Supplementation of a Lacto-fermented Rapeseed-seaweed Blend Promotes Gut Microbial- and Gut Immune-Modulation in Weaner Piglets

Yan Hui
Københavns Universitet https://orcid.org/0000-0002-2388-131X
Paulina Tamez-Hidalgo
Fermentationexperts A/S
Tomasz Cieplak
Kobenhavns Universitet
Gizaw Dabessa Satessa
Kobenhavns Universitet
Witold Kot
Kobenhavns Universitet
Søren Kjærulff Søren
Fermentationexperts A/S
Mette Olaf Nielsen
Arhus Universitet
Dennis Sandris Nielsen
Kobenhavns Universitet
Lukasz Krych (krych@food.ku.dk)
University of Copenhagen

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Abstract

Background

The direct use of medical zinc oxide in feed will be abandoned after 2022 in Europe, leaving an urgent need for substitutes to prevent post-weaning disorders.

Results

This study assessed whether rapeseed meal added two brown macroalgae species (*Saccharina latissima* and *Ascophylum nodosum*) and fermented using lactic acid bacteria (FRS) could improve weaner piglet performance and gut health. From day 28 of life to day 85, the weaned piglets were fed one of three different feeding regimens (n = 230 each) with inclusion of 0%, 2.5% and 5% FRS. The piglets fed with 2.5% FRS manifested superior phenotype with alleviated intraepithelial and stromal lymphocytes infiltration in the gut, enhanced colon mucosa barrier and numerical increase of final body weight. The colon microbiota composition was determined using the V3 and V1-V8 region 16S rRNA gene amplicon sequencing by Illumina Nextseq and Oxford Nanopore MinION, respectively. The two amplicon sequencing strategies showed high consistence between the detected bacteria. Both sequencing strategies indicated that inclusion of FRS reshaped the colon microbiome of weaner piglets with increased Shannon diversity. *Prevotella* stercorea was verified by both technologies to be more abundant in the FRS piglets, and its abundance was positively correlated with colon mucosa thickness but negatively correlated with blood concentrations of leucocytes and IgG.

Conclusions

FRS supplementation improved the gut health of weaner piglets, and reshaped the gut microbiota composition. Increasing the dietary inclusion of FRS from 2.5% to 5% did not lead to further improvements.

Background

A healthy hindgut is essential for the optimal nutrient utilization and host health. Essentially, the gut inhabitants ferment and digest macronutrients such as insoluble fibers and undigested proteins in the colon[1, 2]. Microbial metabolism benefits colonic cells by providing butyrate and the immune system by providing acetate and propionate[1]. The process of microbial colonization, which brings benefits for the host, also the risk of infection-inflammation responses. Thus, microbial communication with the immune system balances on a thin line for preserving homeostasis[3] between the learning of what to attack and what to tolerate[4].

In pig production, the weaning period is characterized by a change in diet from milk to solid feed, separation from the mother and aggregation in a pen with piglets from other litters. It is a stressful period in pig life and has high risk of morbidities like diarrhea. In-feed zinc-oxide used to be a prevalent choice

for prophylaxis, but according to the European Union regulations, zinc-oxide shall no longer be directly used in feed or water from 2022[5]. This leaves an urgent need for new prophylactic substitutes. Inclusion of pre-fermented feeds has been regarded as a promising strategy to ameliorate the post-weaning disorders for its effective act to improve gastrointestinal health and enhance livestock performance in production[6, 7, 8]. The process of microbial fermentation degrades antinutritional compounds and macronutrients in feed, which increases the nutrient bioavailability and nutritional value[9, 10]. Besides, the microorganisms in fermented feed have been proposed to inhibit the overgrowth of opportunistic pathogens, sustain the gut microbiome homeostasis[11] and boost host immune system[12]. A meta-analysis has shown that fermented feed is able to improve the growth and performance of both weaner and growing pigs[13].

Rapeseed meal is a by-product after the oil has been extracted and is generally used as a protein source in animal diets[14]. However, it has a lower protein digestibility as compared to soybean meal. Brown seaweed is acknowledged as a good source of health-promoting phytochemicals[15] with bioactive compounds like laminarin[16], but it has poor digestibility as well. Lacto-fermentation could improve the health and nutritional value of rapeseed-seaweed blend by reducing the naturally present anti-nutritional factors and by hydrolyzing protein and fibers down to a more soluble matrix. We have reported that dietary supplementation with rapeseed-seaweed blend fermented by lactobacilli (FRS) could modulate the gut barrier function of piglets and improve the production performance[5].

In the present study, we tested whether inclusion of FRS to weaner diets could affect the gut microbiome and the host immune function. We chose weaning as the physiological stress situation to reveal the links between the diet induced gut modulation and host health. To trace the gut microbiome shifts, we have profiled the piglet colon content using the V3 and V1-V8 region 16S rRNA gene amplicon sequencing by second (Illumina, NextSeq) and third generation (Oxford Nanopore Technologies, MinION) sequencing platform, respectively.

Methods

Preparation of fermented rapeseed-seaweed feed

The FRS feed was provided by FermentationExperts (Denmark), which was a blend of rapeseed meal (*Brassica napus*), wheat bran (*Triticum eastivum*) and two types of brown seaweed (*Saccharina latissima* and *Ascophylum nodosum*) prepared via a controlled two-step solid state fermentation. The inoculum consisted of three lactic acid bacteria: *Pediococcus acidilactici* (DSM 16243), *Pediococcus pentosaceus* (DSM 12834) and *Lactobacillus plantarum* (DSM 12837). The addition of the inoculant controlled the process by acidifying the blend within the first 24 hours, and assuring an almost entirely anaerobic process. The process continued for 11 days at 38 ˚C. The fermented material was then dried in a spin flash dryer, with a temperature setting and pass-through-speed that preserved the viable bacteria and the microbial thermolabile metabolites.

Animal feeding and performance recording

The feeding trial was carried out on a commercial pig farm (Kawiks Farm, Patoki 23. 98–170 Widawa. Province. Lodz city, Poland) in 2018, where groups of piglets were weaned one day a week over a 5-week period. The trial procedure and sample collection were approved by the Local Ethical Commission of Olsztyn University of Life Sciences (Olsztyn, Poland) with regards to experimentation and animal care. A total of 690 piglets were tested under three different feeding regimens (230 piglets per feeding treatment) from 28 days of age (10 days before weaning) until 85 days of age when the piglets exited the nursing unit. One group was a control group fed a basal feed according to Danish nutritional recommendations[17] (0% FRS), and the other two groups received supplementation of 2.5% or 5% FRS to the basal feed (feed dry matter basis in Table 1). Piglets on each dietary regime were housed in nursing pens holding an average of 48 animals per pen. Each dietary treatment was repeated 5 times (1 repetition per experimental week and 1 pen representing a repetition) and the control was repeated 4 times. None of the diets included growth promoters, prescription antibiotics or zinc oxide. Piglets that experienced diarrhea or any other serious health conditions were removed from the experiment and treated elsewhere and counted as piglets that did not complete the experiment. Feed and fresh water were supplied *ad libitum* throughout the experiment. Litter weights in the nursing period were recorded every week, and feed intake was recorded daily. Performance indicators such as body weight, ADFI (average daily feed intake), average daily weight gain (ADG), feed conversion ratio (FCR) and the completion rate were calculated by pen as previously outlined[5].

Table 1
Feed formulations used for the experiment.

	Pre-starter diet			Starter diet		
	0% FRS	2.5% FRS	5% FRS	0% FRS	2.5% FRS	5% FRS
Ingredients	(g/kg)					
Wheat (11.2%)	613.26	597.88	583.86	539.88	525.11	508.24
Barley (10.6%)	100.00	100.00	100.00	200.00	200.00	200.00
Soybean meal (46.0%)	0.00	0.00	0.00	170.00	170.00	169.69
Digestible soy	79.08	69.35	59.32	10.63	0.77	0.00
Fermented rapeseed-seaweed meal	0.00	25.00	50.00	0.00	25.00	50.00
Potato protein	40.00	40.00	40.00	0.00	0.00	0.00
Fish meal (70%)	40.00	40.00	40.00	14.00	14.00	7.00
Whey protein	50.00	50.00	50.00	0.00	0.00	0.00
Soy bean oil	37.28	38.93	40.17	23.74	25.20	26.50
Limestone (Ca 38.5%)	0.00	0.00	0.00	5.71	5.57	5.66
Calcium formate	5.00	5.00	5.00	0.00	0.00	0.00
Calcium phosphate	9.12	8.68	8.23	8.83	8.38	8.29
Sodium chloride	3.83	3.70	3.58	4.98	4.85	4.89
Summer fruit	2.00	2.00	2.00	2.00	2.00	2.00
Tretracid liquid	5.82	5.83	5.84	5.60	5.61	5.75
Lysine HCl (98%)	5.00	4.00	2.50	5.00	4.00	2.50
Methionine DL (99%)	0.77	0.73	0.69	1.02	0.97	1.01
Threonine L (99%)	1.88	1.83	1.77	1.94	1.88	1.85
Valine (98%)	1.11	1.09	1.06	1.00	0.97	0.96
Tryptophan (99%)	0.65	0.65	0.65	0.37	0.37	0.36

[a]Provided the following per kilogram of feed: Vitamin A 13,000 IU; vitamin D3 2000 IU; Vitamin E 165 mg; vitamin B1 2.5 mg; vitamin B2 7.0 mg; biotin 200 mcg; vitamin B6 4 mg; vitamin B12 50 mcg; vitamin K 3 mg; Niacin 35 mg; folic acid 1.5 mg; pantotenic acid 21.7 mg; vitamin C 100 mg; choline 0 mg; Fe 180 mg; Zn 150 mg; Cu 0 mg; Mn 55 mg; Se 0.4 mg; I 0.6 mg; Mg 0 mg. [b]methionine + cysteine.

	Pre-starter diet			Starter diet		
Microbial phytase	0.15	0.15	0.15	0.15	0.15	0.15
Microbial xylanase, beta-glucanase	0.15	0.15	0.15	0.15	0.15	0.15
Vitamin E (50%)	0.03	0.03	0.30	0.10	0.01	0.01
Vitamin-mineral premix[a]	5.00	5.00	5.00	5.00	5.00	5.00
Calculated nutritive value (%)						
Dry weight	88.50	88.50	88.60	87.50	87.50	87.60
Metabolizable energy (MJ)	14.30	14.30	14.30	13.50	13.50	13.50
Crude protein	19.70	19.70	19.70	18.40	18.40	18.40
Crude fat	5.56	5.75	5.90	4.03	4.20	4.31
Crude fiber	2.20	2.38	2.57	3.11	3.29	3.51
Ashes	5.37	5.42	5.45	5.69	5.71	5.87
Starch (g)	471.40	408.30	399.90	425.60	416.90	406.80
Lactose (g)	36.50	36.50	36.50	0.00	0.00	0.00
Calcium	0.83	0.83	0.84	0.82	0.82	0.82
Total phosphorous	0.65	0.65	0.65	0.58	0.58	0.58
Digestible phosphorous	0.59	0.59	0.59	0.51	0.51	0.51
Sodium	0.23	0.23	0.23	0.22	0.22	0.22
Chlorine	0.57	0.56	0.56	0.51	0.50	0.50
Potassium	0.65	0.65	0.66	0.67	0.68	0.69
Lysine	1.46	1.46	1.46	1.28	1.28	1.28
Methionine	0.45	0.45	0.45	0.41	0.41	0.41
Met + Cyst[b]	0.77	0.77	0.78	0.72	0.73	0.73
Threonine	0.92	0.92	0.92	0.81	0.81	0.81
Tryptophane	0.30	0.31	0.31	0.26	0.26	0.26

[a]Provided the following per kilogram of feed: Vitamin A 13,000 IU; vitamin D3 2000 IU; Vitamin E 165 mg; vitamin B1 2.5 mg; vitamin B2 7.0 mg; biotin 200 mcg; vitamin B6 4 mg; vitamin B12 50 mcg; vitamin K 3 mg; Niacin 35 mg; folic acid 1.5 mg; pantotenic acid 21.7 mg; vitamin C 100 mg; choline 0 mg; Fe 180 mg; Zn 150 mg; Cu 0 mg; Mn 55 mg; Se 0.4 mg; I 0.6 mg; Mg 0 mg. [b]methionine + cysteine.

	Pre-starter diet			Starter diet		
Valine	1.03	1.03	1.03	0.90	0.90	0.90
Isoleucine	0.78	0.78	0.78	0.70	0.69	0.69

[a]Provided the following per kilogram of feed: Vitamin A 13,000 IU; vitamin D3 2000 IU; Vitamin E 165 mg; vitamin B1 2.5 mg; vitamin B2 7.0 mg; biotin 200 mcg; vitamin B6 4 mg; vitamin B12 50 mcg; vitamin K 3 mg; Niacin 35 mg; folic acid 1.5 mg; pantotenic acid 21.7 mg; vitamin C 100 mg; choline 0 mg; Fe 180 mg; Zn 150 mg; Cu 0 mg; Mn 55 mg; Se 0.4 mg; I 0.6 mg; Mg 0 mg. [b]methionine + cysteine.

Biological sample collection

A total of 10 piglets from each treatment (5 in each of two experimental weeks) were randomly selected and euthanized 3 weeks after weaning. The animals were euthanized by stun gunning with a captive bolt immediately followed by de-bleeding at the farm slaughtering facilities under strict sanitary regulations. Whole blood samples and serum for clinical analysis, the digesta from the colon for microbiome analysis, and jejunum and colon tissues for histopathological analyses, were collected in that order immediately after slaughtering.

A blood sample from each piglet was deposited in a tube with the anti-coagulant EDTA and preserved on ice until taken to the laboratory, where it was stored at 2–8 ˚C until analysis. Another blood sample was collected in a tube without anticoagulant, and serum separated by centrifugation, which was then stored at -20 ˚C until analysis. Gut tissues and colon contents were sampled after opening of the abdominal wall, and the stomach, small and large intestines were occluded at both ends and removed. Approximately 2 cm^3 of colon content was collected from the apex of the ascending spiral of the colon with a sterile spatula and deposited in cryotubes with RNAlater™ (Sigma-Aldrich, Munich, Germany). Tubes with colon contents were kept at room temperature for less than 24 h, followed by cryopreservation in the laboratory. Tissue samples (approximate 2 cm long) of the whole transection of the jejunum and colon were excised and carefully rinsed from gut contents by flushing with saline (0.9% NaCl). For each tissue a sterilized blade was used. Tissues were preserved in 10% formaldehyde and kept at room temperature for no longer than 24 hours until further processing[18].

Blood hematology, blood biochemistry and serum immunoglobulin analysis

Full blood counts (erythrocyte, hemoglobin, hematocrit, mean corpuscular volume, mean corpuscular hemoglobin, mean corpuscular hemoglobin concentration, red cell distribution width) and differential white blood cells count (platelets, leucocytes, lymphocytes, monocytes, neutrophils, eosinophils, basophils) were performed using a Sysmex XT 2000i analyzer (Sysmex Corporation, Kobe, Japan). Serum analysis measured concentrations of the following, using standardized quantification methods: alanine aminotransferase, glutamic pyruvic transaminase, aspartate aminotransferase, glutamic-oxaloacetic transaminase, lactate dehydrogenase, lysozyme, glucose, total protein, blood urea nitrogen,

uric acid, phosphorous, total cholesterol, triglycerides, low density lipoprotein, high density lipoprotein and immunoglobulin G (IgG) according to previously described procedures[5].

Histological morphometric analysis of intestinal tissues

The histological analysis of mid-jejunal and colonic tissues was conducted by a commercial analytical laboratory (ALAB Weterynaria, Warsaw, Poland) according to previous procedures[5]. In short, tissue sections fixed in 10% formaldehyde were dehydrated by means of graded ethanol and xylene baths and embedded in paraffin wax, and 3–4 μm section were then stained with haematoxylin and eosin. Histopathological evaluations (at different lens magnifications) measured gut-associated lymphoid tissue (GALT), intraepithelial lymphocytes (IELs) and lymphatic infiltration of the stromal mucosa (stromal lymphocytes, SL) counts. For GALTs, the numbers of lymphoid follicles per millimeter square were counted. For IEL scoring, the following scale was used: 0-normal (0–10 IELs/100 enterocytes), 1-low (10–15 IELs/100 enterocytes), 2-moderate (15–20 IELs/100 enterocytes; this level suggests chronic subclinical inflammation, where the intestinal-blood barrier may be damaged; weak lymphocytic inflammation), 3-severe (> 20 IELs/100 enterocytes; this level indicates chronic inflammation with infiltration damaging the epithelium and intestinal-blood barrier; moderate lymphocytic inflammation). For SL, the visual scoring scale was: 0-normal (single lymphocytes in stromal connective tissues of villus and crypts), 1-low (increased number of lymphocytes, but no damage to the stroma structures), 2-moderate (abundant infiltration of lymphocytes in stroma, damaging blood vessel walls, connective tissue fiber, reducing visibility of stroma structures), 3-severe (lymphocyte infiltration completely disrupts and conceals the stroma). In a blinded fashion, 10 fields of view per piglet at 4 × magnification were used for evaluation of GALT structures and numbers of lymphoid follicles. IEL and SL were evaluated at 40 × magnification. The analysis used a standard light microscope Olympus BX41 and Cell Sens software (Olympus Corporation, Tokyo, Japan). The gut tissues samples which could not reach the requirements for histological analysis were discarded, resulting in n = 9, 8, 10 for the 0%, 2.5% and 5% FRS group, respectively.

16S rRNA gene amplicon sequencing of colon content

Collected colon contents was stored at -60 ˚C prior to the analysis. Two types of 16S rRNA gene amplicon sequencing strategies were adopted to characterize the prokaryotic community: Illumina, NextSeq (Illumina, CA, USA) and MinION (Oxford Nanopore Technologies, Oxford, UK). The genomic DNA was extracted using Bead-Beat Micro AX Gravity Kit (A&A Biotechnology, Gdynia, Poland) according to the manufacturer's instruction. DNA concentration and purity were measured using NanoDrop ND-1000 spectrophotometer (Saveen and Werner AB, Sweden).

Extracted DNA was diluted to 10 ng/μL prior to library preparation. The V3 hypervariable region of 16S rRNA gene was amplified and sequenced with Illumina technology as previously described[19]. Near full-length 16S rRNA gene amplicons were amplified and sequenced with ONT targeting V1-V8 hypervariable region using following primers: ONT_27Fa: GTCTCGTGGG CTCGGAGATG TGTATATAGA TCGCAGAGTT TGATYMTGGCTCAG; ONT_27Fb: GTCTCGTGGG CTCGGAGATG TGTATATAGA TCGCAGAGTT

TGATCCTGGCTTAG and ONT_1540_R: GTCTCGTGGG CTCGGAGATG TGTATACTCT CTATTACGGY TACCTTGTTACGACT. Custom designed barcoding system was developed to tag encode up to 96 samples during the second round of PCR, and the PCR primer sequence is given in Table S1 (Additional file). The PCR1 reaction mix contained 5 µl of PCRBIO buffer and 0.25 µL PCRBIO HiFi polymerase (PCR Biosystems Ltd, London, United Kingdom), 1 µL of primers mix (5 µM of ONT_27Fa and ONT_27Fb, and 10 µM of ONT_1540_R, see above), 5 µL of genomic DNA (~ 10 ng/µL) and nuclease-free water to a total value of 25 µL. The PCR thermal conditions were as follows: denaturation at 95 °C for 5 min; 33 cycles of 95 °C for 20 s, 55 °C for 20 s and 72 °C for 45 s; followed by final elongation at 72 °C for 4 min.

PCR products were verified by agarose gel electrophoresis and then subjected for barcoding (PCR2). The PCR2 mix composed of 5 µL PCRBIO buffer, 0.25 µL PCRBIO HiFi polymerase (PCR Biosystems Ltd, London, United Kingdom), 2 µL of barcode primers (5 µM), 1 µL of PCR1 template and DEPC water up to 25 µL. The PCR2 thermal conditions were as follows: denaturation at 95 °C for 2 min; 13 cycles of 95 °C for 20 s, 55 °C for 20 s, 72 °C for 40 s; final elongation at 72 °C for 4 min. The final PCR products were purified using AMPure XP beads (Beckman Coulter Genomic, CA, USA) and pooled in equimolar concentrations. The pooled barcoded amplicons were subjected to 1D genomic DNA by ligation protocol (SQK-LSK109) to complete library preparation for MinION sequencing. Approximate 0.2 µg of amplicons were used for the initial step of end-prep. And 40 ng of prepared amplicon library was loaded on a R9.4.1 flow cell.

Sequencing data analysis

The raw Illumina derived dataset containing pair-ended reads with corresponding quality scores were merged and trimmed using fastq_mergepairs and fastq_filter scripts implemented in the USEARCH pipeline as described previously[19]. Purging the dataset from chimeric reads and constructing zero radius Operational Taxonomic Units (zOTUs) was conducted using the UNOISE[20]. The Greengenes (13.8) 16S rRNA gene collection was used as a reference database[21].

Data generated by MinION were collected using MinKnow software v19.06.8 (https://nanoporetech.com). The Guppy v3.2.2 basecalling toolkit was used to base call raw fast5 to fastq (https://nanoporetech.com). Porechop v0.2.2 was used for adapter trimming and sample demultiplexing (https://github.com/rrwick/Porechop). The Porechop adapter list was (adapters.py) edited accordingly and is given in Table S1 (Additional file). Sequences containing quality scores (fastq files) were quality corrected using NanoFilt ($q \geq 10$; read length > 1Kb). Taxonomy assignment of quality corrected reads against Greengenes (13.8) database was conducted using uclast method implemented in parallel_assign_taxonomy_uclust.py (QIIME v1.9.1). The uclust settings were tuned on mock communities (–similarity 0.8; min_consensus_fraction 0.51) assuring annotations to the lowest taxonomic level with no false positive annotations. The settings allowed it to treat individual amplicon sequence variants as individual “seeds”. Reads classified to at least phylum level were subjected for further analysis.

Statistics

All the statistical analysis concerning phenotypic data was performed with R (v3.6.2). The difference of piglet production performance was evaluated using linear mixed model as previously outlined[5] and orthogonal polynomial contrast was used to appreciate the effect on increasing dose of the FRS (0%, 2.5%, 5%). The blood hematology and biochemistry data was analyzed by R package compareGroups[22] (v4.0) using “comparaGroups” command and the descriptive table was generated by “createTable” command. In R package compareGroups, the significant differences among groups were determined by anova and Tukey’s procedure for post hoc tests. Wilcoxon rank-sum test was used to evaluate the histological difference between groups.

For microbiome analysis, QIIME 2[23] (v2018.11) combined with R packages (ggplot2, vegan, corrplot, Rhea, rstatix, vennDiagram) were used. Three samples were removed due to inadequate library size (< 1000 counts), resulting in n = 9, 8, 10 for 0%, 2.5% and 5% FRS group, respectively. For both sequencing strategies, all the samples were summarized at the L7 levels (species) and rarefied to the same sequencing depth (11000 reads/sample) for alpha and beta diversity calculations. Rarefaction on the zOTU table (Illumina data) was adopted as comparison for rarefaction on the species-level summarized table. Principal coordinate analysis (PCoA) plots were generated using binary Jaccard and Bray Curtis distance metrics, and PERMANOVA was performed to determine differences between groups and *p* values were adjusted by Benjamini-Hochberg correction. ANCOM[24] was adopted to identify differentially abundant taxa between groups at summarized L7 level. For taxa identified by ANCOM, Wilcoxon rank-sum test was adopted for pairwise comparison. Phenotypic data were integrated with species-level bacterial abundances by Pearson’s correlation analysis using R package Rhea[25]. Rare microbial features were removed with a cutoff of mean relative abundance > 0.1% and minimal presence among 30% of samples. Zeros were regarded as NA. Centered log-ratio transformation was conducted in both the microbial relative abundance and phenotypic data.

Results

Piglet performance, blood hematology, blood biochemistry and systemic immunoglobulin

Piglets fed with 2.5% FRS had numerically increased body weight by the end of the experiment (85 days of age) in comparison with those fed the basal diet. Further, increasing the dose of FRS to 5% did not further increase of the final body weight (Table 2). No significant differences in ADFI, ADG, FCR were found between the three feeding regimens. The completion rate for piglets in the experiment (i.e. not dead or removed due to need for antibiotics treatment) did not differ between treatment groups. In the sub-group of piglets euthanized 3 weeks after weaning, we found no statistical differences in blood hematology, blood chemistry and systemic immunoglobulin parameters between treatment groups, except for the levels of blood urea nitrogen (BUN) and mean corpuscular volume (Additional file: Table 3). Compared with piglets fed without FRS inclusion, 2.5% FRS reduced plasma concentrations of BUN, but

increased mean corpuscular volume. 5% FRS piglets had a similar tendency, but only BUN concentrations significantly declined.

Table 2
Performance of piglets subjected to three dietary regimens.

Parameters	0% FRS	2.5% FRS	5% FRS	SEM	*P* value		
					TG	TG	
						L	Q
Weaning weight (kg, at d 28)	6.07 ± 0.33	6.24 ±0.34	5.99 ± 0.66				
Body weight, at age of interest (kg)							
42	6.77	7.00	6.55	0.406	0.579	0.890	0.428
49	8.24	8.75	8.41	0.418	0.563	0.746	0.314
77	20.5	21.6	20.2	1.18	0.537	0.842	0.341
85	23.2	25.1	23.7	1.82	0.467	0.823	0.269
ADFI (kg/day)							
28 – 42 days	0.162	0.183	0.166	0.015	0.437	0.830	0.231
28 – 49 days	0.233	0.232	0.230	0.020	0.985	0.900	0.967
28 – 85 days	0.516	0.572	0.546	0.037	0.418	0.477	0.236
50 – 77 days	0.626	0.661	0.632	0.031	0.531	0.879	0.299
50 – 85 days	0.671	0.763	0.719	0.058	0.365	0.460	0.199
ADG (kg/day)							
28 – 42 days	0.049	0.063	0.049	0.025	0.835	0.993	0.574
28 – 49 days	0.103	0.122	0.118	0.017	0.681	0.506	0.499
28 – 85 days	0.306	0.339	0.316	0.034	0.584	0.790	0.343
50 – 77 days	0.439	0.456	0.420	0.037	0.623	0.702	0.476
50 – 85 days	0.423	0.467	0.431	0.045	0.486	0.993	0.574
FCR							
28 – 42 days	6.78	7.42	7.13	3.12	0.938	0.878	0.740
28 – 49 days	2.37	2.12	2.09	0.311	0.681	0.486	0.683
28 – 85 days	1.80	1.68	1.76	0.147	0.584	0.834	0.515
50 – 77 days	1.44	1.45	1.55	0.084	0.485	0.365	0.597
50 – 85 days	6.78	7.42	7.13	3.12	0.938	0.878	0.740

Completion rate (%)							
28 – 42 days	95.2	96.9	96.1	2.10	0.773	0.74	0.504
28 – 49 days	94.1	95.1	94.3	2.29	0.847	0.921	0.601
28 – 85 days	94.8	94.4	89.1	3.60	0.472	0.328	0.564

ADFI = average daily feed intake; ADG = average daily gain; FCR = feed conversion ratio; TG = treatment group; L = linear effect; Q = quadratic effect; The significance between different dosage group is based on the result of orthogonal contrasts.

High level of accordance between the short and long amplicon sequencing strategies

Two different sequencing strategies were applied: Illumina NextSeq-based amplicon sequencing of the 16S rRNA gene V3 variable region (Illumina V3) and ONT based sequencing of V1-V8 variable regions (ONT V1-V8). Out of 99 unique taxonomic groups found by the two methods, 78 were shared (Additional file: Figure S1A). The accordance was further improved when abundance threshold was adjusted (Additional file: Figure S1B-D). The taxonomic groups with relative abundance above 3% were identical in the two methods (Additional file: Figure S1E) and the overlapped detections overall showed good positive correlation in between (Additional file: Figure S1F). Both methods revealed that the most dominant bacterial groups belonged to genus *Lactobacillus* and families: *Ruminococcae* and *Lachnospiraceae* independent of treatment (Fig. 1).

Dietary inclusion of FRS induced distinct shifts in the colon microbiota composition

Both sequencing approaches revealed alterations in gut microbial diversity in piglets under different feeding regimens. FRS inclusion resulted in increased Shannon diversity and observed features. The effect was consistent, when the analysis was performed based on the zOTU table (Fig. 2A), and the summarized species-level table (Fig. 2B) from Illumina data and ONT data (Fig. 2C). Increasing the FRS inclusion from 2.5% to 5% did not lead to significant changes of alpha diversity between the two groups (Fig. 2A-C). Beta diversity analysis on binary Jaccard (qualitative) and Bray Curtis dissimilarity metrics (quantitative) indicated that introduction of FRS in the feed influenced the colon microbiota composition of weaner piglets. Surprisingly, the changes were more pronounced in the 2.5% FRS group relative to the 5% FRS (Fig. 2D-F).

Dietary inclusion of 2.5% FRS increased the *Prevotella stercorea* and *Mitsuokella* abundance in colon

The relative abundance of *Prevotella stercorea* and *Mitsuokella spp*. were increased in the 2.5% FRS-feeding group compared to the none-FRS diet and for most comparisons also in 5% FRS as well (Fig. 3A-D). The relative abundance of *Prevotella stercorea* (Illumina V3) was shown to be positively correlated with the colon mucosa thickness and negatively correlated to the blood leucocytes counts and serum IgG concentrations (Fig. 4A), while this observation was only near-significant using ONT (Fig. 4B).

There were 83 significant correlation pairs between bacteria relative abundance by Illumina V3 sequencing and phenotypic indicators of systemic or intestinal immunomodulation or intestinal histopathological parameters, while ONT resulted in 114 significant pairs. Although two methods gave similar associations and trends for the overlapped pairs, only one taxon, *Faecalibacterium prausnitzii* showed identical accordance in correlations to the phenotypic data. The relative abundance of *F. prausnitzii* was positively correlated with colon mucosa thickness but negatively associated with the serum concentrations of aspartate aminotransferase, lactate dehydrogenase and IgG (Additional file: Figure S2).

Dietary inclusion of FRS improved the gut health of weaner piglets

The morphological characteristics of intestinal tissues obtained from piglets in the different treatment groups are shown in Fig. 5A. All animals fed 0%, 2.5% and 5% FRS presented normal ranges for heights and structures of villi and intestinal crypts. The continuity and height of the jejunal and colonic epithelium were more pronounced in both FRS groups compared to the piglets on the basal feed with no added FRS. In the jejunal epithelium and stroma, the piglets fed with FRS had reduced IEL and SL infiltration compared to 0% FRS piglets, with 2.5% FRS showing the best effect (Fig. 5B). We found similar tendency of alleviated focal inflammation in the colon tissues of 2.5% FRS, but with no significant difference between treatment groups (Fig. 5C). Diffuse lymphoid follicles at the base of the mucosa were visible with normal size and structure in jejunum and colon. No clear stimulation of lymphoid follicles was observed in all gut tissues. Neither did FRS inclusion result in the aggregation of jejunal and colonic lymphoid follicles (Fig. 5D). Histological evaluation did not show damaged intestinal epithelial barrier in any group but the mucous membrane was higher with deeper intestinal crypts in the 2.5% FRS relative to 0% FRS (Fig. 5E).

Discussion

In modern pig production, the weaning of piglets is usually conducted at an early age, with physiological stress from changes in diet, environment and social groups. Hence, many weaned piglets experience intestinal and immune dysfunction, elevated risk of infection with enteric pathogens and hence diarrhea,

and lowered weight gain due to reduced feed intake and poorer utilization of ingested nutrients[26]. Reduced weight gain and high mortality rate among weaned piglets are undesirable from a production efficiency point of view. Preventive measures, such as use of in-feed antibiotics have been banned by the European Union (EU) back in 2006, while the commonly used zinc oxide will be banned in 2022. Therefore, there is an urgent need for development of alternative preventive strategies in order to sustain the performance and gut health of weaned piglets. We have previously reported that FRS fed to weaned piglets improved the jejunal villus development, stimulated colon mucosal development and reduced signs of intestinal inflammation[5]. Since FRS was demonstrated to be effective without in-feed zinc oxide, in the present study, we further investigated the dose-dependent influence of FRS on gut microbiome composition and its plausible link with phenotypic indicators.

To study the gut microbial composition, we have adopted two sequencing strategies on different regions of microbial 16S rRNA gene. The short-read 16S rRNA gene amplicon sequencing by Illumina platform was compared with the near-full length 16S rRNA gene sequencing method by ONT. The two strategies showed satisfying accordance and allowed to draw the same overall conclusions incl. the taxonomic detections at the species level, which is challenging even when different hypervariable regions of 16S rRNA gene are profiled with the same sequencing[27, 28, 29].

Data generated with both sequencing strategies confirmed significant changes of gut microbiota composition in response to dietary FRS supplementation. The effect was more pronounced in the 2.5% FRS group relative to the 5% FRS. The colon microbiota of piglets under FRS feeding regimen had increased alpha diversity, suggesting a more diverse and uniformly distributed microbial community than the piglets fed the basal diet. High microbial diversity is generally desirable, as it has been demonstrated to exclude pathogenic microbes, improve immune response and reduce necrotizing enterocolitis and post-weaning diarrhea incidences[30, 31, 32]. FRS supplementation led to increased relative abundance of *Prevotella stercorea* and *Mitsuokella* spp., especially pronounced in the 2.5% FRS group. *Prevotella* is known to be the major contributor to the microbiome of post-weaned piglets given the ability to degrade plant fibers in the solid diet. The species *P. stercorea* has previously been described as a member of the healthy pig gut microbiome[33] and also a potent producer of short-chain fatty acids (SCFA) through fermenting fibers residuals in the hindgut[34], [35]. Our data indicated that the abundance of *P. stercorea* correlated positively with colon mucosa thickness, which is not surprising, since *Prevotella* spp. are recognized colonizers of the mucosal sites[36] and the produced SCFAs maintain the intestinal barrier function through providing energy resources and immunoregulatory regulation[37, 38, 39]. It is also reported that complex hemicelluloses and cellulose most likely enhances mucosal abundance of *P. stercorea*[40, 41]. The negative correlation of *P. stercorea* with the serum levels of leucocytes and IgG could suggest that increased abundance of *P. stercorea* on the more fibrous FRS supplemented diet stimulated gut barrier and immune function, and consequently relieved the risk of host inflammation.

Faecalibacterium prausnitzii is one of the main butyrate producers found in the gastro-intestinal tract[42]. Butyrate plays a vital role in gut physiology and gut health, serving as a main energy source for the colonocytes and a protector against inflammatory disease and colorectal cancer[43, 44]. Many studies

have linked reduced abundance of *F. prausnitzii* with different intestinal disorders, hence it has been proposed that *F. prausnitzii* is a potential biomarker of gut health[45]. Our data indicated that *F. prausnitzii* abundanc in colon correlated positively with colon mucosa thickness and negatively with serum levels of two enzymes released from the liver i.e. aspartate aminotransferase and lactate dehydrogenase and IgG. Increased serum concentration of the hepatic enzymes is a sign of liver malfunction while IgG is systemic indicator of host inflammation. Our findings are in line with studies demonstrating the ability of *F. prausnitzii* to reduce inflammation and improve the liver function in murine models[46, 47] and human trials.

Although the microbiota data suggested there was no distinct impact with regards to the inclusion levels of FRS (2.5% versus 5%), it is important to note that we found significantly alleviated signs of lymphocyte invasion in jejunum and enhanced colon mucosa barrier function solely among piglets receiving 2.5% FRS. Besides, inclusion of 5% FRS tended to numerically worsen production performance (ADG, FCR, completion rate). Possibly, if excessive FRS are added, the piglets are exposed to more bioactive components from either the rapeseed or seaweed. Even though they are beneficial in low amounts, excessive intake could increase the physiological stress in the weaning period and become counterproductive to FCR. Young animals are more sensitive to the anti-nutritional factors e.g. glucosinolates[48] than adult animals, and thereby our results suggest that the optimal inclusion level of FRS was around 2.5%.

Conclusion

Our study demonstrates that dietary supplementation in postweaning piglets with 2.5% FRS reshaped the colon microbiota and led to improved gut health, while further increasing the FRS level to 5% resulted in less pronounced effects. Inclusion of 2.5% FRS induced increased gut microbial diversity and elevated the abundances of *Prevotella stercorea* and *Mitsuokella* spp. Although clear causality cannot be proven, we found clear correlations between the abundance of *Prevotella stercorea* and *Faecalibacterium prausnitzii* and phenotypic biomarkers of reduced intestinal and systemic inflammation, improved liver function, and increased colon mucosal thickness.

List Of Abbreviations

FRS Rapeseed-seaweed blend fermented by lactobacilli

ADFI Average daily feed intake

ADG Average daily weight gain

FCR Feed conversion ratio

IgG Immunoglobulin G

GALT Gut-associated lymphoid tissue

IEL Intraepithelial lymphocyte

SL Stromal lymphocyte

PCR Polymerase chain reaction

PCoA Principal coordinate analysis

PERMANOVA Permutational multivariate analysis of variance

ANCOM Analysis of compositions of microbiomes

QIIME Quantitative insights into microbial ecology.

ONT Oxford Nanopore Technologies

BUN Blood urea nitrogen

SCFA Short-chain fatty acids

Declarations

Ethics approval and consent to participate

Not applicable.

Consent for publication

Not applicable.

Availability of data and materials

Raw sequencing data are available from the corresponding author on reasonable request.

Competing interests

Paulina Tamez-Hidalgo and Søren Kjærulff Søren are employees of FermentationExperts. The authors declare that they have no competing interests influencing the content in this paper.

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Author's contributions

Conceptualization: L.K., D.S.N., S.K.S., M.O.N. and P.T.H.; Investigations: Y.H., T.C., P.TH., G.D.S. and L.K.; Methodology: Y.H., L.K., W.P.K. and G.D.S.; Project administration: P.TH., G.D.S., T.C., and L.K.; Resources: M.O.N., L.K., W.P.K. and D.S.N.; Supervision: P.TH, S.K.S., M.O.N., L.K. and D.S.N.; Software: Y.H., L.K. and G.D.S.; Formal analysis: Y.H., L.K. and G.D.S.; Data curation: Y.H., L.K. and G.D.S.; Visualization: Y.H.; Writing-original draft: Y.H., G.D.S. and L.K.; Writing-review and editing: all authors. Funding acquisitions: S.K.S., M.O.N. and D.S.N. All authors read and approved the final manuscript.

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Table

Due to technical limitations, table 3 is only available as a download in the Supplemental Files section.