in *Lactobacillus*. *Clostridium* did not change significantly from control with IVSF although it was decreased significantly in INU-5 and UFSF-5. *Clostridium perfringens* were found in all groups, but was not changed by addition of IVSF, although it was depressed significantly by INU-5 and UFSF-5. No *clostridium difficile*
were found in any of the groups. *Clostridium* cluster XIVa was only found in the order of 90 reads out of more than a million total reads. *Olsenella* was only elevated in the IVSF-2.5 and IVSF-5 groups. The ratio of genus *Clostridium* to genus *Lactobacillus* was markedly heightened in the control treatment, but was not different between other treatments. Excluding the control group from the analysis to retain homoscedasticity suggested that the IVSF-10 group had a higher ratio of these two genera than UFSF-5, mainly since *Clostridium* here was very low. The genus *Megasphaera* was increased significantly by all fiber treatments except the IVSF-2.5 treatment. *Enterobacteriaceae* was present in all baseline samples, and increased from baseline in all fermentations including the control, although the amounts in INU-5 and UFSF-5 groups were slightly lowered from the control. *Bifidobacteria* were only consistently observed in the INU-5 treatments, and only constituted less than ~0.1 %

On a genus level, the Shannon diversity index was significantly lower in the control and UFSF-5 groups compared to the baseline, IVSF-2.5, IVSF-5, IVSF-10 and INU-5 groups had significantly higher indices. A constrained analysis of coordinates (CAP) on species-level data with the treatment groups as constraints resulted in 71.1 % of the variation being explained on the first two dimensions as seen in Figure 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 unclassified *Turicibacter* had positive coefficients, whereas various species of *Clostridium* and unclassified *Lactobacillus* had negative coefficients. Clustering by the k-means algorithm showed distinct clusters, namely one containing UFSF-5, one with INU5, a third cluster containing IVSF-5 and IVSF-10 treatments and fourth one containing the CON 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. 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 with pH and the OA composition using linear regression (Figure 5). A significant negative association between the score on dimension 1 (CAP1) was observed from pH and acetic acid, whereas there was a positive association of lactic acid, butyric acid and valeric acid. This, in turn, suggest that a gut microbiota rich in species belonging to genera *Lactobacillus* and *Megasphaera* and poor in genera *Clostridium* is associated with a low pH and acetic acid, but high levels of butyric, valeric and lactic acid.

**5 Discussion**

In the present paper, the solubilization of potato galacto-rhamnogalacturonan 1 from potato pulp was attempted in a piglet upper gastrointestinal simulated system. The operating parameters of the system were chosen from direct measurements on piglets. The pH of the stomach and the small intestine was measured to be 4.1-4.6 and 6.0-7.0, respectively, which is in agreement with literature reports (44). A pH of 3.0 was chosen as the stomach-value as this level of acidity is realistic in piglets eating little or after overnight fasting (45, 46). Proteolytic activity, which can potentially degrade the pectinolytic enzymes, was added to mimic the measured values, as literature values are not directly applicable due to differences in methods and definition of units. The release of water soluble fiber from the potato pulp required very little enzyme, in the order of 30 µg of each enzyme (0.03 % E/S) releasing ~21 % of the dry matter from 300 mg FiberBind. Beyond 90 µg of each enzyme, there was no further release.
The two monocomponent enzymes were not purified beyond ultrafiltration before use, so even lower doses may be feasible as the enzyme dose was determined from total protein after Pichia fermentation. Previous reaction schemes have involved a 1 % E/S, which would correspond to 3 mg of each enzyme, approximately 33 times as much (26, 27), making in situ production an attractive possibility.

The monosaccharide composition of the released fractions suggest that this was indeed galactorhamnogalacturonan 1, corresponding to a backbone of rhamnose and galacturonic acid and being highly substituted with galactose chains as well as smaller amounts of arabinose, likely being flanked by domains of homogalacturonan (28, 47). The UFSF was different from the IVSF in that the galactose content was higher and the galacturonic acid content was lower. According to HPSEC-analysis, both fibers encompassed two separate populations of the solubilised pectin, namely high and low molecular weight fractions. The UFSF-fiber, being higher in molecular weight as well as galactose, can then be hypothesized to be richer in galactan-rich RG1-fractions and lower in 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 the IVSF than the UFSF. The existence of a dual population in enzymatically solubilized potato pectin has previously been shown (26). An additional difference is the presence of mannose in the purified fiber, which presumably comes from the enzyme preparation, e.g. as cell wall material from Pichia pastoris. Mannose has been detected (3-5 %) in similarly produced fiber (26, 48), 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 incubated with 10 mg/mL of IVSF for 24 hours. There are few in vitro fermentation studies reported using porcine ileal microbiota, but pH was reported to drop from 6.63 to ~6.2
and ~5.7, respectively, by fermenting 5 or 10 g/L of predigested feed containing various oligosaccharides in pig feces (22). Another study used various human milk oligosaccharides (HMO), inulin and GOS 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 hours (38). Incubations of 10 g/L inulin or transgalacto-oligosaccharides (TOS) in pig GI-contents for 4 hours, showed a drop from 6.5 to 6.3 in distal small intestine content, but a drop onto 5.8 for colonic content (49). pH-values reported from in vivo studies in the terminal ileum has been reported from 7.8-8.3 (48) to 5.7-6.0 (50), suggesting that the low pH-values observed in the in vitro fermentation studies may be lower than physiologically relevant. Colonic pH has been reported as 6.8, regardless of diet (51), suggesting that clearance of fatty acids, which does not occur in vitro but is substantial in the colon (52), has a stabilizing effect on pH.

The fibers in this study showed excellent fermentability, evident by the increase in organic acids (see Table 2). The exact role of the individual fatty acids in piglet health is not entirely clear, but there is general consensus in that butyric acid is both a fuel as well as a trophic factor for colonocytes, whereas propionate and acetate enter circulation and participate in hepatic energy production (53–55). Interestingly, the control sample, which was 24 h incubation without fiber, showed increases in acetate and propionate, probably reflecting fermentation of residual material from digesta or the glycerol added at freezing. Of further interest are the relatively high levels of lactate and the apparent dose-response relationship observed in the IVSF-series and the UFSF-5 group, suggesting that this is a fermentation product of lactic acid bacteria. The high levels of lactic acid bacteria in these treatment groups support this notion (see Figure 3) as well as the correlations of CAP1 and lactate as seen in Figure 5. When fermenting inulin (56) or xylooligosaccharides (21), lactate is present after 11.5 hours, is
markedly lowered after 30 hours, and undetectable after 72 hours, presumably since lactate is metabolized into other organic acids such as propionate through the acrylate pathway (55).

The composition of the microbiota was almost exclusively composed of the phylum *Firmicutes*, which is in contrast to other published pig metagenomics – in a study by Lamendella (57), the *Firmicutes* and the *Bacteroidetes* phylum constituted 40-60 % and 25-40 %, respectively, depending on the sequencing platform and the classification database. This was, however, 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 the phylum *Proteobacteria* in fecal samples. Looft et al. (2012), in an antibiotic feeding study with piglets weaned at 21 days and fed for 21 days, had contrasting results: 20 % *Firmicutes*, 50 % *Bacteroidetes* and ~10 % *Proteobacteria* (58). A study from the same group reported ~20-30 % *Firmicutes* and 50-60 % *Bacteroidetes* in animals weaned at 12 days and tested at 8 weeks (59). One explanation for the dominance of *Firmicutes* in our study is the use of terminal small ileum samples rather than fecal samples, supported by a recent study examining the microbiota composition in several segments of the porcine GI - 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 *Turicibacter* populations, a genus previously found in the ileum of piglets (60), but only isolated from a human patient with acute appendicitis (61), were close to entirely attenuated in all 24 hour fermentations, suggesting that this genus requires host interaction or have specific growth requirements. Merely incubating the bacteria for 24 hours resulted in a shift towards 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 evident 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 genus Lactobacillus, believed to be beneficial (10), along with a significant decrease in genus Streptococcus and a nonsignificant increase in genus Clostridium. Furthermore, Clostridium was substantially and significantly depressed with both INU-5 and UFSF-5 treatment. A point of main interest is the difference between microbial community composition in the IVSF-5 group and the UFSF-5 treatment groups, as these 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, which suggests that the galactan chains of the molecule has a more pronounced effect on 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 Bifidobacterium, whereas Clostridium was increased by polygalacturonic acid as enumerated by FISH (62). In contrast, another study using a pectin fraction with high levels of galacturonic acid showed little growth of clostridia and increased levels of Bifidobacterium also by means of FISH (63).

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 other studies in healthy animals (57, 60, 64, 65). In the absence of genuine illness the actual implications of Clostridium populations are difficult to discern, and whether or not the eradication of Clostridium as seen in the UFSF is a positive effect uncertain as well. Members of Clostridium...
cluster XIVa, previously found in colon of piglets (38), were not found in these samples to any relevant extent, which could be due to this study using the contents of the terminal ileum rather than the colon. Moreover, this cluster appears to preferably colonize the mucus layer (66), whereas this study used the luminal content. *Clostridium perfringens* appear to be a problem in the neonatal animal (67), but less so in the weaning piglet where *E. coli* is the main pathogenic agent (6, 7). In this study, the amount of *Enterobacteriaceae* in the IVSF-series was not different from the control, although the amount was decreased from control in INU-5 and UFSF-5. The observation that the genus *Escherichia* was higher in IVSF-5 than UFSF-5 can possibly be explained by the apparent 3 fold higher levels of monosaccharide in the IVSF-5 group. *Escherichia* is well adapted to catabolism of monosaccharides, including galacturonic acid (68), and may have had an advantage in the early stages of the fermentation owing to the quick replication rate of this genus.

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

*Bifidobacterium*, which are generally considered health-promoting, are ubiquitous in the human gut microbiota, but were not detected in notable numbers in neither the baseline samples nor after fermentation, and is reported to comprise a negligible portion of the pig intestinal microbiota (38, 69, 70). The importance of *Bifidobacterium* in the pig remains to be illuminated.
The genus *Veillonella* grew well in all treatments apart from baseline, likely owing to the fact that this genus metabolizes organic acids rather than carbohydrate (71). Bacteria belonging to the genus *Megaspheara*, including the species *M. elsdenii* suggested to protect the host against the pathogen *Brachyspira hyodysenteriae* (19), was another genus elevated by the fiber treatment in agreement with the fact that this genus feeds on organic acids (72).

With the use of PCoA, the entire microbiota on a species level could be visualized as well as clustered. It was seen that there was marked clustering within each treatment group, but that groups IVSF-5 and IVSF-10 were closer to one another than any other group, and especially that the UFSF-5 group was far away from the IVSF-5 group, with which it should be chemically similar. This could be explained by small, but possibly key differences in the monosaccharide content (3.8 % vs 10.6 %) in the UFSF. The obtained scores, 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, valeric and lactic acid was associated with genera *Lactobacillus* and *Megaspheara* 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 a higher dose, IVSF-10, trended to have levels of *Clostridium* higher than the control. The UFSF fiber 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 simulated *in vitro* digestion. Furthermore, the produced fiber 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 inter-animal variation is too large to verify a consistent response in vivo remains to be determined. For use in animal studies, a dosage corresponding to IVSF-5 may be a valid option.

6 Acknowledgements

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7 References


Figure captions

Figure 1: High performance size exclusion chromatogram of the fibers extracted from FiberBind 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

Figure 2: The pH-value as a function of treatment after 24 hours of in vitro fermentation of in vitro solubilized fiber (IVSF) at 2.5-10 mg/mL, 5 mg/mL inulin (INU-5) or 5 mg/mL ultrafiltered soluble fiber (UFSF-5). N=10, non-similar letters indicate significant differences.

Figure 3: Genus-level composition of the microbial community after sequencing. DNA was sequenced from 24 hours in vitro fermentations of in vitro solubilized fiber (IVSF) at 2.5-10 mg/mL, 5 mg/mL inulin (INU-5) or 5 mg/mL ultrafiltered soluble fiber (UFSF-5). N=9-10 in each group.

Figure 4: Constrained analysis of principal coordinates plot on normalized bacterial reads on a species level using Bray-Curtis dissimilarities. The K-means method was used for clustering (95 % CI). DNA was sequenced from 24 hours in vitro fermentations of in vitro solubilized fiber (IVSF) at 2.5-10 mg/mL, 5 mg/mL inulin (INU-5) or 5 mg/mL ultrafiltered soluble fiber (UFSF-5). N=9-10 in each group.
Figure 5: Associations between pH and fatty acids versus bacterial flora (CAP1). All associations apart from propionate were significant (p<0.05, general linear model). pH and OA was measured and DNA was sequenced from 24 hours \textit{in vitro} fermentations of \textit{in vitro} solubilized fiber (IVSF) at 2.5-10 mg/mL, 5 mg/mL inulin (INU-5) or 5 mg/mL ultrafiltered soluble fiber (UFSF-5). N=9-10 in each group.
Table 1: The composition of the fibers extracted from FiberBind by simulated in vitro digestion (IVSF) and by using an established purification process (UFSF). Total dry matter release was determined by weighing after freeze-drying, and monosaccharide composition was determined by acid hydrolysis followed by HPAEC-PAD analysis. Protein was determined with a bicinchoninic acid-assay. N/A (non-accountable) is the fraction which could not be recovered by HPAEC-PAD or protein determination. All values are in w/w percent of released dry matter, monosaccharides were calculated as dehydrated values, n=4. * denotes significant differences across columns by t-test, p<0.05 after correction for multiple comparisons.

<table>
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<th>Method</th>
<th>Rha</th>
<th>Ara</th>
<th>Gal</th>
<th>Glc</th>
<th>Xyl</th>
<th>Man</th>
<th>GalA</th>
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<td>10.7±0.4</td>
<td>15.2±0</td>
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Table 2: Concentrations of OA as a function of treatment after 24 hours of in vitro fermentation of in vitro solubilized fiber (IVSF) at 2.5-10 mg/mL, 5 mg/mL inulin (INU-5) or 5 mg/mL ultrafiltered soluble fiber (UFSF-5). Values are mmol/L, n=10 for each group. Non-similar letters denotes significant differences for each column by one-way ANOVA, p<0.05 after correction for multiple comparisons.

<table>
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<tr>
<th>Treatment</th>
<th>Lactate</th>
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<th>Acetate</th>
<th>Butyrate</th>
<th>Valerate</th>
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<td>0.11±0.02b</td>
<td>0±0a</td>
<td>3.95±0.09f</td>
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<td>Control</td>
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<td>5.87±0.18d</td>
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<td>8.09±1.69c</td>
<td>22.25±1.92cd</td>
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<td>36.34±1.51d</td>
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