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Dietary supplementation with fermented rapeseed and seaweed modulates parasite infections and gut microbiota in outdoor pigs

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Outdoor pig production systems can increase exposure to helminth infections such as Ascaris suum. Anthelmintic drug treatments are currently the primary strategy for controlling such infections; however, this approach is considered unsustainable due to the risk of parasites developing anthelmintic resistance. This study investigated the potential anthelmintic effect of a 2% w/w fermented rapeseed-seaweed (FRS) dietary supplement administered over an 11-12 week period in growing-finisher pigs. Outcomes assessed included parasitic fecal egg counts (FEC), serology, gut microbiota composition, and systemic inflammation. The FRS supplement consisted of 6% Saccharina latissima, 6% Ascophyllum nodosum, and 88% rapeseed meal (based on dry matter) and was provided in two different batches. Supplementation with FRS tended to lower FEC and reduce the incidence of A. suum infection. However, this effect varied between batches. FRS also influenced gut microbiota composition: pigs that were fed the second batch of FRS showed an increased relative abundance of several Bacteroidetes members (e.g., Prevotella), while unsupplemented pigs were enriched with various taxa from the *Firmicutes* phylum, such as *Clostridium* spp. Furthermore, pigs fed the second batch of FTR showed reduced daily weight gain compared to those fed the control diet. Overall, our results indicate that FRS modulates the gut microbiota toward a composition potentially associated with improved gut health and may have promise as a prebiotic or novel feed additive to help reduce helminth infections.

KEYWORDS

parasite, bioactive forage, gut microbiome, brown seaweed, macroalgae, rapeseed meal, *Ascaris suum*, swine

1 Background

Helminth parasites-primarily the pig roundworm Ascaris suum and the nodular worm Oesophagostomum spp.—are common in pig production systems worldwide, particularly in pigs raised outdoors (1). The eggs of A. suum can survive for years on pastures (2, 3), whereas the external stages of Oesophagostomum spp. are more sensitive to environmental conditions such as drought and frost (4). In northern temperate climates such as Scandinavia, larvae of Oesophagostomum spp. rarely survive outdoors during typical winters (5). As porcine helminths primarily cause subclinical disease, infections are often not perceived by producers as a threat to productivity (1). Nonetheless, negative correlations between helminth infections and pig growth or feed efficiency have been documented for both A. suum (6-9) and Oesophagostomum spp. (10, 11). Infections with the protozoa, Eimeria spp. are also common in weaned pigs. While usually nonpathogenic (12), the long-term effects of Eimeria infections on performance remain poorly understood (13).

Infections with *A. suum* in pigs have been associated with significant changes in the host's gut microbiota (GM) composition and induction of strong local immune responses (14). Thus, helminth infections are considered to influence animal welfare and health and lead to economic loss for producers, with frequent anthelmintic treatment being the obvious solution to limit helminth infections (1). However, due to the risk of anthelmintic resistance, as documented in *Oesophagostomum* spp. (15–18). There is a need for alternative approaches (14, 19). Resistance has also been documented for ascarid helminths in other hosts, e.g., turkeys, horses, and humans (20–22).

The search for alternative treatments has renewed interest in botanicals with anthelmintic properties (23). In ruminants, numerous such botanicals have been identified, including pasture species such as sainfoin and chicory (24, 25) as well as feed additives containing essential oils (26). In contrast, research on botanical alternatives for controlling pig parasites remains limited (27).

However, some evidence suggests that dietary additives such as garlic may hold promise as alternative anthelmintic agents (28). In addition, bioactive compounds—particularly omega-3 fatty acids such as alpha-linolenic acid—from extracts of brown seaweed (*Saccharina latissima*) have shown direct anti-parasitic effects *in vitro* against the larval stages of *A. suum* (29) and the canine hookworm *Uncinaria stenocephala* (30). However, Bonde et al. (31) were unable to demonstrate a consistent anti-parasitic effect in two short-term studies with pigs experimentally infected with *O. dentatum* and *A. suum* and fed a high level of dried whole *S. latissima* (5 or 8% of dry matter intake). Brown seaweeds (BS; *Phaeophyta*) are a recognized source of bioactive compounds, such as high concentrations of soluble polysaccharides (32–34). These complex polysaccharides are not digested and absorbed in the small intestines of the host and are instead fermented in the hindgut by the GM (33, 35). BS polysaccharides have shown anti-infection, anti-inflammatory, and immune-modulating properties in a variety of animal species (35, 36) and could potentially also have antiparasitic properties, as shown for other prebiotic compounds in animals (37, 38). Furthermore, extracts of *Ascophylum nodosum* and other BS have shown promising results in the sustainable control of plant-parasitic nematodes, e.g., *Meloidogyne* spp., in a range of crops (39).

Brown seaweed species such as S. latissima and A. nodosum have been tested in combination with rapeseed (Brassica napus) in feed supplements for pigs (40), where rapeseed has been linked to improved gut health (41). However, as both seaweed and rapeseed contain anti-nutritional components (42, 43), a pre-fermentation process may improve the nutritional value, digestibility (44) and suitability of this product as a pig feed (45) and thereby increase growth performance (46). Pre-fermented rapeseed-seaweed supplements fed to piglets have thus been shown to increase GM diversity, reduce intestinal inflammation, and enhance gut mucosal development (40, 45, 47). Thus, based on these findings and in vitro findings, we hypothesize that long-term consumption of such supplements may help limit intestinal parasite infections; however, to date, no studies have examined the potential anti-parasitic effects of a prefermented rapeseed-seaweed mix fed to pigs under large-scale, on-farm conditions.

Here, we evaluated the effects of a 2% (w/w) supplementation with a pre-fermented rapeseed-seaweed product (FRS) in a standard pelleted diet on naturally acquired parasite infections (predominantly *A. suum*) and gut health in growing finishers, as measured by changes in GM composition and systemic inflammatory parameters.

2 Materials and methods

2.1 Experimental animals and design

In total, 200 weaners/early growers (Landrace/Yorkshire/ Duroc crossbreeds) from a conventional indoor pig unit were introduced, 50 animals at a time, to a free-range farm at four time points during winter season (sub-studies 1-4): November 18th, 2020 (SUB1); January 6th 2021 (SUB2); February 3rd 2021 (SUB3); and February 17th 2021 (SUB4). At arrival, the pigs in each substudy (n = 50) were randomly allocated to two groups of 25 animals after stratification for sex (females or castrated males; Table 1). One group received a standard control diet (group C), while the second group (group S) received a similar diet supplemented with 2% (w/w) FRS. Each group was housed in an individual paddock with a shed, feeding trough, and water system for the fattening period (~11-12 weeks) until slaughter weight was reached. The two feeding groups (control vs. FRS) within a sub-study were kept on neighboring paddocks, which were naturally contaminated with Ascaris suum and other parasites from earlier production. Paddocks were not re-used within the current study. All animals were weighed upon arrival, post-group allocation (week 0) and before slaughter (week 11-12). Animals were monitored daily by farm personnel. The study was conducted after approval by the Danish

Abbreviations: As-Hb, *Ascaris suum* adult hemoglobin; BW, body weight; BS, brown seaweeds; DWG, daily weight gain; EPG, eggs per gram of feces; FRS, fermented rapeseed-seaweed; FEC, fecal egg counts; GM, gut microbiota; IL, interleukin; ODr, optical density ratio; SUB, sub-study; TNF- α , tumor necrosis factor-alpha.

Sub-study	Group	Diet	n (start)	Sex (%F)	<i>n</i> (end)	Trial days	Starting weight Mean \pm SD (Kg)	DWG Mean \pm SD (kg)
SUB1	C1	Control	25	52%	24	84*	29.8 ± 1.8	1.09 ± 0.09
	S1	FRS (batch 1)	25	52%	24	84*	29.9 ± 1.8	1.06 ± 0.06
SUB2	C2	Control	25	56%	25	79	26.7 ± 2.0	1.06 ± 0.11
	S2	FRS (batch 2)	25	56%	24	79	27.7 ± 2.2	1.05 ± 0.08
SUB3	C3	Control	25	44%	23	78	28.1 ± 2.3	1.05 ± 0.09
	\$3	FRS (batch 2)	25	40%	24	78	27.5 ± 1.8	1.02 ± 0.09
SUB4	C4	Control	25	44%	25	78	29.8 ± 2.1	1.12 ± 0.09^{a}
	S4	FRS (batch 2)	25	44%	23	78	29.7 ± 1.4	$1.05\pm0.10^{\rm b}$
SUB1-4	C1-4	Control	100	49%	97	-	28.6 ± 2.4	1.08 ± 0.10
	S1-4	FRS	100	48%	95	-	28.7 ± 2.1	1.05 ± 0.09

TABLE 1 Characteristics of sub-studies and all sub-studies combined (bold figures), in regard to dietary treatments, number of pigs included at the beginning and end of the study, as well as sex distribution (F = females), number of days in the trial, starting live weights and daily weight gain (DWG).

*Five pigs from both C1 and S1 were sent for slaughter after 79 days.

Different superscripts $(^{a,b})$ indicate significant differences (P < 0.05) between control and FRS groups within the same sub-study.

Animal Experimentation Inspectorate (license number 2015-15-0201-00760). Weather data were obtained from the Danish weather service (DMI) from the weather archive.

2.2 Diets

All animals were fed *ad libitum* and had free access to water. C-groups were fed a basal commercial diet (Flex A, complete feed, DLG, Denmark mix) from three batches of similar composition and metabolizable energy [#644690 (SUB1+SUB2+SUB3), #665655, and #681221 (SUB2+SUB3+SUB4)], with a smooth transition between batches. S-groups were fed a similar commercial diet with 2% w/w inclusion of the FRS supplement, which is commercially available (EP1199; provided by Fermentationexperts A/S, Denmark). The inclusion level of the supplement was decided based on previous experience by the company providing it and cost-efficiency. Two different fermentation batches of the FRS supplement diet were produced. Batch 1 was used for SUB1, and Batch 2 was used for SUB2-4. The two FRS supplement batches contained 6% Saccharina latissima, 6% Ascophylum nodosum, and 88% rapeseed [based on dry matter (DM)], which were processed and fermented as described elsewhere (40, 45, 47). Briefly, the fermentation was a solid-state fermentation using an inoculum based on three lactic acid fermentation bacteria: Pediococcus acidilactici (DSM 16243), Pediococcus pentosaceus (DSM 12834), and Lactobacillus plantarum (DSM 12837). The fermentation was a two-step process at 38°C and took a total of 11 days, followed by drying in a spin flash dryer. Both control and FRS diets were pelleted and adjusted to similar levels of protein and energy content (Table 2).

2.3 Fecal egg counts

Fecal samples were initially collected in weeks 0, 5, 7, 9, and 11 or 12 for SUB1 and SUB2 for individual fecal egg counts (FEC) to

characterize parasite transmission patterns based on eggs per gram of feces (EPG). Thereafter, SUB3 and SUB4 were only sampled in weeks 0, 9, and 11. The samples were analyzed using a modified concentration McMaster method (2, 48) with a lower threshold of 20 EPG. Feces were sampled directly from the rectum.

2.4 Antibodies and cytokines

Blood samples were collected from all animals via jugular venipuncture using SSTTM II Advance BD Vacutainer tubes at week 11 (SUB2) or week 12 (SUB1) for antibody detection and cytokine analysis. Samples were centrifuged at RCF 1,000 for 15 min, and serum was stored at -80° C until use. Serum samples were analyzed for specific IgG antibodies against *A. suum* adult hemoglobin [As-Hb; cut-off Optical Density ratio (ODr) = 0.5] and antigens from third-stage larvae isolated from the lungs (cut-off ODr = 0.25) using the ELISA as previously described (49). Further, concentrations of the inflammatory cytokines IL-6 and TNF- α in serum were assessed using commercial antibody pairs (R and D Systems, UK) according to the manufacturer's instructions.

2.5 Gut microbiota analysis: DNA extraction, 16S rRNA gene amplicon sequencing, and data processing

The Bead-Beat Micro AX Gravity Kit (cat# 106-100-M1; A&A Biotechnology, Gdynia, Poland) was used to extract DNA from fecal samples from the experiments SUB1 (week 0 and 12) and SUB2-4 (week 0 and 11) based on the manufacturer's guidelines. DNA purity and concentration were determined using a Nanodrop 1000 Spectrophotometer (Thermo Fisher Scientific, USA) and Varioskan Flash (Thermo Fisher Scientific, USA).

A two-step PCR was carried out to amplify the near fulllength 16S rRNA gene with multiple forward and reverse primers (Supplementary Table 1). Reaction conditions for the first PCR

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TABLE 2 Main ingredients and composition analysis of control diet and experimental diet with 2% w/w fermented rapeseed-seaweed (FRS) supplement.

Ingredients (%)	Control	FRS batch 1	FRS batch 2					
Barley	35.79	35.00	35.00					
Wheat	25.00	26.31	26.31					
Soybean meal	10.47	10.71	10.71					
Wheat bran	6.10	-	-					
Rye	5.30	10.00	10.00					
Oats	4.00	2.00	2.00					
Sunflower meal	7.23	8.00	8.00					
FRS*	-	2.00	2.00					
Dried sugar beet	2.00	2.00	2.00					
Feed chalk	-	1.17	1.17					
Vegetable oil and fat (palm)	0.90	1.01	1.01					
Calcium format	0.50	-	-					
Calcium carbonate	0.80	_	-					
Mono calcium phosphate	0.20	0.31	0.31					
Sodium chloride	0.54	0.48	0.48					
L-lysine sulfate	0.60	0.59	0.59					
Vitamin mix	0.20	0.23	0.23					
Analyzed composition	Analyzed composition							
Crude fat (%)	3.2		3.2					
Crude protein (%)	16.1		16.3					
Water (%)	12.2		12.9					
Crude ash (%)	4.8		4.9					
Crude fat (%)	3.2		3.2					
Metabolisable energy (MJ/kg)	13.22		13.15					

*FRS, fermented rapeseed-seaweed.

were as follows: 95°C for 5 min, two cycles of 95°C for 20 s, 48°C for 30 s, 65°C for 10 s, 72°C for 45 s, and a final extension at 72°C for 4 min. First PCR products were next barcoded by a second PCR reaction with the following conditions: 95°C for 2 min, followed by 33 cycles of 95°C for 20 s, 55°C for 20 s, 72°C for 40 s, and a final extension at 72°C for 4 min. After each PCR reaction, PCR products were cleaned using SpeedBeadsTM magnetic carboxylate (obtained from Sigma Aldrich). A 1.5% agarose gel electrophoresis was performed to check the size of barcoded PCR products.

The Nanopore sequencing library was prepared following the ligation sequencing kit SQK-LSK109 and SQK-LSK110 protocols and sequenced on a GridIONX5 platform (Oxford Nanopore Technologies, Oxford, UK).

Sequence data collection was conducted using the Nanopore sequencing software GridION version 21.02.5 (https:// nanoporetech.com). ONT's Guppy version 4.5.2 (https:// nanoporetech.com) was used for base calling and demultiplexing. Demultiplexed sequences were filtered and trimmed (min = 1,300 bp, max = 1,600 bp, q score \geq 10) using Nanofilt version 2.7.1 (50). Taxonomy assignment was conducted by the parallel_assign_taxonomy_uclust.py script of Quantitative Insights into Microbial Ecology (Qiime) 1 version 1.8.0 (51). The Greengenes database version 13.8 (52) was used as a reference database.

2.6 Statistical analysis

Data from eight animals were removed during the study (see Section 3.1 for listed reasons), and data were excluded from the analysis. Due to the change in FR's batches, all SUB1 data were analyzed separately, while SUB2-4 data were analyzed together. Infection levels were based on raw FEC, expressed as accumulated FEC based on the sum of egg excretion from each animal on sample days, whereas A. suum prevalence and incidence were determined based on FEC >200 EPG to estimate likely patent infections and exclude potentially false-positive pigs (53). Incidence refers to the number of initially uninfected pigs becoming infected during the study, i.e., the proportion of a group having a positive FEC (>200 EPG) at least once after initial sampling. All data were checked for normality using the Shapiro-Wilks test using GraphPad Prism (7.00). Data that could be normalized through log transformation were analyzed using an unpaired t-test, with Welch's correction applied where needed. Data that could not be normalized were analyzed using a Mann-Whitney test. A chi-squared or Fisher's exact test was performed using R Studio (R version 3.5.2) to estimate differences in infection incidence between groups for all three different parasites. Data were also analyzed using a linear model (LM, Type III) using R to test factors affecting DWG and A. suum FEC. The model included the infection status of A. suum, feed, sex, sub-study, and body weight at arrival (BW0) and was tested for interactions. The infection status of Eimeria spp. and Oesophagostomum spp. was not included due to sporadic, low-level infections. Similarly, effects on A. suum cumulative FEC were analyzed as a linear model for factors: sub-study, diet, sex, and DWG for weeks 9 and 11/12. The linear model for FEC was considered both with and without log transformation. The Spearman rank correlation test examined correlations between log-transformed FEC and antibody responses.

16S rRNA gene amplicon sequencing data were analyzed separately for SUB1 (n = 90) and SUB2–4 (n = 282). Data were rarefied to 11,000 reads per sample using QIIME 2 (54). After rarefaction, four samples from SUB1, three from SUB2, six from SUB3, and one from SUB4 were excluded from the analysis since they had fewer than 11,000 reads. RStudio version 1.3.1073 (55) using R version 4.0.2 (55) and R packages phyloseq (56), tidyverse (57), ggpubr (58), reshape2 (59), and ggprism (60) were used for data analysis. Observed features and Shannon index measures were calculated for alpha diversity evaluation. Beta diversity analysis was performed by generating principal coordinate analysis (PCoA) plots based on Bray-Curtis dissimilarity and Jaccard distances. Bacteria differentially abundant between groups were identified using the DESeq2 package (61), and a heatmap of taxa found to be significantly differently abundant between groups by DESeq2

(adjusted *P*-value < 0.05) was drawn by using the pheatmap (62) and RColorBrewer (63) packages.

For GM analysis, adjusted *P*-values for alpha diversity measures were obtained by pairwise Wilcoxon rank-sum tests with the Benjamini–Hochberg correction from the R stats package (55). Adjusted *P*-values for beta diversity were calculated by pairwise comparisons using permutation MANOVAs on a distance matrix with *P*-value correction using the Holm method from the R package RVAideMemoire (64).

3 Results

3.1 Observations and performance

The weather conditions varied during the study, as the average daily temperatures during SUB1, SUB2, SUB3, and SUB4 were 4.63, 2.27, 3.73, and 5.45°C, respectively (data not shown). February was particularly cold, thus affecting SUB2 and SUB3, with the lowest temperature during the study measured at -11.9° C, and the mean temperature for the month was 1.1°C. In March, the second lowest temperature was recorded at -6.2° C, with a mean for the month at 4.6°C, affecting the outcome of SUB2–4. Both diets were well accepted by the pigs. Eight pigs were removed from the study: five individuals died from unknown reasons (C1, S2, C3, S3, and S4), two pigs were withdrawn due to a rectal prolapse/stricture (S4 and C3), and one pig was excluded after it managed to jump the fence into another paddock (S1 \rightarrow F0E0C1).

We found no difference in initial body weight between the feeding groups in each sub-study (Table 1) and between male (castrated) and female pigs (28.18 vs. 28.35 kg, P = 0.77). During the study, FRS-fed pigs tended to gain less weight than their respective control groups (Table 1), though only significantly in SUB4 (P = 0.02). Applying a linear model to SUB2–4 confirmed that mean daily weight gain (DWG) was significantly affected by diet (P < 0.01), sub-study, and sex, with males growing 0.07 kg/day more than females (P < 0.0001; Figure 1). Moreover, for SUB2–4, the model showed that pigs with patent *A. suum* infection tended to have a lower DWG than uninfected pigs (-0.03 kg/day; P = 0.11). There were no interactions

between the parameters. For SUB1 sex, it did not significantly affect DWG, but male pigs tended to grow 0.05 kg/day more than female ones (P = 0.07). Within all four sub-studies, the largest difference between males and females was in the FRS groups (Figure 1). In summary, DWG was 0.04 kg/day lower for FRS-fed animals, but the difference was only significant in SUB4.

3.2 FRS supplementation did not significantly reduce parasite infections

Eggs of A. suum and Oesophagostomum spp., as well as Eimeria oocysts, were detected in the fecal samples. Due to sporadic detection, data on Cystoisospora suis were excluded from further analysis. Based on the sampling schedule for SUB1 and SUB2, patent A. suum egg excretion (i.e., >200 EPG) was first detected in week 7 (Figure 2), indicating that the infections were acquired on pastures and not in the herd of origin. Patent A. suum infections occurred in all subgroups (Table 3). Specifically, in SUB1, the prevalence was initially lower for the FRS group in week 7 but was higher in week 12 than in the control group (Figure 2). In contrast, the FRS groups in SUB2-4 consistently had a lower prevalence than the control groups at all time points. This was also reflected in lower incidence levels in FRS groups compared to controls in SUB2-4 (Table 3), although the difference was not statistically significant (p = 0.19). With respect to the accumulated A. suum FEC, calculated as the sum of egg excretion from each individual animal across sample days, the FRS groups in all four sub-studies excreted fewer A. suum eggs overall compared to the control groups; however, the difference was not statistically significant. The outcome was similar (P = 0.16) when combining SUB2-4, even though the total accumulated egg excretion by FR's pigs was 45.3% lower than that of control pigs (Figure 3). In SUB1, the total accumulated egg excretion was 29.1% lower in FRS-fed animals. The linear model identified that SUB2-4 FECs at weeks 9 and 11 were significantly influenced by sex (Figure 4, Supplementary Table 2), with diet trending toward significance in weeks 9 and 11 (P = 0.08and P = 0.10, respectively). However, FEC in SUB1 was not affected





TABLE 3 Incidence, i.e., new infections picked up during the whole study, and fecal egg excretion accumulated over sample days (Acc. FEC) of parasites of individual groups and for sub-study 2–4 combined (figures in bold).

Sub-study	Group	Asca	ris suum	Oesophag	<i>Eimeria</i> spp.	
		Incidence	Acc. FEC (epg)	Incidence	Acc. FEC (epg)	Incidence
SUB1	C1	20.8%	19,860	29.2%	220 ^a	100.0%
	S1	33.3%	14,090	8.3%	30 ^b	100.0%
SUB2	C2	40.0%	44,150	24.0%	8,150	100.0%
	S2	25.0%	16,040	12.5%	50	100.0%
SUB3	C3	34.8%	45,485	8.7%	20	100.0%
	\$3	25.0%	22,780	0.0%	0	83.3%
SUB4	C4	36.0%	63,580	0.0%	0	60.0% ^a
	S4	30.4%	44,931	0.0%	0	21.7% ^c
SUB2-4	C2-4	37.0%	153,215	11.0%	8,170	86.3 % ^a
	\$2-4	26.8%	83,751	4.2%	50	69.0% ^c

Different superscripts (^{a,b,c}) indicate significant differences (a-b: P = 0.05, a-c: P = 0.01) between control and FRS groups within the same sub-study.

by either diet or sex (P > 0.15) despite lower excretion in females compared to males (Figure 4).

Oesophagostomum spp. egg excretion was not detected in SUB3 and SUB4, except for two animals with low egg counts (10 EPG) in group C3, week 9, and the incidence rates were generally low (Table 3). No significant difference in incidence rates between diet groups was thus found within SUB1 (P = 0.14) or SUB2–4 (P = 0.13). The total accumulated FEC was significantly lower in the FRS group than in the control group in SUB1 (P = 0.05) and showed a

trend of being lower in the FRS group than in the control group in SUB2 (non-significant). However, the higher accumulated FEC count in the C2 group compared to the S2 group was likely due to an outlier—one pig that exhibited a high FEC of 6,600 EPG at week 0.

Eimeria spp. were detected in pigs from all groups after arrival (Table 3), with incidences much higher in SUB1–3 than SUB4 for both diet groups. The incidence was significantly lower for FRS pigs compared to controls for SUB2–4 (P = 0.01), which likely reflects





a large difference in incidence within SUB4 (Table 3). Only a few oocysts were present in positive pigs in SUB4.

In general, we found that FRS inclusion significantly reduced or showed tendencies of reduced incidence of infection for all three parasites and tendencies of reduced FEC and prevalence each week for both *A. suum* and *Oesophagostomum* spp. However, we again saw a batch difference in that FRS batch 1 in SUB1 increased *A. suum* incidence and prevalence.

3.3 High anti-*A. suum* antibody levels in all groups (SUB1 and SUB2)

All animals were highly positive for specific antibodies against both adult and larval *A. suum* in these sub-studies, even pigs not excreting *A. suum* eggs. Thus, there was no correlation between ODr values and FEC for SUB1 or SUB2. There were also no significant differences in As-Hb OD values (Supplementary Figure 1). However, S1 had significantly lower ODr values for antibodies against larval antigens compared to C1. Furthermore, no significant differences were found in the levels of IL-6 and TNF- α (data not shown). Serum samples from SUB3 and SUB4 were not analyzed.

3.4 FRS but not parasite infection levels modulate gut microbiota diversity and composition

The microbial community richness and diversity were determined in SUB1 and SUB2–4 based on two alpha diversity indices, i.e., observed features and the Shannon index (Figure 5). Before starting the diet intervention, baseline alpha diversity indices did not significantly differ between C- and S-groups at week 0 for SUB1 and SUB2–4 (P > 0.05). In SUB1, feeding the control diet did not result in any significant changes in alpha diversity indices over time (P > 0.05), while we observed a significant increase in observed features (P = 0.0002), and the Shannon index (P = 0.004) over time after FRS diet feeding. In SUB2–4, we observed increases in both alpha diversity indices over time for pigs fed both control feed and the FRS diet (P < 0.01). In addition, we examined the FRS intervention effect by comparing



was flagged on the plots if P > 0.05.

C and S groups at week 12 or 11. We found that FRS intervention increased the number of observed features in SUB1 (P = 0.02), both observed features (P = 0.002) and the Shannon index in SUB2-4 (P = 0.0008).

Subsequently, Bray-Curtis dissimilarity metrics (Figures 6A, C) and Jaccard (Figures 6B, D) distances were determined to investigate the influence of time, feed, and parasites on the overall GM composition. Bray-Curtis dissimilarity metrics showed, as expected, no clustering according to diet at baseline (P >0.05). On both diets, the GM significantly changed over time (P = 0.006 for both Bray-Curtis and Jaccard). However, the FRS supplementation had different effects in SUB1 and SUB2-4. We observed a significant FRS intervention effect based on Jaccard in SUB1 (P = 0.022) (Figure 6E), whereas this effect was also significant in SUB2-4, both based on Bray-Curtis and Jaccard (P = 0.006) (Figure 6F).

We next analyzed beta diversity in C and S groups at week 12 or 11, according to their infection status. We found that infection affected only the GM composition within the different feeding groups based only on Jaccard distances in SUB1 (C1-w12infection vs. C1-w12-no-infection; P = 0.05) and SUB2-4 (C2-4w11-infection vs. C2-4-w11-no-infection and S2-4-w11-infection vs. S2-4-w11-no-infection; P = 0.02).

To assess bacterial GM composition, we initially analyzed the phyla with a median relative abundance >1% in each group of SUB1 and SUB2-4 (Figure 7). We found Firmicutes and Bacteroidetes to be the most abundant phyla in all groups, with Tenericutes also detected in most pigs. Moreover, the phylum Actinobacteria was present in SUB2-4 but not in SUB1.

We finally assessed differentially abundant bacteria by performing DESeq2 analysis at the species level. Prior to diet intervention, only one bacterium significantly differed in the baseline GM of pigs in SUB1 (Supplementary Figure 2), and there was no significant difference in baseline in SUB2-4. In line with the observations above, DESeq2 analysis also showed that the effect of including FRS in the feed differed between SUB1 and SUB2-4. Only Bifidobacterium thermacidophilum was more abundant in the C-group than the S-group at week 12 in SUB1 (Supplementary Figure 3A). But, in SUB2-4, 18 bacterial species significantly differed in abundance by week 11 (Supplementary Figure 3B). In SUB2-4, FRS led to increased relative abundance of a range of Bacteroidetes members, e.g., Prevotella and S24-7, while the unsupplemented pigs were enriched in various Firmicutes, such as Clostridium and Turicibacter spp.

In general, we found that the FRS supplementation in SUB2-4 (batch 2) induced major alterations to the GM compared to



FIGURE 6

Development in gut microbiota composition of pigs fed with FRS supplemented diet and control diet. Principal coordinates analysis (PCoA) based on (A, C) Bray-Curtis dissimilarity (left) and (B, D) Jaccard distances (right) were performed for SUB1 (upper panel) and SUB2 (lower panel). Each data point on PCoA plots indicates samples that are depicted based on diet, FEC > 200, and time. Ellipses denote a 95% confidence interval, and the percentage in brackets is the percentage of variation explained by each PCoA axis. Statistical pairwise group comparisons for beta diversity using permutation MANOVAs on a distance matrix with *P*-value correction using the Holm method for (E) SUB1 and (F) SUB2-4. P < 0.05 was considered as significant.



Phylum-level relative abundance of gut microbiota in pigs fed with FRS supplemented diet (S1 and S2-4) and control diet (C1 and C2-4). Stacked bar plots show the phyla with a median relative abundance above 1% for (A) SUB1 at weeks 0 and 12 and (B) SUB2-4 at weeks 0 and 11. "<1%" represents the phyla in each group with a median relative abundance below 1%.

controls, contrasting with the more moderate changes in SUB1 with the FRS from batch 1.

4 Discussion

This large on-farm study, which investigated the effects of 2% FRS dietary inclusion on naturally acquired parasite infections and GM composition, showed a clear tendency of reduced incidence of infection of parasites in SUB2-4 using the FRS batch 2. The pigs were primarily infected with A. suum, and all sampled pigs (both control and FRS-fed pigs) were highly positive for antibodies against A. suum, indicating a high level of exposure on all pastures in SUB1 and SUB2. Furthermore, we found increased diversity in the microbiota of FRS-fed pigs compared to control-fed pigs; however, we also observed a decrease in daily weight gain (DWG). As this was a field study and pasture infectivity levels were not quantified, we cannot exclude the possibility that varying infection pressures at the start may have influenced the results. However, we consider this unlikely, as paddocks were randomly allocated to the different dietary treatments, and the study was replicated across multiple substudies.

The decreased incidence and reduced FEC indicate that FRS may contain anthelmintic phytochemicals. We previously found that S. latissima extracts killed >90% of third-stage A. suum larvae in vitro (29), supporting the idea that bioactive compounds in brown seaweed may contribute to the observed antiparasitic effects in this study. Thus, we observed tendencies toward reduced accumulated FEC and incidences of A. suum (SUB2-4) and Oesophagostomum spp. (SUB1 and SUB2), and Eimeria spp. (SUB3 and SUB4), suggesting that inclusion of FRS in the diet may exert an anti-parasitic effect, potentially by affecting parasite establishment and/or fecundity. In addition to direct antiparasitic activity, FRS may also improve gut barrier integrity and mucosal immune responses, thereby supporting host defense against infection (40, 65). Further studies will be necessary to elucidate these potential mechanisms of action. Moreover, due to the generally low prevalence and infection levels observed, it remains inconclusive whether FRS had a definitive impact on Oesophagostomum spp. infections.

The general declining incidence levels observed for Oesophagostomum spp. and Eimeria spp. from SUB1 to SUB4 during winter are most likely due to weather conditions since SUB3 and SUB4 were introduced in February 2021 (3rd and 17th), which was the coldest month recorded during this study. Eggs and free-living larvae of Oesophagostomum spp. are considered very susceptible to extreme weather conditions (4, 5). A reduction in sporulation has been reported for the porcine E. debliecki and E. scabra exposed to freezing, thawing, and continuous freezing (66). Moreover, freezing has been found to inactivate chicken Eimeria spp. (67) and reduce bovine Eimeria spp. oocysts when kept at -18° C (68). In contrast, the transmission of A. suum appeared unaffected by weather conditions during this study, as judged by accumulated FEC over the sub-studies. A. suum eggs are very resistant to environmental conditions (2), especially when unembryonated, and can survive freezing temperatures down to -27° C for 10 days when containing embryos, although freezing usually diminishes infectivity (69). Due to low temperatures during the study, it is unlikely that any embryonation of excreted eggs took place in the paddocks.

All tested pigs were positive for specific *A. suum* serum antibodies at the end of the study, even though they did not all have patent infections. This indicates that all pigs were exposed to the parasite on the pastures, but that acquired immunity resulted in the expulsion of the parasites before the infections reached patency (70). Additionally, some low infection levels may have remained undetected. Studies have shown that worm burdens are dose-independent and highly variable, as 10% of pigs may harbor 80% of the burden (71).

The seroprevalence of *A. suum* infections is normally found to be higher than coproprevalence (49, 72), as in our study with 100% seroprevalence. However, our results question whether these serology tests can overestimate infection prevalence within a farm since the antibody response only indicates exposure level and not individual patent infections (72). A positive correlation between FEC and anti-AsHb-IgG antibodies has been established (49), as well as in naturally infected weaners and fatteners (72). However, this was not present in our study, and other studies found no correlation between ELISA OD levels and worm burdens in naturally infected pigs (73).

Prebiotics have also been recognized to have a significant impact on helminth infections (38), as observed for *Oesophagostomum* spp. repeatedly (37) and *Trichuris suis* occasionally (74). The effects of FEC and transmission could be due to interactions with the microbiota or a direct effect by fermentation products (75) since both BS and rapeseed meal have been shown to contain compounds with prebiotic properties (76–78).

The fecal microbiota analysis showed overall large changes during the 11-12 weeks of the two sub-studies, which is in agreement with previous observations (79). The inclusion of FRS in the feed in our study influenced GM composition in all sub-studies, but it had an overall stronger effect in SUB2-4. This effect of FRS to modulate the pig GM is also in agreement with previous studies (40, 45), including our previous study with pigs co-infected with A. suum and Oesophagostomum (31). FRS contains various compounds influencing microbiota, such as polysaccharides, glycosinolates, and other bioactives. The difference in the effects on the microbiota of FRS diet inclusion in SUB1 and SUB2-4 indicates the batches were not completely similar. Fermentation is a complex biological process, and batch-tobatch variation occurs randomly or if variables such as temperature, pressure, pH, dissolved oxygen, and feed flow are not thoroughly controlled (80).

While the effect of FRS inclusion on specific GM members (as determined by DESeq2 analysis) was subtle in SUB1, the effect was considerably stronger in SUB2–4, where FRS was associated with increased relative abundance of mainly *Bacteroidetes* members such as *Prevotella* spp. Members of this genus are potent degraders of various undigestible carbohydrates and may thereby stimulate the ability to extract energy from feed components (79). Notably, *Prevotella* abundance has consistently been shown to increase in response to supplementation with seaweed-derived polysaccharides in pigs (81), calves (82), and bulls (83). *Prevotella* spp. have also been associated with increased fat accumulation in Duroc pigs; however, the implications of this relationship for gut

health in pigs remain unclear (84). In contrast to this study, Bonde et al. (31) showed no overall change in *Bacteroidetes* but a decrease in specifically *Prevotella copri* in both infected and uninfected animals fed a substantially higher level of fermented *S. latissima* (8% dry weight).

In one sub-study (SUB4), the FRS diet had a significant negative impact on weight gain. The FRS feed had a slightly lower metabolizable energy (-0.5%) than the control feed, but probably not enough to explain the differences in weight gain (-3%). A negative effect of FRS on DWG may be related to the effects on microbiota, lower palatability, or anti-nutritional factors in both the rapeseed (41) and BS (40). Rapeseed has a high content of condensed tannins (6% in rapeseed hulls) (85). Fermentation of FR's supplement has been applied to mitigate antinutritional effects and has been shown to generally increase the weight performance in growing finishers (44, 46, 47). However, as tannins may form complexes with macromolecules such as proteins (86), they may reduce the overall digestibility of the feed (87). In our case, fermentation may not have been sufficient to eliminate all anti-nutritional factors. Furthermore, tannin-protein complexes have been coupled to a bitter taste (88), which could have reduced the feed intake and, thus, the weight gain of the pigs. It also remains speculative whether rapeseed or seaweed is responsible for antinutritional effects. The inclusion of seaweeds (S. latissima and A. nodosum) in the supplement to weaners indicated that the seaweed inclusion may have imposed a negative effect on weight gain compared to weaners fed fermented rapeseed alone (45). This may be partly caused by the high content of fermentable/indigestible polysaccharides of BS (34, 89) since growing finishers have a fully developed hindgut around the end-time of the fattening period (79), but in weaner piglets their hindgut is not yet well developed for fermentation (90).

That castrated male pigs grow better than female pigs in our study has previously been seen for growing finishers, with males also having a higher daily feed intake (91, 92). However, in other studies, the growth between male and female finishers was similar, though males did have a significantly better feed conversion ratio than females (93, 94). The current higher DWG in males may explain part of the observed higher egg excretion in males, as larger animals generally are considered to have a higher ingestion of parasite eggs and a larger intestinal surface area to host them (95). However, hormonal differences cannot be ruled out (96).

5 Conclusion

Trends in *A. suum* infection dynamics indicated that the inclusion of a 2% FRS supplement in the basal diet had a moderate effect, reducing egg excretion by 45% and lowering the number of new infection cases. This indicates that the FRS product may, depending on the batch, contain anthelmintic phytochemicals capable of reducing nematode infection levels. The 2% FRS supplement also modulated GM composition, notably increasing the relative abundance of *Prevotella* spp., but was associated with a slight reduction in weight gain. Thus, while the observed modulation of GM composition supports the potential use of FRS as a prebiotic dietary supplement, a deeper understanding of the mechanisms underlying its effects on gut function is

needed to refine this strategy. Such refinement is essential to ensure health benefits while optimizing productivity. Furthermore, standardization of the production process to achieve consistent bioactivity across FRS batches will likely be crucial to realizing its full potential in future applications.

Data availability statement

The datasets presented in this study can be found in online repositories. The names of the repository/repositories and accession number(s) can be found at: https://www.ncbi.nlm.nih. gov/, PRJNA854963.

Ethics statement

The animal studies were approved by Danish Animal Experiments Inspectorate (national body) License number 2015-15-0201-00760. The studies were conducted in accordance with the local legislation and institutional requirements. Written informed consent was obtained from the owners for the participation of their animals in this study.

Author contributions

CB: Conceptualization, Data curation, Formal analysis, Investigation, Methodology, Visualization, Writing - original draft. FD: Data curation, Formal analysis, Investigation, Writing original draft. NB: Data curation, Formal analysis, Investigation, Writing - original draft. LK: Data curation, Formal analysis, Writing - review & editing. DN: Data curation, Formal analysis, Writing - review & editing. HP: Investigation, Methodology, Writing - review & editing. RM: Conceptualization, Methodology, Resources, Writing - review & editing. NP: Conceptualization, Resources, Writing - review & editing. PG: Investigation, Methodology, Writing - review & editing. AW: Conceptualization, Funding acquisition, Investigation, Supervision, Writing review & editing. HM: Conceptualization, Funding acquisition, Investigation, Supervision, Writing - review & editing. ST: Conceptualization, Funding acquisition, Investigation, Project administration, Supervision, Writing - review & editing.

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Conflict of interest

The study was performed in collaboration with a commercial project partner, Fermentationexperts A/S, using their commercially available product EP1199. NP and RM are employees of Fermentationexperts A/S.

The remaining authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

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Supplementary material

The Supplementary Material for this article can be found online at: https://www.frontiersin.org/articles/10.3389/fvets.2025. 1565686/full#supplementary-material

SUPPLEMENTARY TABLE 1

UMI containing multiple forward and reverse primers used in this study.

SUPPLEMENTARY TABLE 2

P-values for the final model of fecal egg count (FEC), based on logarithmic transformation for weeks 9 and 12 or 11. Includes Sub-study 1 (SUB1) and combined Sub-studies 2–4 (SUB2–4). P-values are from the final model, whereas "Excl." refers to factors excluded from the final model (>0.15).

SUPPLEMENTARY FIGURE 1

Mean (+ SD) optical density ratio (ODr) as measurement of serum IgG antibodies against adult (As-Hb) and larvae (L3 lung) Ascaris suum in pigs from for (A) sub-study 1, group C1 and S1; (B) Response when divided into FEC positive or FEC negative based on FEC >200 for sub-study 1; and (C) sub-study 2, group C2 and S2; (D) Response when divided into FEC positive or FEC negative based on EPG >200 for sub-study 2 (****P < 0.0001). The stippled line represents the cut-off value for As-Hb (0.50) and L3 lung test (0.25).

SUPPLEMENTARY FIGURE 2

Heatmap of bacteria found to be significantly differently abundant by DESeq2 analysis for SUB1 at week 0. The heatmap shows the comparison of control-fed (C) and FRS-fed (S) groups. Significantly different bacteria (Adjusted *P*-value < 0.05) at the species level are demonstrated in the heatmap.

SUPPLEMENTARY FIGURE 3

Heatmap of bacteria found to be significantly differently abundant by DESeq2 analysis for (A) SUB1 at week 12 and (B) SUB2–4 at week 11. Heatmap shows the comparison of control-fed (C) and FRS-fed (S) groups for (A) SUB1 and (B) SUB2–4. Significantly different bacteria (Adjusted *P*-value < 0.05) at the species level are demonstrated in heatmaps.

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