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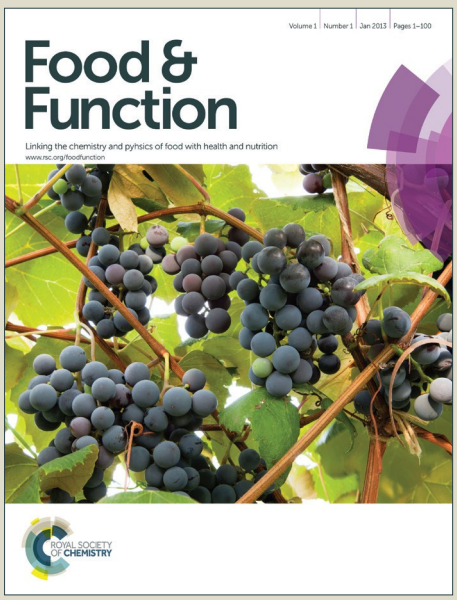

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Extrusion of barley and oat influence the fecal microbiota and SCFA profile of growing pigs

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ABSTRACT

The effect of extrusion of barley and oat on the fecal microbiota and the formation of SCFA was evaluated using growing pigs as model system. The pigs were fed a diet containing either whole grain barley (BU), oat groat (OU), or their respective extruded samples (BE and OE). 454 pyrosequencing showed that the fecal microbiota of growing pigs was affected by both extrusion and grain type. Extruded grain resulted in lower bacterial diversity and enrichment in operational taxonomic units (OTUs) affiliated with members of the *Streptococcus, Blautia* and *Bulleidia* genera, while untreated grain showed enrichment in OTUs affiliated with members of the *Bifidobacterium* and *Lactobacillus* genera, and the butyrate-producing bacteria *Butyricicoccus, Roseburia, Coprococcus* and *Pseudobutyrivibrio.* Untreated grain

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resulted in a significant increase of n-butyric, i-valeric and n-valeric acid, which correlated with an increase of *Bifidobacterium* and *Lactobacillus*. This is the first study showing that cereal extrusion affects the microbiota composition and diversity towards a state generally thought to be less beneficial for health, as well as less amounts of beneficial butyric acid.

INTRODUCTION

Epidemiological studies suggests that diets high in whole grain and cereal fiber reduces the risk for developing several Western diseases such as cardiovascular diseases, hypertension, diabetes, obesity and certain types of cancer like colon cancer [1-3]. Chemically, dietary fiber (DF) consists of non-starch polysaccharides such as arabinoxylans, cellulose, and many other plant components such as resistant starch, resistant dextrins, inulin, lignin, waxes, chitins, pectins, beta-glucans, and oligosaccharides [4-6]. One of the mechanisms behind the observed health effects of whole grain and cereal fiber might be related to the viscosity forming properties of DF in the small intestine, where specifically soluble beta-glucans have got health claims by the European Food Safety Authority (EFSA), and documented to reduce blood cholesterol and blood glucose rise after consumption [7]. However, it has become more evident that the health benefits of DF also are due to their impact in the large intestine, where DF is one major factor shaping the composition and physiology of the gut microbiota [8, 9]. Microbial fermentation in the large intestine transforms the indigestible DF into short chain fatty acids (SCFAs), mainly acetate, propionate and butyrate [10], which have profound effects on health, e.g. as inflammation modulators and as energy source for colonic epithelium (butyrate) and peripheral tissues (acetate and propionate) [11]. Butyrate also exerts an anti-inflammatory [12] and anti-carcinogenic effect [13, 14] in the colon. Acetate, butyrate and propionate are absorbed into the bloodstream and travel to the liver where acetate and propionate are incorporated into lipid and glucose metabolism, respectively [15, 16]. The colonic epithelium utilizes a high

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proportion of the butyrate leaving only a small proportion of that produced in the gut to be recovered in the portal vein, where it flows to liver, the heart and the lungs [16, 17]. Studies have also shown that increased gut production of butyrate raises the circulation level of butyrate [17]. A vegetarian diet high in DF has been shown to result in increased SCFA production [18]. In contrast, a typical "Western" diet has less amounts of DF, but high in sugar and fat, and is associated with gut dysbiosis [19], i.e. microbial imbalance in the gut. Dysbiosis affects disease susceptibility and is observed in several disease states, like obesity and metabolic syndrome [20-25], diabetes [26, 27], inflammatory bowel disease [28], inflammatory bowel syndrome [29, 30] and colorectal cancer [31]. Although the benefits of an increased intake of DF are becoming evident, the intake of DF in the Western countries is less than the recommended levels. The major sources of DF in Western countries are especially whole grain cereals of wheat, rye, oat and barley. To produce cereals with desired sensory properties processing of the cereal grains are common, affecting the physical, chemical, and nutritional status of the cereal constituents in processed food. As a result, the interest in the nutritional aspects of extruded cereals has lately increased due to the possibility to produce new cereal products rich in DF (e.g. arabinoxylan and beta-glucan). Extrusion is a thermal processing that involves the application of high heat, high pressure and shear forces to an uncooked mixture such as cereal foods [32]. Extrusion of cereal-based products has advantages over other common processing methods because of low cost, speed, high productivity, versatility, unique product shapes, and energy savings [33]. The extrusion process results in a number of chemical changes and physical changes, including gelatinization of starch molecules, crosslinking of proteins, inactivation of endogenous enzymes, increased content of soluble DF and phenolic acids, a destruction of antinutritional factors such as trypsin inhibitors and phytates, mechanical damage to the cell walls [34-36] and the generation of flavour [37]. In addition it has been shown that extrusion of cereal grains can lead to enhanced mineral bioavailability [35] and protein digestibility [38]. Recently it has been

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shown that extrusion of barley and oat improved bioaccessibility of phenolic acids [39]. To our knowledge, the impact of cereal extrusion on the gut microbiota is scarce. However, some effects of extrusion of the gut microbiota have been indicated in growing pigs [40, 41]. The pig has been shown to be a useful model organism for studying how diets affect the human gut microbiota [42]. Both pigs and humans are colon fermenters, and they have similar composition of the colonic microbiota [43], and mainly consists of the *Firmicutes* and *Bacteroidetes* phyla [44]. However, it is important to state that although pigs and humans have many similarities in the gastrointestinal tract anatomic structure, function, metabolism, nutrient requirements and major phyla, there are also considerable differences in the bacterial composition between pigs and humans [42]

The aim of this study was to investigate the effect of extrusion of whole grain barley and oat groat on the fecal microbiota (454 pyrosequencing) and the formation of SCFA using growing pigs as model system. The hypothesis was that extrusion processing of whole grain barley and oat groat would cause chemical, structural and enzymatic changes of the cereals that would affect the composition of the fecal microbiota and metabolism.

MATERIALS AND METHODS

Experimental design

The fecal samples used in this study were collected from the feeding experiment previously published by Hole *et al*. [39] where the bioaccessibility of dietary phenolic acids were reported. The feeding experiment was performed as previously been described by Hole *et al*.[39]. All pigs were cared for according to laws and regulations controlling experiments with live animals in

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Norway (Animal Protection Act of December 20, 1974, and the Animal Protection Ordinance concerning experiments with animals of January 15, 1996).

Four different types of grain were used in the study: (1) whole grain barley with hull, untreated (BU); (2) whole grain barley with hull, extruded (BE); (3) oat groat, untreated (OU); (4) oat groat, extruded (OE). Details about the feed production have been described previously [39].

The total experimental period lasted for 21 days. The pigs were fed twice daily (at 8 a.m. and 2 p.m.) according to a restricted Norwegian feeding scale [45], and they had free access to drinking water. They were kept in pens designed for individual feeding in a room with an average temperature of 18 °C.

A total of 16 female pigs [(Landrace × Yorkshire) × (Landrace × Duroc)] from four litters were used in the experiment. The average initial weight was 46.5 kg, and the average final weight was 57.6 kg. The pigs were blocked by litter and by live weight, and four animals were fed each dietary treatment. Live weight and feed intake were measured for each pig at every week in the experiment and have been reported previously by Hole *et al*. [39].

Total tract digestibility (TTD)

The four diet samples were analyzed for dry matter (DM; EU Dir. 71/393), ash (EU Dir. 71/250), crude protein (Kjeldahl-N x 6.25; EU Dir. 93/28), crude fat (EU Dir. 98/64), crude fiber (EU Dir. 92/89) and starch (AOAC 996.11). The four diets were also analyzed for Yttrium by inductively coupled plasma mass spectrometry (ICP-AES) analysis, Perkin Elmer Optia 3000DV; Perkin Elmer, Wellesley, MA, USA) at 371 nm, after mineralization and solubilization in acid of the pooled sample. From each feed group of four pigs the final fecal

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samples were pooled and analyzed for dry matter, ash, crude protein, crude fat, crude fiber, starch and for Yttrium as reported previously by Hole *et al*. [39].

Fecal collection and analysis of SCFAs

Fecal samples were collected from each of the pigs at the beginning and during the final two days of the experiment (in total 32 samples). The samples were frozen immediately at −20 °C. Thawed fecal samples were centrifuged (12000 rpm for 5 min) and 50 µl of the supernatant was added to 650 µl of a mixture of formic acid (20 %), methanol and 2-ethyl butyric acid (internal standard, 2 mg/ml in methanol) at a ratio of 1:4,5:1. A 0.5 µl sample was injected on the GC-column and the content of SCFAs were analyzed using the method described by Anson *et al*.[46]. A portion of the fecal samples were freeze-dried and ground and used in the DNA isolation.

DNA isolation

Feces were prepared for analysis by adding 300µl of Solution A (25 mM Tris-HCl of pH 8.0, 10 mM EDTA of pH 8.0) per 0.1 g of freeze dried feces (0.1-0.18g). The samples were mixed by vortexing and left for 30 min on ice before 200 µl of the supernatant was mixed by vortexing with 400µl of 4 M guanidinium thiocyanate (GTC). Five hundred microliters of samples was transferred to a sterile FastPrep®-tube (Qbiogene Inc., Carlsbad, CA, USA) containing 250 mg of glass beads (106 microns and finer; Sigma-Aldrich, Steinheim, Germany), and samples were homogenized at 4m/s for 40 s using a FastPrep® Instrument (Qbiogene Inc.). Wells in a 96-well Greiner U-plate (Greiner Bio-One, Frickenhausen, Germany) were filled with 170 µl of sample and 10 µl of silica particles (Merck, Darmstadt, Germany). One percent of Sarkosyl was added, and the plate was incubated at 65°C for 10

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min and at room temperature for 10 min. The supernatant was removed, and the paramagnetic beads were washed twice with 50% ethanol. DNA was eluted from the silica particles by suspension of the particles in 100 µl of Buffer C (1 mM EDTA of pH 8.0, 10 mMTris-HCl of pH 8.0) at 65°C for 30 min. Amount of purified DNA was measured by NanoDrop ND-1000 (NanoDrop Technologies Inc., Wilmington, DE) and diluted to a concentration of 10-20 ng/µl.

Microbiota analyses

All samples were submitted for pyrosequencing. Two µl of DNA was amplified by PCR using 16S rRNA gene primers, forward primer (5'-AYTGGGYDTAAAGNG-3') and reverse primer (5'- TACNVGGGTATCTAATCC-3') (RDP (Ribosomal Database Project) website: http://pyro.cme.msu.edu/pyro/help.jsp), producing a 240 bp fragment covering the variable region V4 of the 16S rRNA genes [47, 48]. PCR reactions were performed using 50 µl (final volume) mixtures containing 1× FastStart Buffer #2 (Roche Ltd., Basel, Switzerland), 0.2 mM dNTP mix, 0.4 µM of each primer and 2.5 U FastStart HiFi Polymerase (Roche). The amplification protocol was 94°C for 3 min, followed by 35 cycles of 94°C for 50 s, 40°C for 30 s and 72°C for 1 min, and a final elongation step at 72°C for 5 min. PCR products were purified using Agencourt AMPure PCR purification (Beckman Coulter Inc., Danvers, MA). DNA concentration was measured with use of Quant-iT PicoGreen dsDNA Assay Kit (Invitrogen), and the samples were pooled before running an emulsion-based clonal amplification (emPCR amplification, Roche). All samples were run as multiplex on the same picotiter plate in the GS Junior System (Roche) using nucleotide barcodes on primers as described on the RDP website.

The output sequences and the quality score file was processed together with the mapping file using the QIIME 1.3.0 (Quantitative Insights Into Microbial Ecology) pipeline. QIIME is an open source software package for comparison and analysis of microbial

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communities, primarily based on high-throughput amplicon sequencing data. QIIME allows analysis of high-throughput community sequencing data [49]. The multiplexed reads were assigned to starting samples based on their nucleotide barcode, key tag, and primers were trimmed and sequences of low quality were removed.

A total of 125378 raw sequences were obtained by pyrosequencing. Filtering in QIIME (default settings) resulted in 73178 sequences that were distributed on 32 samples (number of sequences per sample ranging from 1468 to 2712, with an average of 2121). QIIME open reference analyses identified 4199 Operational Taxonomic Units (OTUs) divided over 67879 sequences. The sequences were clustered into OTUs based on their sequence similarity using a 97% similarity threshold, where representative sequences for each OTU was identified and assigned to taxonomic identities using the RDP classifier. This OTU table was further used in the statistical analyses described below.

Statistical analyses

The OTU data was filtered prior to analyses. To avoid modeling noise, only OTUs present in at least 2 samples within each diet group and in at least 16 samples in total were included. This filtering ensures focus on the most abundant bacteria in the statistical analyses. In total 261 OTUs passed the filter, each of these represents a phylotype and may be a representative of a bacterial species.

Data from the phenolic acids and their metabolites in plasma, previously published by Hole *et al.* [39] was also analyzed to see if there was a correlation between these data and the microbiota data presented here. No correlation was found and these results are summarized in supplementary file S1 (Phenolic acid-derived metabolite profile in blood).

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Multivariate analysis by principal component analysis (PCA) was applied for explorative studies of the data. For each data set (OTU, SCFA and blood metabolites) analysis of variance (ANOVA) applying a two way model with interaction was fitted to the data to test the effect of grain type. Because normality cannot always be assumed for these data, effects were also tested by Friedman test. The results were, however, similar to the results with ANOVA. We report variables where effect of grain, treatment or interaction is significant ($p<0.05$).

To visualize the results for the OTU data, hierarchical cluster analysis was applied for the OTUs where grain, treatment or interaction had a significant effect ($p<0.05$). The cluster analysis was based on spearman correlation and weighted average distance (WPGMA). The results for the cluster analysis are presented by heatmaps constructed by the matlab function clustergram (matlab bioinformatics toolbox, mathworks).

The relation between OTU and SCFA were investigated by partial least squares regression (PLSR) using OTU as the explanatory data and the SCFA as the dependent variables. Different models were tested. We report results from the best model which used the OTU and SCFA data from the end of the experiment with n-butyric and i-valeric acids as the response variables and the OTU data from the end point as the independent variables. Cross validation with four segments were used for validation.

All analyses were conducted in Matlab (v.7-12, R2013b).

RESULTS

Total tract digestibility (TTD)

Results showed that TTD for crude fiber in extruded barley and oat groat (71.2 % and 71.5 % respectively) was lower than untreated barley and oat groat (79.2 % and 83.7 % respectively).

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No difference in TTD between untreated and extruded barley and oat groat for protein, fat, starch, ash and dry matter was observed.

Chemical composition of the grains and diets and the growth performance of the pigs have been reported previously by Hole *et al*.[39]

Microbiota

The overall microbiota

The microbiota across all samples was dominated by phylum *Firmicutes* (80%), followed by "Unclassified bacteria" (10%), *Bacteriodetes* (7%), *Spirochaetes* (3%), *Acinetobacter* (0.3%) and *Proteobacteria* (0.2%). Most *Firmicutes* belonged to the class *Clostridiales* (64%). Other phyla that were represented in the data, but under 0.05% were *Euryarchaeota*, *TM7*, *Fibrobacteres*, *Verrucomicrobia* and *Cyanobacteria*.

Effect of extrusion and grain on the microbiota

The *Firmicutes/Bacteroidetes* ratio was not significantly different between the different treatments at the end of the experiment (data not shown). Extrusion and grain type had effects on the alpha diversity (observed species) and microbial composition (Figure 1 and 2). Highest alpha diversity was observed for pigs fed BU followed by OU, BE and OE (Figure 1A). The diversity was significantly higher in untreated than extruded ($p<0.01$), and in barley than oat ($p<0.05$) (Figure 1B). Principal Component Analysis (PCA) of the complete data revealed that the highest variation in the dataset (PC1, 15.3%) was related to the time changes, whereas PC2 (7.1%) spanned the individual variations in the start of the experiment. PC3 (5.4%) and PC4 (4.8%) were related to differences between samples from pigs fed extruded and non-extruded grains, together they gave a complete separation of these two groups. Figure 2 shows the score plot with PC1 and PC4, which gave better separation than PC3. Further statistical

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analyses were based on data from the end of the experiment. The results from the ANOVA for all OTUs are summarized in supplementary table S2.

OTUs with significant effects of treatment or grain ($p<0.05$) are presented by heatmaps in Figure 3 and 4, respectively. Thirty seven OTUs were significantly different between extruded and untreated grain (Figure 3), whereas 27 OTUs were significantly different between barley and oat (Figure 4). Only six OTUs were significantly affected by both grain and treatment: OTU3595 (p *Bacteroidetes*), OTU2901 (g *Streptococcus*), OTU3455 (o *Clostridiales*), OTU1016 (g *Pseudobutyrivibrio*), OTU885 (f *Ruminococcaceae*) and OTU2159 (g *Mitsuokella*). The interaction between grain and treatment were significant for only four OTUs: OTU2812 (p *Firmicutes*), OTU997 (g *Eubacterium*) and OTU2808/OTU3453 (f *Lachnospiraceae*). Hence, with a few exceptions different OTUs were affected by grain and treatment.

Among the OTUs that were enriched in the groups fed untreated grain were OTUs affiliated with members of the *Roseburia*, *Sporobacter*, *Butyricicoccus*, *Pseudobutyrivibrio*, *Lactobacillus*, *Bifidobacterium* and *Mitsuokella* genera (Figure 3). Among the OTUs that were enriched in the groups fed extruded grain were OTUs affiliated with members of the *Streptococcus*, *Bulleidia* and *Blautia* genera. The OTU2901 (g *Streptococcus*) was the most abundant OTU in the extruded group and accounted for 3.7 and 9.9% of the microbiota in the untreated- and extruded group, respectively.

The effect of barley compared to oat is shown in Figure 4. Among the OTUs that were enriched in the group fed barley were OTUs affiliated with members of the *Coprococcus*, *Butyricicoccus*, *Clostridium*, *Turicibacter* and *Blautia* genera, whereas OTUs affiliated with the *Streptococcus*, *Oscillibacter*, *Peptococcus*, *Pseudobutyrivibrio* and *Mitsuokella* genera were enriched in the group fed oat groat.

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SCFA profile

Effect of extrusion and grain on the SCFA profile

PCA of the SCFA profiles of all samples showed that the variation was mainly related to the time difference (Figure S3). After the experimental period, significant differences in the SCFA profiles could be seen. More specifically propionic acid ($p<0.01$) and acetic acid ($p<0.05$) were significantly higher in pigs fed barley than oat. Whereas n-butyric, i-valeric and n-valeric ($p<0.01$ for all three) was higher for pigs fed untreated grain. The total amount of SCFAs was also higher in untreated grain compared to extruded grain. Note that the SCFAs with similar effects were highly correlated. This analysis is summarized in table 1.

Relation between microbiota and SCFA

The relation between OTUs and SCFA from the end of the experiment was investigated by partial least squares regression (PLSR). Figure 5 shows the regression coefficients for n-butyric and OTUs with the PLS model with one component. i-valeric acid was also included in the model, but the regression coefficients for i-valeric were not included in the figure since they were highly correlated with n-butyric acids. The OTUs with positive regression coefficients are the OTUs that correlated with the two acids (the more positive the higher concentration). The results showed that n-butyric and i-valeric acid are positively correlated ($p<0.01$) with OTUs affiliated with the *Bifidobacterium* and *Lactobacillus* genera. OTU455, affiliated with the *Butyricicoccus* genus, was also positively correlated with the two acids, but not shown significant at 5% level due to large individual variations. The n-valeric acid was not included in the model due to large individual variation, and the other acids did not have a high correlation with the OTU data (data not shown).

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DISCUSSION

The extrusion process is known to result in a number of chemical and structural changes of the cereal, which has been shown to affect their digestibility and bioavailability [33, 35-38]. However, limited knowledge exists on how these changes affect the composition of the fecal microbiota and metabolism. In the present study, we have investigated the effect of extrusion of whole grain barley and oat groat on the fecal microbiota and SCFA content by using growing pigs as model system. The fecal samples originated from a pig intervention study that previously showed that extrusion improved the bioaccessibility of dietary phenolic acids in the pigs [39]. Chemically changes of the cereal grains used in the diets were also observed, where extrusion increased the crude protein content (Kjeldahl-N x 6.25; EU Dir. 93/28) and caused a shift from insoluble beta-glucan to soluble beta-glucan for both grains. However, no significant effect on NSP, starch and crude fiber were observed [39].

Here we show clear effects by extrusion of whole grain barley and oat groat on the fecal microbiota (composition and diversity) and the SCFA content, where extrusion resulted in lower species diversity and lowered the amount of SCFA. Generally, high microbial diversity is thought to be associated with a healthy gut microbiota, while loss of diversity seems to correlate with disease [50]. We suggest two possible reasons for the impact of extrusion on the microbiota and SCFA, where one is due to the chemical and structural changes of the cereal and the other is due to heat inactivation of endogenous enzymes (e.g. endo xylanase and endo beta-glucanase) in the grain [51], which might affect fermentability of DFs in the grains. It could be hypothesized that the nutritional changes by extrusion might increase the bioavailability of a limited number of nutrients (e.g. soluble beta-glucan), favoring only a few species. In contrast, the untreated grains are likely to contain a structural more complex fiber matrix,

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where numerous microbes are thought to be required in a step-wise breakdown and use of complex substrates [8]. Interestingly, higher bacterial diversity has been observed in feces of children from Burkina Faso, who consumed a diet high in whole grain, compared to Europeans [52] and high nutrient availability has been shown to reduce the diversity of the equine cecal microbiota [53]. Whether a microbiota with lower diversity is less resilient to environmental challenges and is less "healthier" for the host is not yet known [54]. Extrusion also lowered the total tract digestibility (TTD) of the crude fiber, which might reflect chemical changes due to the extrusion or lower fermentability in the large intestine, resulting from a higher digestibility of carbohydrates and other nutrients in the small intestine and thereby reducing the supply of fermentable substrate to the large intestine. This reduced supply of fermentable substrates to the large intestine may also explain the lower amounts of SCFA detected in the feces of the pigs feed extruded grains. The microbiota and to some extent the SCFAs were also affected by grain type, where BU had a higher diversity and higher amounts of the measured SCFAs than OU. Both barley and oat contain beta-glucan, ranging from 3-7% in oat, and 5-11% in barley depending on the genotype [55]. In pigs, it has been shown that beta-glucan is easily fermentable for microbes along the gastro intestinal tract and stimulate bacterial fermentation already in the stomach [56]. Insoluble beta-glucan may however escape digestion and flow into the large intestine available for fermentation [57]. Untreated grains (e.g. BU and OU) contain arabinoxylan and beta-glucan hydrolyzing enzyme systems [58]. However, in the production of food grade oat groat (OU) heat treatment (kilning) is used to inactivate all oat enzyme systems especially fat-hydrolyzing enzymes, but kilning also inactivates arabinoxylan and beta-glucan hydrolyzing enzyme systems [58-61]. BU was not heat treated and both arabinoxylan and beta-glucan hydrolyzing enzyme systems will therefore be active. It is therefore likely that the intact endo beta-glucanases and xylanases in whole grain barley (BU) will affect the production of fermentable oligosaccharides from beta-

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glucan and arabinoxylan in the gut. The degree of hydrolysation of beta-glucan and arabinoxylan has previously been shown to have impact on the prebiotic effectBeta-glucan oligomers/oligosaccharides have been shown to stimulate growth of lactobacilli and/or bifidobacteria [62, 63]. Oligosaccharides of arbinoxylan (arabinoxylooligosaccharides (AXOS) and xylooligosacchariedes (XOS)) has been shown to stimulate bifidobacteria in numerous trials in humans and animals [64], and xylooligosacchariedes has been shown to be utilized by some lactobacilli [65, 66]. This is in agreement with our observation of an enrichment of OTUs affiliated with the *Bifidobacterium* and *Lactobacillus* genera in the feces of pigs fed untreated grain. Species of *Bifidobacterium* and *Lactobacillus* are regarded as health beneficial bacteria, thus indicating a potential prebiotic effect with the untreated grains. We also observed a positive correlation between the genera *Bifidobacterium* and *Lactobacillus* and butyric acid, which is known to be important for gut health since it serves as the main energy resource to colonocytes and prevents inflammation [12, 67]. Further, we observed an enrichment of *Butyricicoccus, Roseburia* and *Pseudobutyrivibrio* genera in pigs fed untreated grain, genera known to include butyrate-producing species [68]. A previous study in humans has also linked *Bifidobacterium* and *Roseburia* to whole grain barley intake [69]. Interestingly, species of *Roseburia* and *Bacteroides* are identified as the main endoxylanase producing bacteria in the human intestine [70] with capacity to hydrolyze arabinoxylan into AXOS and XOS. Thus, a co-operation mechanism between several gut bacteria could be indicated with untreated grain.

Extrusion stimulated especially one OTU (OTU2901) affiliated with the *Streptococcus* genus. The representative sequence of this OTU is in close phylogeny to *S. pasteri*, *S. gallolyticus*, *S. alactolyticus* , *S. bovis* and *S. macedonicus* in the RDP database (data not shown), and not the emerging pig pathogen *S. suis* [71]. Interestingly, *S. bovis* is known to possess 1,3-1,4 beta-glucanase activity on barley beta-glucan [72]. It could be hypothesized that the effect of extrusion on *Streptococcus* was a direct effect of the increased soluble beta-glucans in the diet

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caused by the extrusion process. A more thorough analysis on species/strain level would however be needed to understand why this OTU was elevated in the feces of pigs fed extruded feed.

In conclusion, cereal extrusion results in chemical- and structural changes of the grain resulting in changes in the fecal microbial composition and diversity, as well as the SCFA profile of growing pigs towards a state generally thought to be less beneficial for gut health. This stands in contrast to the fact that extrusion is a promising procedure to produce products rich in DFs and that extrusion has been shown to improve the bioaccessibility of phenolic acids in grains. It is important to state that although pigs and humans have many similarities in the gastrointestinal tract anatomic structure, function, metabolism, nutrient requirements and major phyla, there are also considerable differences in the bacterial composition between pigs and humans [42]. Further studies are required to understand the implications that these changes have on the human gut health, as well as the gut health of pigs.

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TABLES

Table 1. ANOVA results for the SCFA. P-values for grain effect (p-grn), treatment effect (p-trt), and least squares means (n=4) for each group with standard error of the mean within parenthesis. Significant ($p<0.05$) results are highlighted.

SCFA	p-grn	p-trt	BE	BU	OE	OU
Acetic	**0.028**	0.367	31.83 (0.97)	34.26 (2.20)	27.67 (1.74)	28.84 (2.43)
Propionic	**0.005**	0.145	11.75 (0.25)	12.80 (0.52)	9.29 (0.96)	10.42 (0.83)
n-butyric	0.237	**0.002**	4.52 (0.13)	6.42 (0.45)	4.33 (0.43)	5.59 (0.52)
i-valeric	0.176	**0.007**	1.49 (0.13)	2.20 (0.29)	1.30 (0.17)	1.84 (0.12)
n-valeric	0.111	**0.01**	1.94 (0.08)	2.70 (0.14)	1.95 (0.18)	2.15 (0.20)
Caprioic	0.593	0.755	0.39 (0.05)	0.51 (0.09)	0.53 (0.06)	0.46 (0.08)
Heptanoic	0.479	0.309	0.14 (0.02)	0.14 (0.02)	0.20 (0.04)	0.12 (0.04)

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FIGURES

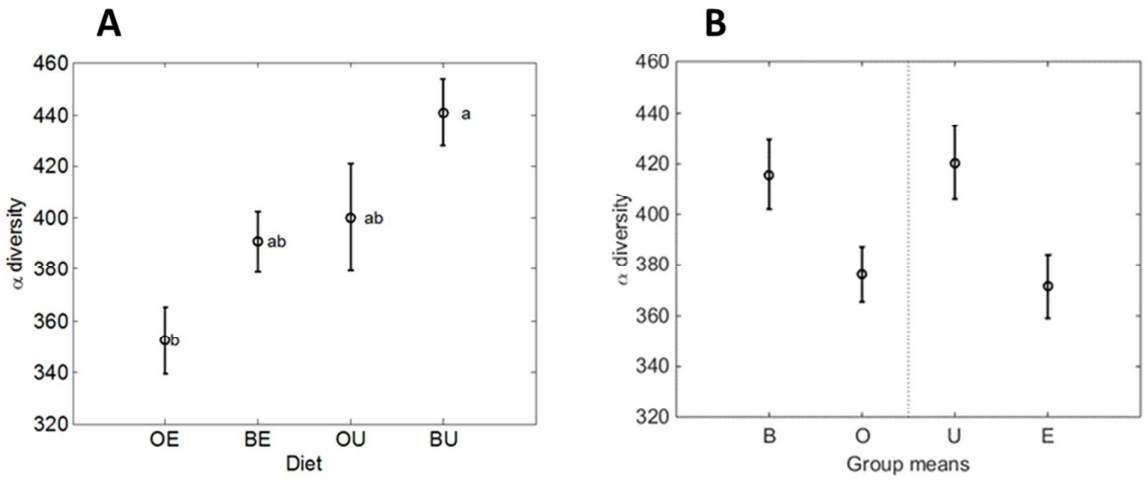
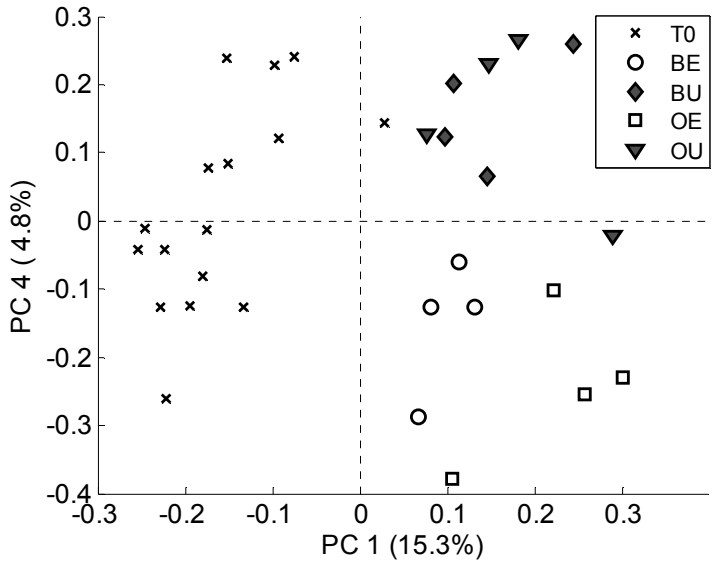


Figure 1. Alpha diversity (observed species) of the four diets (OE; BE; OU and BU) (**A**) and group means; grain type (barley (B); oat (O)) and treatment (untreated (U); extruded (E))) (**B**). The diversity was significantly higher in OE than BU ($p<0.01$) (**A**). The diversity was also significantly higher in untreated than extruded ($p<0.01$), and in barley than oat ($p<0.05$) (**B**). There was no significant effect of interaction between grain type and treatment.



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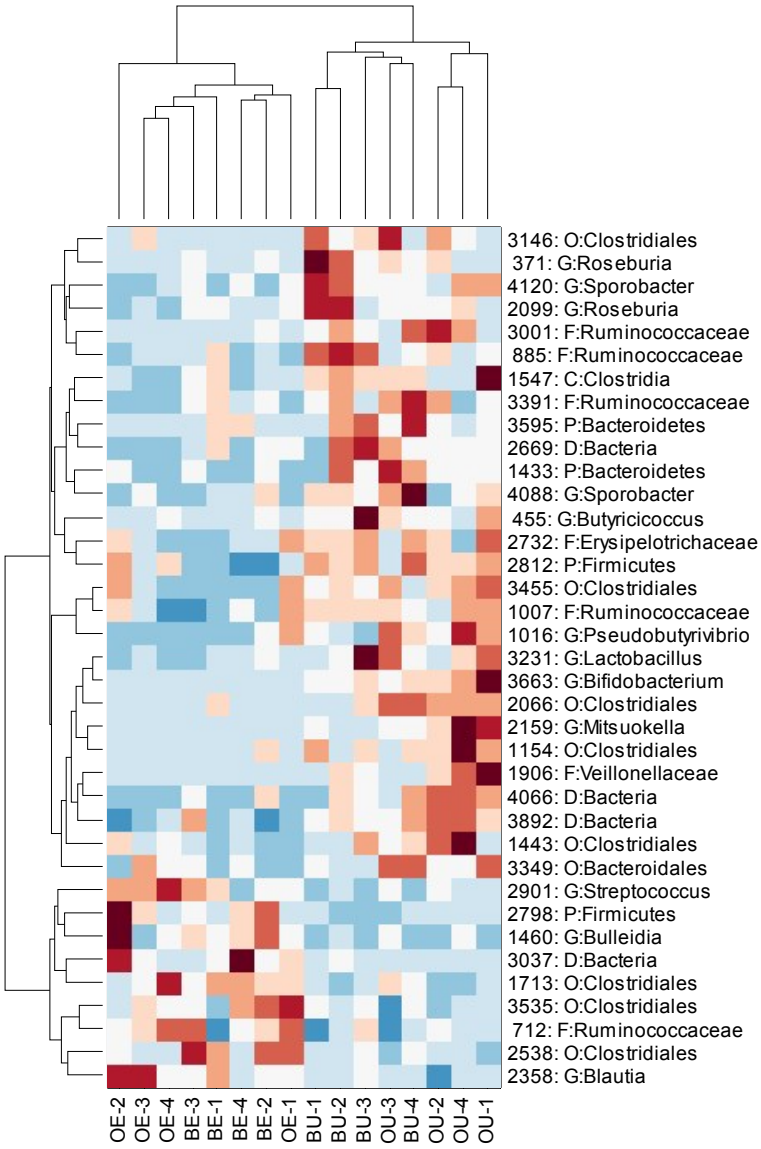
398 **Figure 2.** PCA score plot of OTU data (PC1 and PC4). PC1 separates start and end of
399 experiment, whereas PC4 is related to treatment. For the extruded grain, barley and oat (OE
400 and BE) is separated. More variation is observed in the start than in the end.

401



402

403 **Figure 3**. Heatmap showing the relative abundances of the OTUs significantly different
404 between treatments. The OTUs with high relative abundances are red, intermediate

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abundances white and low abundances blue.. D=domain; P= phylum; C= class; O= order; F= family and G= genus. Note that numbers are different animals within each group (OE-1 is not same animals as OU-1).

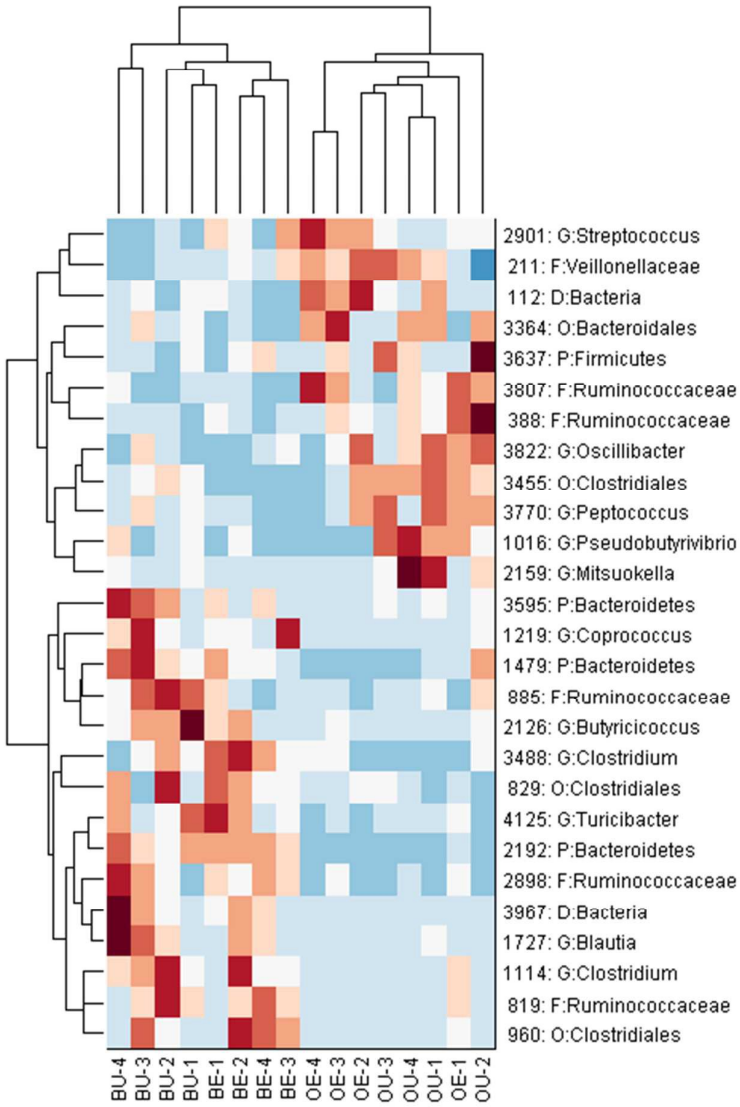

Figure 4. Heatmap showing the relative abundances of the OTUs significantly different between grainsD=domain; P= phylum; C= class; O= order; F= family and G= genus. Note that numbers are different animals within each group (OE-1 is not same animals as OU-1).

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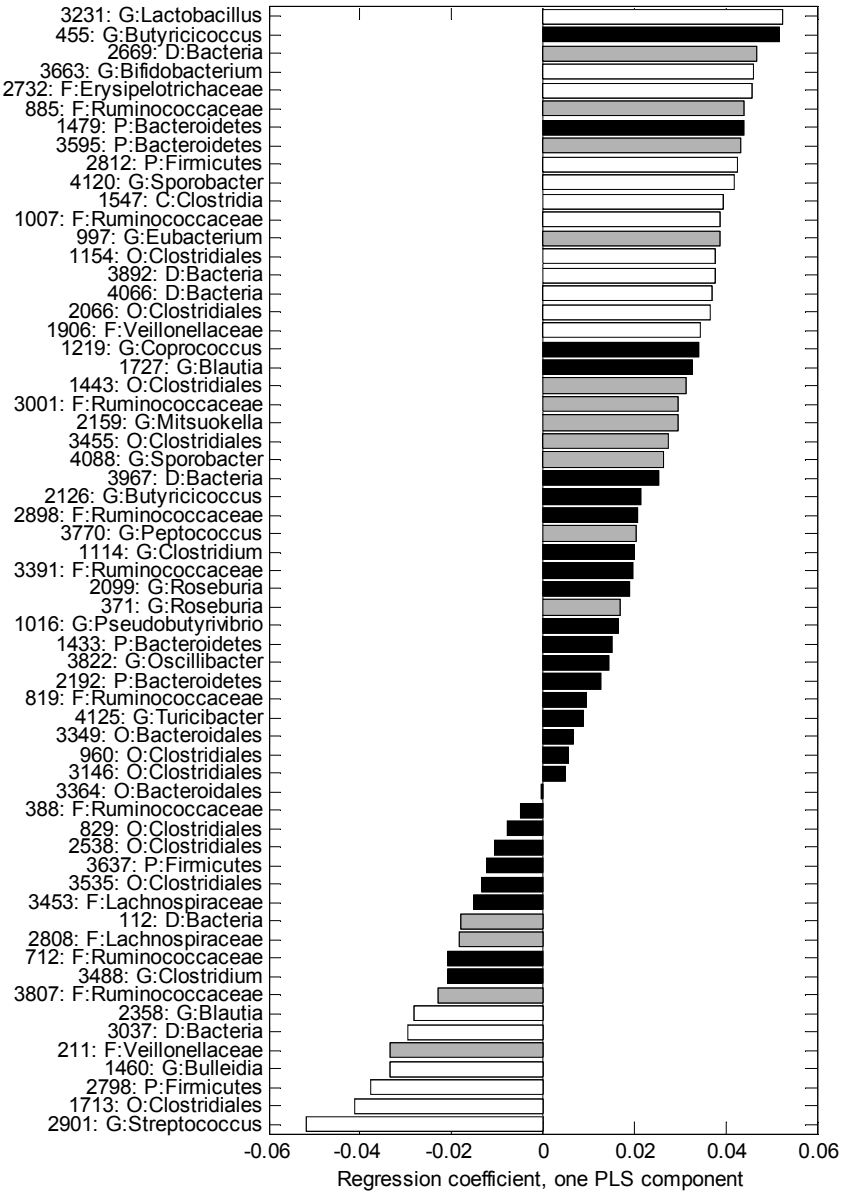

413

414 **Figure 5**. Regression coefficients for n-butyric for the PLS model with one component. Since

415 i-valeric and n-butyric acid are highly correlated the regression coefficients are almost

416 identical for both acids for the one component model. P-values was obtained by jack-knifing,

417 p-value < 0.05 (gray), p-value < 0.01 (white), black: not significant. The more positive the

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418 regression coefficients are (point to the right), the higher is the correlation with n-butyric and
419 i-valeric acid.

420

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