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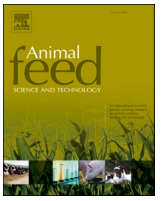

Comparing a seaweed blend to pharmacological levels of zinc oxide in weaner pig diets: The benefit to pig performance and inflammatory response

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ABSTRACT

After the ban of pharmacological zinc oxide (ZnO) in EU pig diets, alternative ways to improve health and growth performance of pigs after weaning are being sought. Seaweed blends have been an area of interest given their potential prebiotic effects. This work compared two levels of a seaweed blend, added to a control diet and a diet with pharmacological levels of ZnO, on feed intake, growth, feed efficiency and the inflammatory response. A total of 240 pigs ((Large White x Landrace) x Danish Duroc) were weaned (8.5 ± 0.31 kg) into pens of five pigs per pen and allocated to one of four dietary treatments. Across treatment groups, pens of pigs were balanced for the sex ratio within a pen, a pig's litter of origin and weight at weaning. Pens of pigs were fed one of four diets: 1) positive control (PC) - standard diet with 3.1 g/kg ZnO; 2) negative control (NC) - standard diet without ZnO; 3) NC+5 g/kg seaweed blend (SWB); 4) NC+10 g/kg SWB, across three phases: days 0–7, 7–20 and 20–42 after weaning. Feed refusals per pen and individual pig weights were recorded on days 7, 20 and 42 to determine average daily gain (ADG), average daily feed intake (ADFI) and gain:feed (G:F) per phase. On day 20, six pigs per treatment were euthanised and dissected. Peripheral plasma samples were collected for ELISA analysis of interleukin-6 (IL-6), IL-1β and IL-10 and the ileal mucosa was scraped for qPCR analysis of relative mRNA quantification of IL-6, IL-1β and tumour necrosis factor-alpha (TNF-α). During the first seven days after weaning, PC fed pigs tended to have higher ADG than other treatments ($P<0.10$) and higher G:F compared to NC (0.888 vs. 0.764, $P<0.001$, NC+5 g/kg SWB (0.888 vs. 0.753, $P=0.019$) and NC+10 g/kg SWB (0.888 vs. 0.762, $P=0.034$). Between 8 and 20 days, PC fed pigs tended to have higher ADFI than the NC and the NC+10 g/kg SWB ($P<0.10$). Average daily gain between 8 and 20 days was not different for PC and NC+5 g/kg SWB pigs (0.368 and 0.308 kg/day) and G:F was higher for PC compared to both NC (0.830 vs. 0.721, $P=0.021$) and NC+10 g/kg SWB pigs (0.830 vs. 0.702, $P=0.005$). Between 21 and 42 days, there were no differences in ADG or ADFI. Gain:feed for PC (0.755) and NC+5 g/kg SWB (0.768) fed pigs were not different but were lower than NC (0.804, $P=0.002$ and $P=0.038$, respectively). There were no

Abbreviations: ZnO, zinc oxide; PC, positive control; NC, negative control; SWB, seaweed blend; ADG, average daily gain; ADFI, average daily feed intake; G:F, gain:feed; IL, interleukin; TNF-α, tumour necrosis factor-alpha; ETEC, enterotoxigenic *E. coli*; PWD, post-weaning diarrhoea; GIT, gastrointestinal tract.

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differences in mRNA levels of IL-6, IL-1β or TNF-α in the ileum. In plasma, PC and NC+5 g/kg SWB tended to have lower IL-6 concentrations compared to NC and NC+10 g/kg SWB (1.7 and 1.1 vs. 4.7 and 5.0 pg/ml; P<0.10). Given the intermediate performance of pigs fed 5 g/kg inclusion of SWB it could be beneficial to investigate this inclusion level further, or lower inclusion levels, given 10 g/kg did not show any beneficial effect.

1. Introduction

The weaning process in pig production is considered one of the most stressful events in a pig's lifetime and is characterised by intestinal and immune dysfunctions as a result of abrupt changes in their environment and feed format (Pluske et al., 2003; Campbell et al., 2013). These changes can compromise the pig's feed intake, growth, and health, often due to an increase in pathogenic, enterotoxigenic *E. coli* (ETEC), one of the major causes of post-weaning diarrhoea (PWD) in pigs (Pluske, 2013). Until recently, the widespread use of pharmacological levels of zinc oxide (ZnO) has been common practice, since the ban on antibiotic growth promotors in 2006, as a way of improving feed intake and growth performance of pigs immediately after weaning (Hill et al., 2001; Stensland et al., 2015; Hazelden, 2021) as well as reducing the incidence of PWD (Højberg, et al., 2005). However, the recent EU ban on these levels of ZnO arose due to antibiotic resistance and environmental concerns, with increasing concentrations of Zn in soil and groundwater that could affect soil organisms, lead to toxic concentrations in aquatic environments or both (Monteiro et al., 2019). As a result of the ban on pharmacological levels of ZnO, there has been a focus on identifying natural alternatives to prevent PWD and the reduction in growth performance frequently seen immediately after weaning, in its absence.

One such alternative that is receiving increased attention is the use of seaweed extracts and blends (Sweeney and O'doherty, 2016: Vigors et al., 2021). Seaweeds are rich in indigestible polysaccharides and are a potential source of soluble dietary fibres, with laminarin, fucoidan and alginic acid being the most abundant polysaccharides in brown seaweeds (Zvyagintseva et al., 2003; O'Doherty et al., 2010). Polysaccharides and oligosaccharides originating from seaweeds are reported to regulate intestinal metabolism and fermentation (Lean et al., 2015), enhance growth performance of pigs and reduce enteric *Enterobacteriaceae* numbers (Reilly et al., 2008; Gahan et al., 2009). Several seaweed polysaccharides have also shown benefits including anti-inflammatory, antiviral and antioxidant activities (Lopez-Santamarina et al., 2020).

Following a lipopolysaccharide (LPS) challenge to illicit an immune response, piglets that suckled from sows that had received a gestation and lactation diet containing laminarin (1 g/d) and fucoidans (0.8 g/d), had reduced gene expression of the inflammatory cytokines interleukin (IL)-6, IL-8 and IL-10 in the ileum at weaning, compared to piglets that had suckled from sows receiving a basal diet (Heim et al., 2015). The cytokines IL-6 and IL-8 have been associated with a response to ETEC, with McLamb et al. (2013) demonstrating that ETEC challenge induced an innate immune response, with the increase of IL-6 and IL-8. It could therefore be expected that their down-regulation, or the down-regulation of other pro-inflammatory cytokines improves health. Although many report health and performance benefits of including seaweed extracts in the diet of pigs, there can be variations in the polysaccharide yield depending on the species of seaweed, growing conditions and extraction methods (Bilan et al., 2006). Ocean Harvest Technology have created Oceanfeed™ Swine by drying and blending a selected mix of brown, red and green seaweeds (Ocean Harvest Technology, Galway, Ireland). Previously, a shift in the microbiota of piglets reared from sows fed OceanFeed™ Swine has been observed with a higher number of species detected from the *Ruminococcaceae* and *Lachnospiraceae* families, that are generally considered beneficial, as well as a reduction in *Fusobeteriaceae*, which are typically considered to be pathogenic (Del Tuffo et al., 2019). Although Del Tuffo et al. (2019) did not find growth performance benefits to nursery pigs when their sow had received OceanFeed™ Swine, there was an interaction for pigs weaned from OceanFeed™ Swine fed sows and then fed OceanFeed™ Swine themselves, having firmer faecal scores compared to those not receiving OceanFeed™ Swine at all. Given the potential health benefit, the aim of this research was to determine whether including a seaweed blend (SWB; OceanFeed™ Swine, Ocean Harvest Technology, Galway, Ireland), at two different inclusion levels, directly to pigs after weaning could improve growth performance and reduce inflammatory responses.

2. Materials and methods

The experimental procedures were carried out at the University of Leeds, National Pig Centre. The protocol used for the experiment was reviewed and approved by the University of Leeds Animal Welfare and Ethical Review Body.

2.1. Animals, experimental design and diets

A total of 240 pigs ((Large White X Landrace) x Danish Duroc) were weighed at weaning (~28 days of age) and allocated to a 42-day trial. Before weaning, all pigs were ear tagged for individual identification. At weaning, all pigs were allocated to pens (5 pigs per pen) and pens were randomly assigned to one of four dietary treatments, creating 12 replicate pens per treatment. Across the treatment groups, pens of pigs were balanced for sex ratio within a pen, litter of origin and their live weight at weaning. Pigs were housed in fully slatted accommodation throughout the trial. Pens were allocated one of four dietary treatments: Positive control (PC): standard nursery diet, including 3.1 g/kg ZnO; Negative control (NC): Standard nursery diet without pharmacological levels of ZnO; NC plus SWB at 5 g/kg; NC plus SWB at 10 g/kg. All diets were formulated to meet or exceed the requirements for the pigs after weaning (NRC, 2012). Diets were fed in three phases: stage 1 diets were fed from days 0–7, stage 2 diets were fed between 7 and 20 days after weaning

Table 1
Ingredient content and calculated energy and nutrient contents of stage 1, stage 2 and stage 3 of the positive control (PC), negative control (NC) and 2 seaweed blend (SWB) inclusions.

	STAGE 1 DIETS				STAGE 2 DIETS				STAGE 3 DIETS			
Ingredients (g/kg)	PC	NC	NC+5 g/ kg SWB	NC+10 g/ kg SWB	PC	NC	NC+5 g/ kg SWB	NC+10 g/ kg SWB	PC	NC	NC+5 g/ kg SWB	NC+10 g/ kg SWB
Micronised barley	100.0	100.0	100.0	100.0	150.0	150.0	150.0	150.0	150.0	150.0	150.0	150.0
Raw wheat	199.2	199.2	191.7	184.3	373.9	373.9	366.5	359.1	484.8	484.8	477.3	470.0
Micronised wheat	100.0	100.0	100.0	100.0	50.0	50.0	50.0	50.0	0.0	0.0	0.0	0.0
Wheat	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	10.3	10.3	10.3	10.3
Micronised oats	100.0	100.0	100.0	100.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0
Fishmeal	72.5	72.5	72.5	72.5	57.7	57.7	57.7	57.7	0.0	0.0	0.0	0.0
Hipro soya	190.0	190.0	190.0	190.0	240.0	240.0	240.0	240.0	260.0	260.0	260.0	260.0
Vitamin & Mineral premix[a]	5.0	5.0	5.0	5.0	5.0	5.0	5.0	5.0	2.5	2.5	2.5	2.5
Dried skimmed milk	40.0	40.0	40.0	40.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0
Whey powder	114.1	114.1	114.1	114.1	72.5	72.5	72.5	72.5	36.2	36.2	36.2	36.2
L-Lysine-HCL	3.1	3.1	3.1	3.2	2.4	2.4	2.4	2.4	4.8	4.8	4.8	4.9
L-Methionine	1.9	1.9	1.9	1.9	1.2	1.2	1.2	1.2	1.9	1.9	1.9	1.9
L-Threonine	1.9	1.9	1.9	1.9	1.2	1.2	1.2	1.2	2.2	2.2	2.2	2.2
L-Tryptophan	0.3	0.3	0.3	0.3	0.0	0.0	0.0	0.0	0.1	0.1	0.1	0.1
L-Valine	0.7	0.7	0.7	0.7	0.0	0.0	0.0	0.0	1.0	1.0	1.0	1.0
Vitamin E	0.2	0.2	0.2	0.2	0.1	0.1	0.1	0.1	0.3	0.3	0.3	0.3
Phytase	0.1	0.1	0.1	0.1	0.1	0.1	0.1	0.1	0.1	0.1	0.1	0.1
Flavour	0.2	0.2	0.2	0.2	0.2	0.2	0.2	0.2	0.2	0.2	0.2	0.2
Sweetener	0.1	0.1	0.1	0.1	0.1	0.1	0.1	0.1	0.1	0.1	0.1	0.1
Seaweed[b]	0.0	0.0	5.0	10.0	0.0	0.0	5.0	10.0	0.0	0.0	5.0	10.0
Benzoic acid	5.0	5.0	5.0	5.0	5.0	5.0	5.0	5.0	5.0	5.0	5.0	5.0
Copper sulphate	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.3	0.3	0.3	0.3
Dicalcium phosphate	8.4	8.4	8.4	8.4	9.7	9.7	9.7	9.7	19.5	19.5	19.5	19.5
Salt	0.0	0.0	0.0	0.0	1.6	1.6	1.6	1.6	4.1	4.1	4.1	4.1
Soya oil	57.4	57.4	59.9	62.2	29.4	29.4	31.8	34.1	16.8	16.8	19.3	21.5
Energy and nutrient content[c] (g/Kg, unless otherwise stated)												
Dry Matter	909.0	909.0	905.0	900.9	896.5	896.5	892.4	888.3	889.3	889.3	885.3	881.2
NE (MJ/kg)	10.81	10.81	10.81	10.81	9.90	9.90	9.90	9.90	9.45	9.45	9.45	9.45
Crude Protein	224.8	224.8	223.9	223.2	224.1	224.1	223.3	222.5	203.6	203.6	202.8	202.0
Crude Fibre	18.7	18.7	18.5	18.3	22.6	22.6	22.4	22.3	26.2	26.2	26.0	25.9
Ash	55.0	55.0	54.9	54.8	52.3	52.3	52.2	52.2	51.6	51.6	51.5	51.5
Calcium	8.7	8.7	8.7	8.7	7.8	7.8	7.8	7.8	7.2	7.2	7.2	7.2
Digestible phosphorus	4.5	4.5	4.5	4.5	4.0	4.0	4.0	4.0	4.1	4.1	4.1	4.1
Zinc (mg/kg)	136.32[d]	136.32	136.17	136.02	136.37[d]	136.37	136.22	136.07	0.83	0.83	0.83	0.82
SID Lysine	14.1	14.1	14.1	14.1	12.7	12.7	12.7	12.7	12.4	12.4	12.4	12.4
SID Methionine	5.6	5.6	5.6	5.6	4.6	4.6	4.6	4.6	4.5	4.5	4.5	4.5
Total Lysine	15.4	15.4	15.4	15.5	14.1	14.1	14.1	14.1	13.5	13.5	13.5	13.6
Total Methionine	6.0	6.0	6.0	6.0	5.0	5.0	5.0	5.0	4.8	4.8	4.8	4.8
Ash (g/kg, analysed)	61.0	53.0	49.0	57.0	56.0	51.0	58.0	56.0	56.0	55.0	57.0	58.0
Crude Fibre (g/kg, analysed)	18.0	18.0	19.0	19.0	21.0	24.0	23.0	21.0	25.0	24.0	23.0	22.0
Crude Protein (g/kg, analysed)	205.0	224.0	211.0	195.0	208.0	216.0	212.0	199.0	190.0	189.0	179.0	174.0
Moisture (g/kg, analysed)	101.0	99.0	97.0	100.0	100.0	102.0	101.0	100.0	106.0	108.0	111.0	107.0
Oil (g/kg, analysed)	57.1	68.6	73.0	74.4	54.3	55.9	58.1	57.3	37.3	40.4	37.1	42.5

[a] Vitamin and mineral mix (per kg) contained: Vitamin A = 13750 IU in stage 1 and 2 and 10550 IU in stage 3, Vitamin D_3 2100 IU in stage 1 and 2 and 2250 IU in stage 3, Vitamin E 250 IU in stage 1 and 200 IU in stage 2 and 3, Na = 2 g in all stages, Zn ($ZnSO_4$) = 136 mg in stage 1 and 2 and 0.8 mg in stage 3, Cu ($CuSO_4$) = 145 mg in stage 1 and 2 and 128 mg in stage 3, Se (sodium selenite) = 0.42 mg in stage 1, 0.39 mg in stage 2 and 96.20 mg in stage 3.

[b] Composition: Moisture 150 g/kg, crude fat 3 g/kg, crude ash 320 g/kg, crude protein 90 g/kg, carbohydrates 450 g/kg, crude fibre 45 g/kg, total polysaccharides 380 g/kg.

[c] Calculated content unless otherwise stated, analysis conducted by Sciantec Analytical Services Ltd. Stockbridge Technology Centre, UK.

[d] Additional 2500 ppm added

and stage 3 diets were fed between days 20–42 after weaning (Table 1). Pharmacological levels of ZnO were removed from the diet after day 20, but the SWB remained in the feed at the same inclusion levels throughout the 42-day trial, the composition of these diets are shown in Table 1. Diets were analysed based on Association of Official Analytical Chemists (AOAC) methods by Sciantec Analytical Services Ltd. (UK). Pigs had free access to feed and water throughout the trial.

Pigs were individually weighed at weaning (day 0), day 7, day 20 and day 42 after weaning, to determine average daily gain (ADG, g/d). Feed refusals were measured at the same time points to determine pen average daily feed intake (ADFI, g/d). Average daily gain and ADFI were used to determine the pen average gain:feed (G:F) for each dietary period and overall. Pig health was recorded based on the number of pigs within the pen showing signs of poor health, which included but was not limited to diarrhoea, ear infections, lameness, or respiratory problems. Poor health was typically noted if a pig required medication. Faecal scores were recorded (1 – firm; 2 – soft and spreads easily; 3 – very soft, mild diarrhoea; 4 – watery liquid, diarrhoea) and cleanliness scores (1 – clean; 2 – some indication of faecal contamination; 3 – contamination with faecal material; 4 – heavily contaminated with faecal material) were determined by the same personnel each morning throughout the entire experiment. The number of deaths, pigs medicated or removed from the trial were also recorded throughout.

2.2. Sample collection

On day 20 after weaning, six pigs per treatment were euthanised under Schedule 1 of the Animals (Scientific Procedures) Act 1986, followed by collection of blood and removal of the gastrointestinal tract (GIT) for sample collection. Pigs were selected for euthanasia by randomly choosing 6 of the 12 replicates. Within each replicate, one pig from each pen was selected based on their performance being as close to the mean for that treatment replicate as possible. Immediately following euthanasia, blood plasma samples were collected from peripheral circulations into heparinised tubes. Samples were centrifuged at 1000 *x g* at 4°C for 20 minutes and aliquots of the supernatant were frozen at −80°C until analysis, to determine the concentration of IL-1β, IL-6, IL-10 and TNF-α as indicators of an inflammatory response. A 10-cm section of the terminal ileum (determined as 65 cm prior to the ileocecal valve, with the most terminal 25 cm discarded) was cut longitudinally, washed with PBS and scraped by microscope slide into Trizol™, snap-frozen in liquid nitrogen, and stored at −80°C for the determination of IL-1β, IL-6 and TNF-α gene expression within the ileum.

2.3. Quantitative PCR to measure inflammatory cytokines in the ileum

Of the ileal samples collected, ~0.025 mg was homogenised with 0.7 ml of 2 mm lysis beads by bead beating for 60 s at 50 rps (Tissue Lyser, Qiagen). Particulates were removed by centrifugation at 12,000 *x g* for 1 min and 500 µl of the supernatant was mixed with 600 µl 99% ethanol. Total RNA was then isolated using the Direct-zol™ RNA Miniprep with Zymo-Spin™ ICC Columns (Cambridge Bioscience, UK), following the 'Tough-to-lyse' tissue samples protocol. The protocol used incorporated an in-column Dnase I (6 U/µl) digestion step to minimise DNA contamination. RNA was eluted in 35 µl DNase/RNase free water. RNA quality and quantity was assessed using a NanoDrop-ND1000 Spectrophotometer and gel electrophoresis.

Isolated RNA was converted to complimentary DNA (cDNA) using the First Strand cDNA Synthesis Kit (Thermo Scientific, USA) according to the manufacturer's instructions. Random Heximer primers were used and minus reverse transcriptases (-RT) were run for each sample. Complimentary DNA synthesis product and the corresponding -RT for each sample was assessed on a 1% agarose gel following standard end point PCR to amplify a 496 base pair section of the housekeeper gene, Glyceralehyde 3-phosphate dehydrogenase (GAPDH). Primers for all genes of interest (IL-6, TNF-α and IL-1β) and housekeeper genes (GAPDH and beta-actin) were purchased from Bio-Rad Laboratories (USA) as predesigned PrimePCR™ Assays. A serial dilution of 1:10 (IL-6) and 1:6 (TNF-α and IL-1β) were determined as best fit for primer efficiencies and subsequent qPCR (Supplementary Table A). After primer efficiencies were determined, relative expression of selected genes of interest were measured in relation to the two housekeeper genes by qPCR on a CFX-96™ Real Time PCR Detection system (Bio-Rad Laboratories, USA).

2.4. Plasma sample analysis of inflammatory cytokines

Plasma concentrations of IL-6, TNF-α, IL-1β and IL-10 were also measured by sandwich ELISA using commercially available ELISA kits (R&D Systems for IL-6, TNF-α and IL-1β; MyBioSource for IL-10), according to the manufacturer's instructions. Selection of cytokines for analysis in plasma was based on qPCR analysis results. This included the addition of IL-10 (anti-inflammatory cytokine), which was not possible to analyse retrospectively in the ileum with qPCR analysis, due to sample quantities available. Circulating antigen levels were measured using ELISAs compared to snapshot levels of cytokine expression in the GIT using qPCR analysis. The absorbance of the ELISAs were read using an ELISA plate reader (SPECTAmax™ 340; Molecular Devices, San Jose, USA) and the software GraphPad Prism version 9.4.1. Concentrations were calculated based on the standard curve created from the concentrations and absorbance of the respective standards. Inter and intra assay CVs were $< 10\%$ for all kits.

2.5. Statistical analysis

Pig performance and plasma inflammatory cytokine ELISA data were analysed according to a randomised complete block design using the General Linear Model function of IBM SPSS Statistics (version 26), using pen as the experimental unit for performance data and individual pig as the experimental unit for ELISA and qPCR analysis. Data were checked for normality of residuals using the Kolmogorov-Smirnov test. When significant differences were found, data were subjected to Tukey's HSD test to separate the means at a

significance level of $P < 0.05$. P-values of 0.05–0.10 were noted as trends.

All qPCR data analysis was performed using the qbase+ software, v.3.2 (Biogazelle, Zwijnaarde, Belgium), which uses a generalised model of the ΔΔCt approach. Stability of the housekeeper genes was determined using the geNorm expression stability value of the reference gene (M) and the coefficient of variation (CV) of the normalised reference gene relative quantities, as calculated within qbase+. The Cq values generated by qPCR were converted into normalised relative gene expression values according to the formulas described by Hellmans et al. (2007). Exported data were tested for normality using the Shapiro-Wilk test and homogeneity of variance using the Levene's test prior to statistical analysis using a one-way ANOVA in SPSS (v.26).

3. Results

3.1. Growth performance

Although there were no differences in ADFI between treatment groups for the first seven days after weaning, there was a tendency for pigs fed the PC to have a higher ADG than pigs fed the NC diet (0.156 vs. 0.121 kg/day; P=0.070; Table 2). As a result, during the first seven days after weaning, G:F was higher for pigs fed the PC diet compared to the NC (0.888 vs. 0.764, P<0.001), the NC plus 5 g/kg SWB (0.888 vs. 0.753, P=0.019) and the NC plus 10 g/kg SWB (0.888 vs. 0.762, P=0.034).

Between days 8 and 20 after weaning, pigs fed the PC diet tended to have higher ADFI compared to those fed the NC diet (0.440 vs. 0.363 kg/day; P=0.068) and the NC diet with 10 g/kg SWB (0.440 vs. 0.370 kg/day; P=0.109). In terms of growth, pigs fed the PC diet had higher ADG than pigs fed the NC diet (0.368 vs. 0.264; P=0.013) and NC plus 10 g/kg SWB (0.368 vs. 0.265 kg/day; P=0.014). Pigs fed the NC diet plus 5 g/kg SWB had an intermediate ADG (0.308 kg/day), which was not significantly different from the PC, NC or NC plus 10 g/kg SWB. Gain:feed between 8 and 20 days was highest for pigs fed the PC diet compared to the NC (0.830 vs. 0.721, P=0.021) and NC plus 10 g/kg SWB (0.830 vs. 0.702, P=0.005). Overall, during the first 20 days after weaning, there was a tendency for treatment to effect ADFI, with PC fed pigs eating the most (0.348 kg/day) and NC fed pigs eating the least (0.298 kg/day), although the Tukey test did not reveal significant differences. Positive control-fed pigs also had higher ADG compared to both the NC (0.294 vs. 0.213; P=0.006) and NC plus 10 g/kg SWB (0.294 vs. 0.218 kg/day; P=0.009). The ADG of pigs fed the NC diet plus 5 g/kg SWB was intermediate and not different from either the PC, NC or NC plus 10 g/kg SWB. As a result, G:F was higher for pigs fed the PC diet than all other treatments (P<0.05).

During the latter half of the trial, between days 21–41 after weaning, ADG and ADFI was not different between treatments. However, G:F was higher for pigs fed the NC diet (0.804) compared to the PC diet (0.755, P=0.002) and the NC plus 5 g/kg SWB (0.768, P=0.038), with pigs fed the diet with 10 g/kg SWB having an intermediary G:F. When looking at the performance of pigs over the entire 42 days, there was no overall difference in rate of gain, feed intake or feed efficiency between any of the treatments.

There were no differences in health scores between treatment groups throughout the trial (Table 3), with good health for the duration. Faecal scores for pigs fed the PC diet were lower (firmer) than NC and NC plus 5 g/kg SWB for the first seven days (P<0.05)

Table 2
Estimated marginal means for average weight, average daily feed intake (ADFI), average daily gain (ADG) and gain:feed (G:F) of pigs fed one of four experimental diets (PC- Positive control; NC- Negative Control) from weaning through to 42 days.

	PC	NC	NC + 5 g/kg Seaweed blend	NC + 10 g/kg Seaweed blend	SEM	P-value
Wean weight (kg)	8.48	8.48	8.48	8.49	0.304	1.000
Day 7 wt (kg)	9.57	9.32	9.39	9.40	0.311	0.951
Day 20 wt (kg)	14.35	12.75	13.40	12.84	0.583	0.147
Day 42 wt (kg)	30.74	30.35	31.13	29.77	1.145	0.855
Days 0 – 7						
ADG (kg/day)	0.156	0.121	0.131	0.130	0.010	0.072
ADFI (kg/day)	0.174	0.176	0.172	0.169	0.007	0.881
G:F	0.888[a]	0.764[b]	0.753[b]	0.762[b]	0.031	<0.001
Days 8 – 20						
ADG (kg/day)	0.368[a]	0.264[b]	0.308[ab]	0.265[b]	0.023	0.008
ADFI (kg/day)	0.440	0.363	0.409	0.370	0.022	0.061
G:F	0.830[a]	0.721[b]	0.743[ab]	0.702[b]	0.026	0.005
Days 0 – 20						
ADG (kg/day)	0.294[a]	0.213[b]	0.247[ab]	0.218[b]	0.016	0.003
ADFI (kg/day)	0.348	0.298	0.326	0.299	0.015	0.078
G:F	0.844[a]	0.712[b]	0.746[b]	0.716[b]	0.021	<0.001
Days 21 – 42						
ADG (kg/day)	0.751	0.796	0.805	0.767	0.030	0.556
ADFI (kg/day)	0.996	0.995	1.050	0.989	0.042	0.698
G:F	0.755[a]	0.804[b]	0.768[a]	0.773[ab]	0.009	0.003
Days 0 – 42						
ADG (kg/day)	0.531	0.523	0.540	0.507	0.022	0.753
ADFI (kg/day)	0.668	0.643	0.684	0.640	0.028	0.633
G:F	0.796	0.818	0.789	0.788	0.014	0.365

n=12 per parameter; SEM- Standard error of the mean; rows with different superscripts indicate significant differences.

and remained lower compared to all other treatments between days 8 and 20 (P<0.001). These pigs also had a lower cleanliness score (clean) compared to all other treatments between days 8 and 20. Between days 21–42, pigs fed the PC diet tended to maintain their lower faecal scores compared to NC plus 10 g/kg SWB pigs and had the lowest cleanliness score during this period.

3.2. mRNA expression and plasma concentrations of inflammatory cytokines

There were no differences in mRNA expression of the pro-inflammatory cytokines TNF-α, IL-6 and IL-1β in ileal mucosa samples between treatments, when compared to expression levels of housekeeper genes, using a generalised model of the ΔΔ-CT approach (Table 4; Biogazelle, 2017). Mean plasma levels for TNF-α, IL-10, IL-6 and IL-1β are also shown in Table 4. Plasma IL-6 levels tended to be lower for pigs fed the PC diet and the NC diet plus 5 g/kg SWB compared to the NC and NC plus 10 g/kg SWB (P<0.10) at day 20, when pigs were sampled. There were no other differences between treatments for the other cytokines analysed.

4. Discussion

The objective of this experiment was to investigate the effect of including 5 g/kg or 10 g/kg SWB (blend of green, red and brown seaweeds) in pig feed, on pig performance and markers of inflammation within their GIT, in comparison to pharmacological levels of ZnO (3.1 g/kg). Health scores showed an average of less than one pig per pen showing signs of illness, with cleanliness scores averaging clean or some indication of faecal contamination and faecal scores showed soft faeces that did not indicate diarrhoea. As a result, the performance and overall health of the pigs used within this experiment can be considered good. Despite this, a response to pharmacological levels of ZnO was still observed, as these pigs had higher ADG and had a higher G:F compared to the NC and NC with 10 g/kg SWB, for the first 20 days after weaning. These improvements with pharmacological levels of ZnO are in line with a number of previous studies that have found improvements in growth rate, daily feed intake and feed conversion ratio immediately after weaning (Hill et al., 2001; Stensland et al., 2015; Hazelden, 2021). Although the mode of action of ZnO is still unclear, there is evidence suggesting that it enhances intestinal function due to improved morphology (Højberg, et al., 2005), and shifts in microbial diversity within the GIT to confer a beneficial microbiome (Yu et al., 2017; Hazelden, 2021). This is also supported by a reduction in ETEC shedding when pigs have been fed diets containing pharmacological levels of ZnO and deliberately challenged with ETEC after weaning (Slade et al., 2011). Although bacterial load was not investigated in this research, firmer faeces indicate less diarrhoea caused by ETEC.

In partial support of the overall hypothesis of this research, pigs that were given a NC diet containing 5 g/kg SWB had an intermediate ADG between the NC and PC groups. This indicates a potential benefit to pig performance when included at the lower rate, although no differences were seen in G:F between the two inclusion levels of SWB and the NC group. The inclusion of SWBs to weaner pig diets has previously been reported to have prebiotic effects, by modifying the GIT microbiota including reducing faecal *E. coli* counts and increasing nutrient digestibility. Previous studies have also reported improved feed efficiency and subsequent growth rate with seaweed extracts (SWE) (Dillon et al., 2010; McDonnell et al., 2010; Smith et al., 2011). Rattigan et al. (2020) found that the addition of 300 ppm (0.3 g/kg feed) laminarin increased rate of gain compared to a basal diet (without ZnO) and feed intake compared to a basal diet as well as lower inclusion levels of laminarin. They found that lower than 0.3 g/kg laminarin had no benefit to the pig,

Table 3
Means for Health, Faecal and Cleanliness score for all four dietary treatments (PC- Positive control; NC- Negative Control) from weaning to 42 days after weaning.

	PC	NC	NC + 5 g/kg Seaweed blend	NC + 10 g/kg seaweed blend	SEM	P-value
Days 0 – 7						
Health	0.06	0.00	0.04	0.00	0.017	0.563
Faecal	2.18^{a}	2.42^{b}	2.50^{b}	2.33^{ab}	0.035	0.010
Cleanliness	1.22	1.29	1.31	1.35	0.046	0.238
Days 8 – 20						
Health	0.02	0.03	0.20	0.05	0.027	0.308
Faecal	2.17^{a}	2.53^{b}	2.50^{b}	2.70^{b}	0.041	<0.001
Cleanliness	1.49^{a}	1.83^{b}	2.13^{c}	1.92^{bc}	0.066	<0.001
Days 0 – 20						
Health	0.03	0.02	0.14	0.03	0.019	0.312
Faecal	2.18^{a}	2.49^{b}	2.50^{b}	2.57^{b}	0.034	<0.001
Cleanliness	1.40^{a}	1.64^{b}	1.85^{c}	1.72^{bc}	0.049	<0.001
Days 21 – 42						
Health	0.004	0.03	0.004	0.02	0.007	0.799
Faecal	2.07	2.17	2.15	2.28	0.028	0.088
Cleanliness	1.82^{a}	1.98^{ab}	2.18^{b}	2.19^{b}	0.067	0.001
Days 0 – 42						
Health	0.02	0.02	0.07	0.03	0.010	0.521
Faecal	2.12^{a}	2.32^{b}	2.31^{b}	2.42^{b}	0.026	<0.001
Cleanliness	1.62^{a}	1.82^{b}	2.02^{c}	1.96^{bc}	0.05	<0.001

n=12 per parameter; SEM- Standard error of the mean; rows with different superscripts indicate significant differences.

Table 4
Normalised Relative Quantities (NRQs) of Interleukin (IL)-6, IL-1β and Tumor necrosis factor (TNF)-α in the ileum of pigs fed one of four diets (PC-Positive control; NC- Negative Control) from qPCR analysis. Normalised relative quantities are relative to housekeeper genes (GAPDH and Beta-actin) and mean plasma levels (pg/ml) of IL-6, IL-1β, TNF-α and IL-10 (ELISA) from pigs 20 days after weaning, after being fed one of four diets.

		PC	NC	NC + 5 g/kg Seaweed blend	NC + 10 g/kg Seaweed blend	SEM	P-value
Ileal mRNA expression	IL-6	1.93	0.94	0.97	0.70	0.692	0.952
	IL-1β	0.66	1.92	1.57	0.49	0.687	0.848
	TNF-α	1.50	0.39	1.30	1.29	0.974	0.931
Plasma antigens	IL-6	1.7	4.7	1.1	5.0	1.21	0.066
	IL-1β	4.3	8.1	3.9	6.4	1.35	0.135
	TNF-α	128.0	152.9	163.1	148.7	13.69	0.348
	IL-10	410.6	312.3	369.6	381.4	30.14	0.775

n=6 per parameter; SEM- Standard error of the mean

potentially indicating an optimal level of inclusion. Conversely, at higher inclusion than Rattigan et al. (2020), Reilly et al. (2008) found no differences in performance between control fed pigs or pigs fed a diet containing 1.5 g/kg *L. hyperborean* SWE (brown seaweed), 1.5 g/kg *L. digitata* SWE (brown seaweed) or 1.5 g/kg of a combination of *L. hyperborean* and *L. digitate* SWE's, when fed from 24 days of age (weaning), for seven days. Differences in performance results across studies may be a result of the period of time the extract is fed as well as seaweed species used or the conditions in which they were extracted, making direct comparisons more challenging (Bilan et al., 2006. Nonetheless, results of the present study indicated the 10 g/kg inclusion of this SWB may to be too high, given these pigs had a lower ADG compared to the PC treatment for the first 20 days of the trial. Previous reports have found that diets or ingredients containing a higher ash and non-starch polysaccharide content, such as seaweed, can reduce dietary nutrient levels and compromise voluntary feed intake and nutrient digestibility, thus negatively affecting growth performance of weaned pigs, which could be why 10 g/kg inclusion of this SWB was not beneficial compared to the NC diet (Owusu-Asiedu et al., 2006; Agyekum and Nyachoti, 2017). It may be beneficial to consider inclusion levels of 5 g/kg SWB, or lower, in future studies as inclusion at 5 g/kg provided intermediate growth, with similar, lower levels of looseness and higher cleanliness of pigs, that were not different to the PC group receiving pharmacological levels of ZnO. Despite initial differences in performance, beyond day 20, and when looking at the entire 42-day trial, there were no significant differences between treatment groups, although PC fed pigs were still 976 g heavier than NC plus 10 g/kg SWB at the end of the trial. The absence of a long-term benefit of ZnO has been seen previously (Hazelden, 2021) and could be as a result of the pigs on this trial not facing an obvious health challenge, as recorded with daily health, cleanliness and faecal scores. Furthermore, as pharmacological levels of ZnO have frequently been reported to improve feed intake of pigs, its removal from the diet after day 20 may have slowed down intake, leading to intakes that were not different across the treatment groups (Heo et al., 2010; Slade et al., 2011; Sales, 2013; Hazelden, 2021).

Collection of blood and tissue samples occurred after euthanasia. It has been suggested that stress can affect the levels of IL-6 and IL-10 (Zhang and Zhao, 2009), however all animals were treated and euthanized in the same way to minimize any confounding effects. The current study found no significant effect of pharmacological levels of ZnO, or either inclusion levels of SWB on ileal mucosa levels of inflammatory markers. However, there was a tendency for IL-6, an inflammatory cytokine that is produced by a number of cell types such as macrophages and B cells and is up-regulated in most inflammatory states (Dalrymple et al., 1996), to be reduced in pigs fed the PC or NC plus 5 g/kg SWB diets. As feed intake tended to be higher for PC fed pigs, with NC plus 5 g/kg SWB having the second highest intake, there would have been an increase in luminal nutrients within the GIT of pigs on these diets, which reduces the likelihood of changes to the structure and function of the GIT that cause an inflammatory response (McCracken et al., 1999; Pie et al., 2004). This could explain why pigs fed the PC and NC plus 5 g/kg SWB tended to have reduced IL-6 levels and improved growth performance. Given none of the other pro- (IL-1β and TNF-α) or anti- (IL-10) inflammatory cytokines differed in the blood, and IL-6 can have both pro- and anti-inflammatory properties, depending on signaling pathway (Scheller et al., 2011), it is hard to definitively interpret the effect of IL-6 in this instance. However, given the health and performance of the pigs it is perhaps more likely to have been a beneficial change.

The effects of seaweed on cytokine expression have previously been reported. Bouwhuis et al. (2017) saw benefits of including 0.18 g laminarin/kg feed and 0.34 g fucoidan/kg feed to 30 kg live-weight pigs, with reduced mRNA expression levels of IL-6, IL-22 and TNF-α in the colon of pigs fed SWE supplementation. However, this was observed after pigs had been orally challenged with *Salonella typhimurium* to deliberately elicit an immune response. A bacterial challenge was not carried out during the current research so detection of immune markers was under standard health conditions. However, similar benefits have also been observed by Heim et al. (2015), whereby the inclusion of a combination of laminarin and fucoidans in a sow diet reduced gene expression of cytokines IL-6 and IL-8 in the ileum and colon of her piglets at weaning. Heim et al. (2015) found an up-regulation of TNF-α and IL-1 at weaning in the ileum of piglets suckling from sows provided a seaweed-derived polysaccharide-supplemented diet. Tumor necrosis factor-alpha is one of the cytokines produced by epithelial cells in response to various pathogens as it can block pathogen replication, therefore an up-regulation of this cytokine can be beneficial but also indicate a pathogenic invasion within the gut. As the levels of TNF-α were not different between treatment groups in the current study, it could be that the response seen with IL-6 were resulting from an inflammatory response closer to weaning, which may not have been associated with a pathogenic invasion, particularly given faecal scores did not indicate diarrhoea presence across the study. Cytokines such as IL-6 can be released as part of an acute phase response that is not just caused by pathogenic infection, but also general inflammation or trauma, which may explain why cytokines such as

TNF-α did not respond the same (Gruys et al., 2005). Perhaps earlier sampling, prior to day 20, would have seen a more defined response with IL-6 and other cytokines, if the inflammation was due to a bacterial invasion. For example, de Groot et al. (2021) found that under normal health conditions, pro-inflammatory cytokines were upregulated 15 days after weaning, but not at 30 days after weaning.

In conclusion, as expected, including pharmacological levels of ZnO in the diet improved pig performance immediately after weaning. The performance of pigs fed 5 g/kg inclusion of the seaweed blend was intermediate, and thus not different from either the PC or the NC diets between days 8 and 20 and 21–42 days after weaning. Given this and the tendency towards reduced levels of IL-6 in pigs fed the PC and NC, it could be beneficial to investigate this inclusion level further, or investigate lower inclusion levels, given 10 g/kg SWB did not show any beneficial effects.

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CRediT authorship contribution statement

Sophie C Hazelden: Writing – review & editing, Writing – original draft, Visualization, Investigation, Formal analysis, Data curation. **Hadden Graham:** Writing – review & editing, Methodology, Funding acquisition, Conceptualization. **Steven Laird:** Writing – review & editing, Resources. **Ryan Clarkson:** Writing – review & editing, Project administration, Data curation. **Katie McDermott:** Writing – review & editing, Methodology, Conceptualization. **Amy E Taylor:** Writing – review & editing, Supervision, Project administration, Methodology, Formal analysis, Data curation, Conceptualization.

Declaration of Competing Interest

The authors declare no conflict of interest or competing financial interests that could influence the work reported within this paper.

Appendix A. Supporting information

Supplementary data associated with this article can be found in the online version at doi:10.1016/j.anifeedsci.2024.115928.

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