Article

Effect of Red Beetroot Supplemented Diet on Gut Microbiota Composition and Metabolite Profile of Weaned Pigs – A Pilot Study

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Simple Summary: Weaning causes gut microbiota disruption that results in dysbiosis and post-weaning diarrhoea. The recent ban on pharmacological doses of in-feed zinc oxide in weaned pig diet has made exploration of alternative dietary supplements to improve post-weaning condition of pigs imperative. Plants (e.g., red beetroot) containing bioactive compounds, have shown great potential in this regard, favorably abating gut microbiota dysbiosis, promoting gut metabolite production and health.

Abstract: Red beetroot, is a well-recognized and established source of bioactives (e.g., betalains and polyphenols) with anti-inflammatory and antimicrobial properties. It is proposed as a potential alternative to zinc oxide, with a focus on gut microbiota modulation and metabolite production. In this study, weaned pigs aged 28-days were fed either a control diet, diet supplemented with zinc oxide (3,000 mg/kg), or 2% and 4% pulverized whole red beetroot (CON, ZNO, RB2 and RB4; respectively) for 14 days. After the pigs were euthanized, blood and digesta samples were collected for microbial composition and metabolite analyses. Results showed, red beetroot supplemented diet at 2% improved the gut microbial richness relative to other diets, but marginally influenced the caecal microbial diversity compared to zinc oxide supplemented diet. Further increase in red beetroot levels (4% -RB4) lead to loss of caecal diversity, decreased short chain fatty acids and secondary bile acid concentrations. An increased Proteobacteria abundance, presumably due to increased lactate/lactic acid producing bacteria was also observed. Summarily, red beetroot contains several components conceived to improve the gut microbiota and metabolite output of weaned pigs. Future studies investigating individual components in red beetroot will better elucidate their contributions to gut microbiota modulation and pig health.

Keywords: weaned pig; gut microbiota; red beetroot; short chain fatty acids; bile acids

1. Introduction

Weaning is a stressful phase in pig production, characterized by reduced feed intake, poor growth rate, gut microbiota disruption and diarrhoea [1,2]. It is a transitionary phase of the pig life, associated with compositional and functional alterations of the gut microbiota, resulting in enteric infections. Several measures to prevent dire economic losses at weaning in pig production are currently being explored. Diet provided to weaned pigs have been demonstrated to modulate significant gut microbiota changes leading to increased population of beneficial bacteria species with remarkable changes observed 10 to 14 days post-weaning [3,4].

Pathogenic colonization of the gut, a leading cause of diarrhea and death of young pigs at weaning is thus avoidable via dietary modulation of a healthy gut microbial composition [5,6]. Notably, the composition and diversity of the gut microbiota can be altered

 doi:10.20944/preprints202305.1372.v1

by the source and level of protein and fibre, causing increased or depleted gut microbiota metabolite production and corresponding biological responses [7-9]. The gut microbiota metabolite levels in return enhance or inhibit the growth of certain bacteria phyla (e.g., Bacteroidetes, Firmicutes and Proteobacteria) in the gastrointestinal tract [10,11].

Similarly, in-feed antibiotics and zinc oxide (ZnO) have been reported to reduce piglet mortality during weaning [12,13]. Their capacity to suppress post weaning diarrhoea, alter host-gut microbiota metabolism, improve feed intake and energy production for growth is well known [14-16]. However, despite these advantages, they have been found to destabilize the gut microbial diversity, alter short chain fatty acid (SCFA) levels and support the emergence of harmful and antibiotic-resistant bacteria species [17-19].

Evident from past literature, gut microbiota changes to in-feed ZnO have been characterized with increased coliforms [20,21], reduced anaerobic and lactic acid bacteria [22,23] and reduced commensal bacteria population [24,25]. Consequently, the functional potential of the gut microbiota and production of health promoting metabolite (e.g., short chain fatty acids and bile acids) may be compromised.

Additionally, there are concerns about severe environmental pollution from high fecal excretions of zinc [26] linked to in-feed pharmacological doses of zinc oxide, coupled with increasing trends of multidrug-resistant *E. coli* [27,28]. This signifies a risk to the animal-environment food chain and highlights an urgent need for alternatives to in-feed ZnO. Therefore, there has been an increased research interest in plants containing bioactive compounds e.g., red beetroot with health promoting properties, as possible replacement for in-feed ZnO with emphasis on modulating a healthy gut microbiota thus preventing pathogenic colonization post-weaning [29,30].

Red beetroot (*Beta vulgaris subsp. vulgaris conditiva*) contains bioactive compounds such as betalains, polyphenols, inorganic nitrate (NO_3), fibre and minerals (e.g., potassium, sodium, phosphorus, calcium, magnesium, copper, iron, zinc, and manganese) [31-33]. These bioactives (i.e., betalains, polyphenols, nitrate and fibre) have been indicated in the potential prebiotic effect of red beetroot, driving gut microbiota modulation and metabolite production, with impact on host metabolism, physiology and immune functions [34-36].

Red beetroot is one of the top ten plants with high antioxidant, anti-inflammatory, antimicrobial, anticarcinogenic and hepatoprotective characteristics [37,38]. It is currently being considered as a therapeutic ingredient, in the treatment of conditions caused by oxidative stress, inflammation and metabolic disorders (e.g., hypertension, diabetes, insulin resistance and kidney dysfunction) [39-41]. The health benefits of red beetroot in humans, rodents [42,43] and rainbow trout [44,45] have been widely studied and reported in literature, but studies using pigs have not been considered, and research demonstrating the potential of red beetroot supplementation on the gut microbiota is yet lacking. This study therefore examines the effect of red beetroot on the gut microbiota composition and metabolite output of weaned pigs.

2. Materials and Methods

The animal trial was conducted at the National Pig Centre, UK under an ethical approval granted by the University of Leeds Animal Welfare and Ethical Review Committee (AWERC) with the approval number; 070510HM. All husbandry practices were set by the farm in accordance with the Welfare of Farmed Animals, England Regulation 2007, and all procedures were conducted following the amended Animals (Scientific Procedures) Act 1986. For ethical reasons, the number of piglets per treatment was reduced and determined based on previous studies [46-48] focusing on gut microbiota diversity. The basal diet was provided by Primary diets, UK and whole red beetroot powder was purchased from Buy Wholefoods online Ltd (Ramsgate, UK). Reference bile salts for bile acid quantification were purchased from Sigma-Aldrich (Steinheim, Germany) and Cayman (Cambridge, UK). A mixed short chain fatty acid standard solution containing acetate, propionate, butyrate, valerate, isobutyrate and isovalerate was obtained from Supelco - Merck

 doi:10.20944/preprints202305.1372.v1

life science Ltd (Dorset, UK). Chemicals, solvents, and other reagents were purchased from Sigma-Aldrich (Germany) and Fischer Scientific (Loughborough, UK) accordingly.

2.1 Experimental animals and experimental design

Forty-eight piglets (Large White X Landrace X Duroc) weaned on day-28 (average body weight: 7.58±0.69 kg) were randomly allocated to one of four diet (n = 12), balancing for body- weight, sex, and litter origin, for a 14-day feeding experiment. The pigs were housed in a temperature-controlled flat deck with open feed troughs and nipple drinkers allowing easy access to feed and water *ad libitum*. The experimental diets comprised a basal control diet (CON) and diet supplemented with 3000 mg/kg zinc oxide (ZNO), both formulated according to the National Research Council (2012) recommendations (Table 1). Red beetroot supplemented diets (RB2 and RB4) were obtained by adding 2% (20 g/kg) and 4% (40 g/kg) pulverized whole red beetroot to the basal diet respectively, thoroughly mixed with an electric mixer on-site.

2.2 Sample collection

At the end of the experiment period, eight animals per diet (n = 8) were euthanized by captive bolt and exsanguination. Blood samples were collected from the jugular vein into heparinized tubes, from which plasma was obtained after centrifugation at 2000 x g, 4°C for 10 min. Fecal samples were collected from the rectum into designated tubes. The abdominal cavity was immediately opened, each intestinal segment (duodenum, jejunum, ileum, caecum, and colon) was identified, separately cut, and emptied into a sterile beaker. Digesta from each segment was mixed, aliquoted into sterile 2 mL Eppendorf tubes. All samples were snapped frozen in liquid nitrogen and stored at -80°C for analysis of gut bacterial composition, short chain fatty acids and bile acids.

2.3 Gut microbiota analyses and bioinformatics

Pig gut microbial composition was examined using digesta samples from the jejunum, ileum, and caecum. Genomic DNA was extracted from (approx. 1.0 g) digesta sample with QIAamp Power fecal DNA kit (Qiagen, Hilden, Germany) according to the manufacturer's instructions. The concentration and purity of DNA samples were measured spectrophotometrically with Nano Drop® ND-1000 (Nano Drop Technologies Inc., Dover, USA), absorbance ratio at 260/280 nm observed were within the range of 1.8 - 2.0. DNA samples were submitted to the University of Leeds, Next Generation Sequencing facility, St. James Hospital Leeds, UK, for quality screening, 16S rRNA gene library preparation and sequencing. According to a previous study [49], the V4 hypervariable region of the 16S rRNA gene was amplified in a two-step polymerase chain reaction (PCR) with specific primers (564F, 806R) and Illumina adaptor overhang. Following the Illumina 16S metagenomics sequencing library preparation protocol, the final libraries were pooled, and pair-end sequenced on the Illumina MiSeq platform (Illumina, San Diego, CA, USA).

Sequence reads were processed in Mothur v.1.43.0 with the MiSeq standard operation procedure developed by the Schloss group [50,51]. Chimera-free and unique sequences were identified and aligned to the SILVA (v.138) database, then sequences with 97% similarity were clustered into operational taxonomic unit. The "Biome" file generated from Mothur was transferred to R (v 3.6.2 and 4.0.0) environment for further analyses including the alpha and beta diversity indices.

 doi:10.20944/preprints202305.1372.v1

Table 1. Composition of experimental diets and result of analyzed nutrients

Ingredients (%)	Control (CON)	Diet with ZnO (ZNO)	2% red beetroot supplemented diet (RB2)	4% red beet root supplemented diet (RB4)
Barley	15.00	15.00	14.70	14.40
Wheat	28.17	28.17	27.51	26.95
Micronized maize bulk	2.50	2.50	2.45	2.40
Micronized oats	5.00	5.00	4.90	4.80
Fishmeal bulk	6.00	6.00	5.88	5.76
Soya hypro	18.16	18.16	17.80	17.43
Full fat soybean	2.50	2.50	2.45	2.40
Pig weaner premix	0.50	0.50	0.49	0.48
Whey powder bulk	13.89	13.89	13.61	13.33
Potato protein	1.60	1.60	1.57	1.54
Sugar/sucrose	0.63	0.63	0.61	0.60
L-Lysine	0.28	0.28	0.28	0.27
DL-Methionine	0.19	0.19	0.19	0.19
L-Threonine	0.15	0.15	0.15	0.15
L-Tryptophan	0.02	0.02	0.02	0.02
L-Valine	0.04	0.04	0.04	0.04
Vitamin E	0.04	0.04	0.04	0.04
Pan-tek robust	0.02	0.02	0.02	0.02
Sucram	0.01	0.01	0.01	0.01
Benzoic acid	0.50	0.50	0.49	0.48
Pigzin (zinc oxide)	0.00	0.31	0.00	0.00
Di-calcium phosphate	1.13	1.13	1.11	1.08
Sodium carbonate	0.05	0.05	0.05	0.05
Sipernat 50	0.31	0.00	0.30	0.30
Red beetroot	0.00	0.00	2.00	4.00
Soya oil	3.40	3.40	3.33	3.26
Total (%)	100	100	100	100
Dry matter (%)	89.93	89.65	89.47	89.01
Analysed nutrient				
Ash (%)	6.80	7.50	6.70	6.60
Ether extract (%)	6.73	6.99	6.62	5.92
Crude protein (%)	21.30	21.30	20.70	20.40
Crude fibre (%)	1.90	1.50	1.80	2.20
Zinc (mg/kg)	422.00	2252.00	193.00	187.00

The alpha diversity of the gut microbial community was evaluated using Chao1, Shannon and Simpson indices, variables were compared using ANOVA evaluating the effect of diet, gut location, and their interaction with the lmerTest (linear mixed effects). Differences between gut samples (beta diversity) were determined by a permutational multivariate analysis of variance (PERMANOVA) of the non-phylogenetic distance matrix (Bray Curtis) and visualized on a non-metric multidimensional scale (NMDS) plot. The diet effect on each gut location was computed by a paired comparison of distance matrices with pairwise Adonis function (adonis2) in vegan package (v. 2.6.4).

Differentially (distinct) abundant taxa between gut locations per diet was identified in a two-sided Welch's *t*-test and Benjamin Hochberg false discovery ratio (FDR) correction in STAMP (Statistical Analysis of Metagenomics and other Profiles) software [52]. Further analysis employed DESeq2 (v. 1.27.32) in R [53] with Wald hypothesis testing for

 doi:10.20944/preprints202305.1372.v1

distinct genera in each gut location comparing multiple diet groups. Differences were estimated as fold change (Log 2- fold change) between diet and with FDR-corrected *p* values.

2.4. Predicted functions of pig gut microbiota

To predict the functional pathways mediated by the gut microbiota, OTU abundance and representative sequences processed in Mothur were submitted to Piphillin (https://piphillin.secondgenome.com/). Gene sequences were matched against KEGG (Kyoto Encyclopedia of Genes and Genomes) database as described in Iwai, *et al.* [54], using USEARCH version 8.0.1623 with global alignment setting for sequence identification fixed to 90% cut-off (a level significantly associated with PICRUSt - phylogenetic investigation of communities by reconstruction of unobserved states) [55].
Pathways differentially mediated by diet in the different gut locations were computed with DESeq2 in R using Wald test and *p* values adjusted for multiple inter-diet comparisons.

2.5. Quantification of short chain fatty acids (SCFA) and bile acids

Short chain fatty acids (acetate, propionate, butyrate, valerate, isobutyrate and isovalerate) in plasma, jejunum, ileum, caecum, colon digesta and fecal samples were determined with gas chromatography (Varian 3400; Varian Ltd., Oxford, UK). Method used was as described in Taylor, *et al.* [56] with slight modification. Briefly, 1.0 g or 1 mL sample was mixed with an equal volume of distilled water in an Eppendorf tube and centrifuged at 12,000 x *g*, at 4°C for 10 min. Phosphoric acid (50 µL, 85% v/v) was added to the supernatant (500 µL) collected and 150 µL caproic acid (150 mM/L) as internal standard. The mixture was made up to 1 mL with distilled water, centrifuged at 14,000 x *g* for 20 min, after which the supernatant was collected for SCFA analyses. Individual short chain fatty acid in processed sample was quantified using a standard curve obtained from a mixed volatile fatty acid prepared in the concentration range 0 to 125 mM.

Bile acids in samples were determined as described in Zhang, *et al.* [57], briefly, 0.3 g digesta was mixed with acetonitrile (final conc. 80% v/w), incubated for 20 min at room temperature and centrifuged at 15,000 x *g* at 4°C for 20 min. The supernatant collected was passed through Strata-X 33µm polymer based solid phase extraction cartridges (Phenomenex, Torrance USA) after cartridges had been conditioned with methanol and water. Subsequently, bile acids were eluted in 1.5 mL methanol, concentrated, and dried using a solvent evaporator (SP Genevac EZ-2 Series, Pennsylvania, USA), then reconstituted in 150 µL methanol before subjecting to HPLC-MS (Shimadzu, Kyoto, Japan). The mobile phase: A and B was a mixture of 5 mM ammonium acetate in water and methanol respectively, both acidified with 0.012% formic acid. A mixed standard reference (0 - 0.1 mM) containing; taurohyodeoxycholic acid (THCA), glycohyodeoxycholic acid (GHDCA), taurocholic acid (TCA), glycocholic acid (GCA), taurochenodeoxycholic acid (TCDCA), taurodeoxycholic acid (TDCA), glycochenodeoxycholic acid (GCDCA), glycodeoxycholic acid (GDCA), cholic acid (CA), glycolithocholic acid (GLTHCA), chenodeoxycholic acid (CDCA), deoxycholic acid (DCA) and lithocholic acid, was prepared for quantification of bile salts.

2.6. Statistical analyses

SCFA and bile acid concentrations were analyzed in R environment (v. 4.2.2), zero inflated data was analyzed using negative binomial with square root link function, multiple comparison of means was computed using Tukey *post hoc* test with significance level of $p < 0.05$. Results were expressed as mean and standard error of mean (SEM) and presented in tables. A Spearman correlation analysis was conducted between SCFA levels, bile acids and the mean relative abundance of top twenty-five genera in each gut location per diet using the "Psych" [58] and "Pheatmap" [59] packages in R (v. 3.31).

 doi:10.20944/preprints202305.1372.v1

3. Results

3.1. Effect of diets on gut microbial diversity and taxonomic composition

In the present study, there was no difference in the growth performance of the pigs, however, the diets ($P = 0.01$) significantly influenced the species richness and diversity of the gut microbiota with respect to the gut locations ($P < 0.001$; jejunum, ileum, and caecum) examined. Diet RB2 increased the jejunal species richness ($P = 0.02$) compared to other diets according to the Chao1 index, but caecal species abundance was comparable for all the diets (Figure 1a). From the Shannon index of alpha diversity, the gut microbial community was diverse, but this was not influenced by the diets ($P = 0.07$), howbeit a pairwise diet comparison showed, ZNO diet was different from CON and RB4 in the caecum (Figure 1b). No significant species abundance, divergence, or evenness (i.e., dominance) was observed between the diets and in the gut locations for the Simpson index (data not shown).

The beta diversity is as shown in the non-metric multi-dimensional scaling (NMDS) plots (Figure 2a and 2b). Samples from the caecum clustered distinctively away from the ileum and jejunum, depicting the caecum had a significantly different ($P = 0.013$) microbial composition from the ileum and jejunum, while the ileum and jejunum microbial community are marginally different ($P = 0.051$). A subset analysis of the caecal biome with inter-diet comparisons indicated CON pigs had more similar species in the caecum than ZNO ($P = 0.03$) and RB2 ($P = 0.05$) pigs but related to RB4 pigs ($P = 0.35$). Hence, ZNO pigs contained more dissimilar species than RB4 pigs, whereas RB2 and RB4 pigs were not different ($P = 0.09$).

From the digesta samples analyzed, 15 phyla and 310 genera were observed with approximately 99% of total sequences (17,573,278) assigned. The mean relative phyla and genera abundance is presented in Figure 3a and 3b. Dominant bacteria phyla with mean relative abundance >1% were Firmicutes, Actinobacteriota, Bacteria unclassified, and Bacteroidota. The mean relative phyla and top genera abundance in the gut were compared across the diet groups and have been presented in Table 2 and S1 respectively. The gut locations mainly influenced ($P < 0.05$) the relative mean phyla distribution with increase in the caecum compared to other regions examined, however Firmicutes abundance reduced. An increase in phylum Actinobacteriota and Proteobacteria abundance in CON and RB4 pigs was also observed.

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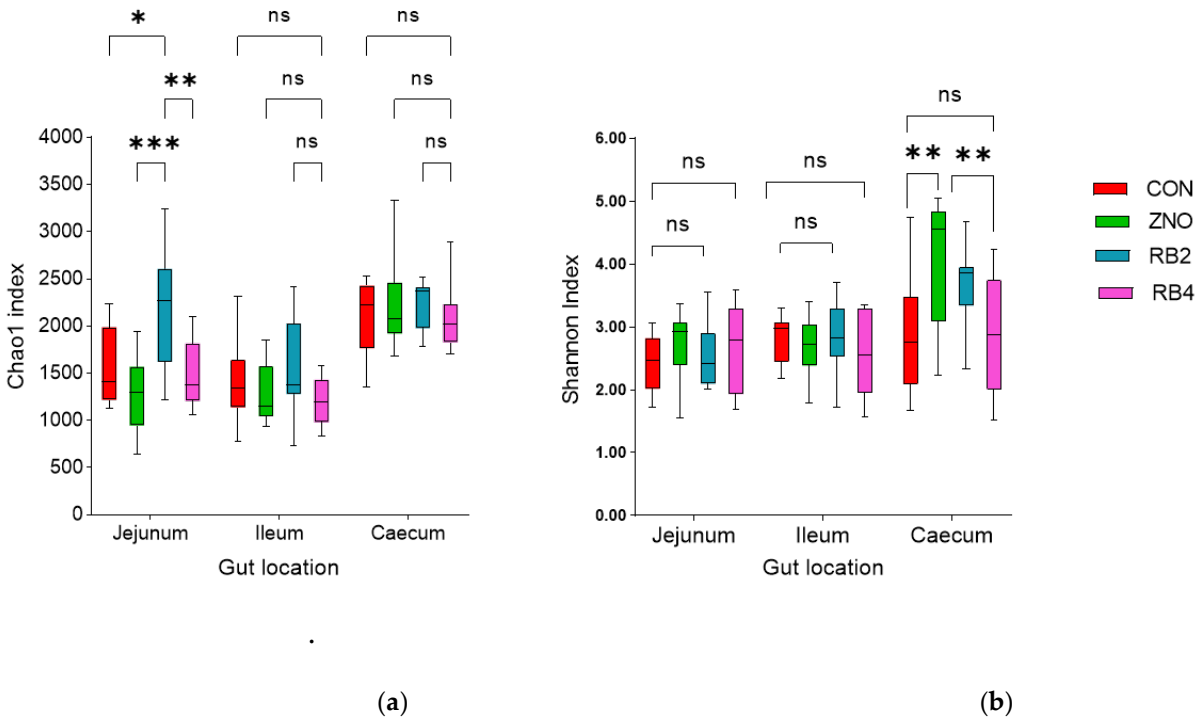

(**a**) (**b**)

Figure 1. Alpha diversity indices of pig gut microbiota; (a) Chao1 index (b) Shannon index, showing diet effect on gut species richness and/or diversity. Boxplot represents mean (minimum to maximum) species richness and or evenness from each diet in the gut locations evaluated. Significant difference between the diets linked by a line is indicated by * $P < 0.05$, ** $P < 0.01$, *** $P < 0.001$, ns; - not significant. CON, ZNO, RB2, and RB4 represent the control diet and diet supplemented with zinc oxide, 2% and 4% red beetroot respectively.

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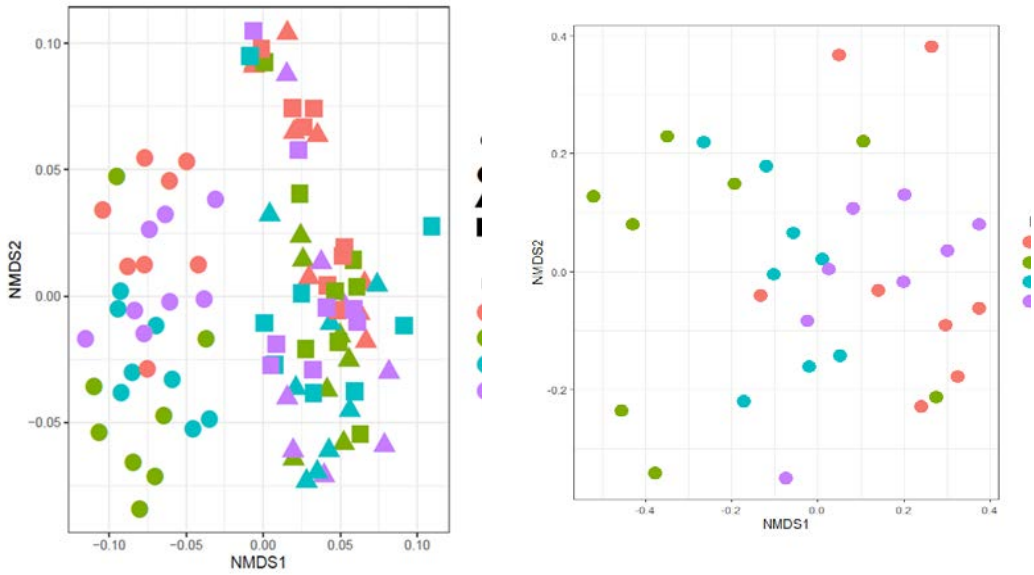

(**a**) (**b**)

Figure 2. Non-metric multidimensional scaling (NMDS) plots of Bray Curtis non-phylogenetic distance matrices of gut microbial community of weaned pigs fed different diets (a) distribution of samples by diet and gut location (b) distribution of samples from the caecum. Diets: CON, ZNO, RB2, and RB4 represent; control diet, diet supplemented with zinc oxide, 2% and 4% red beetroot supplemented diets respectively.

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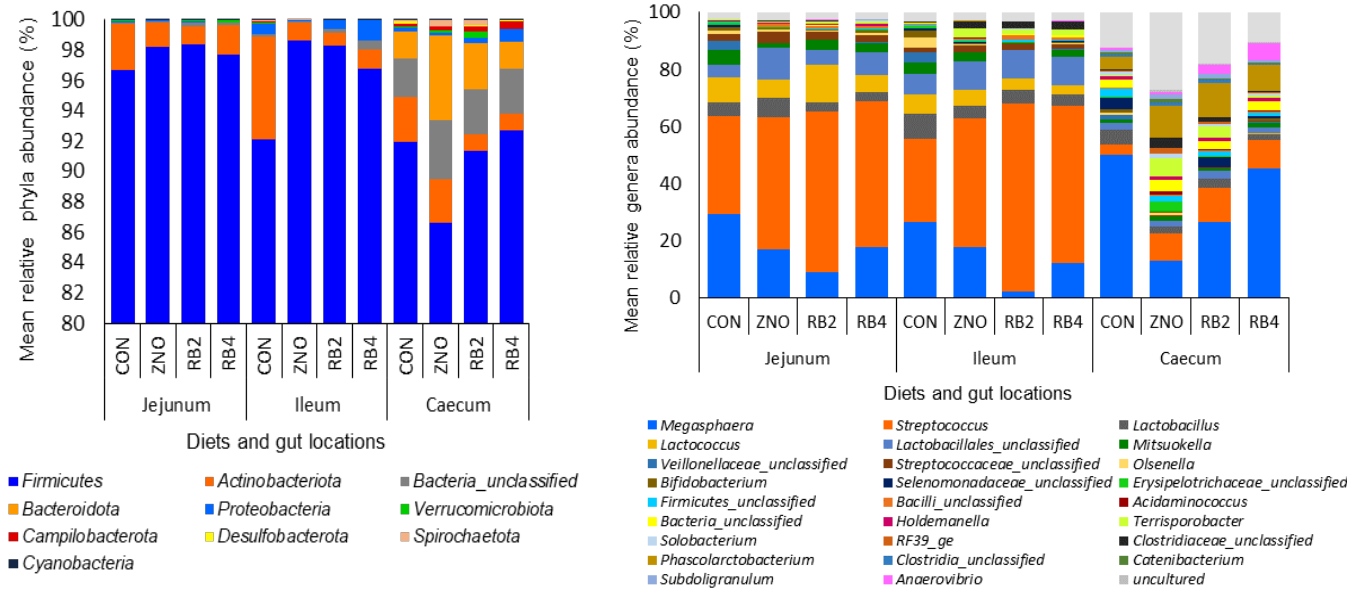

(a) (b)

Figure 3. Mean relative abundance of (a) phyla and (b) genera in pig gut locations with respective diets. CON, ZNO, RB2 and RB4 represent control diet and diets supplemented with zinc oxide, 2% and 4% red beetroot respectively.

Table 2. Comparative analyses of mean relative phyla abundance between diet and gut locations

Phylum	Diets				Gut locations				*P* value		
	CON	ZNO	RB2	RB4	Jejunum	Ileum	Caecum	[d]SEM	[f]L	[e]D	[g]L*D
Firmicutes	93.58[a]	94.48[a]	96.01[a]	95.72[a]	97.74[a]	96.46[a]	90.64[b]	0.617	0.000	0.312	0.062
Actinobacteriota	4.25[a]	1.88[ab]	1.06[b]	1.42[b]	1.93[a]	2.51[a]	2.02[a]	0.347	0.733	0.004	0.224
Bacteria unclassified	0.95[a]	1.37[a]	1.13[a]	1.26[a]	0.17[b]	0.28[b]	3.09[a]	0.206	0.000	0.796	0.800
Bacteroidota	0.61[a]	1.86[a]	1.03[a]	0.59[a]	0.003[b]	0.005[b]	3.06[a]	0.248	0.000	0.067	0.029
Proteobacteria	0.31[ab]	0.11[b]	0.31[ab]	0.71[a]	0.03[b]	0.66[a]	0.39[a]	0.068	0.000	0.005	0.126
Verrucomicrobiota	0.09[a]	0.06[a]	0.18[a]	0.08[a]	0.11[a]	0.04[a]	0.16[a]	0.026	0.187	0.389	0.132
Campilobacterota	0.09[a]	0.08[a]	0.13[a]	0.16[a]	0.01[b]	0.01[b]	0.32[a]	0.041	0.002	0.916	0.921
Desulfobacterota	0.07[a]	0.01[a]	0.03[a]	0.04[a]	0.004[b]	0.001[b]	0.11[a]	0.010	0.000	0.230	0.213
Spirochaetota	0.05[a]	0.13[a]	0.11[a]	0.01[a]	0.001[b]	0.03[b]	0.19[a]	0.026	0.003	0.259	0.058

Data represents mean phyla abundance in each gut location with the different experimental diets, significant difference was indicated by different superscript between diet groups and the gut location. [d] Standard error of the group mean, [e] *p* value for gut location, [f] *p* value for diet, [g] *p* value for interaction between the gut location and diet. CON, ZNO,

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RB2 and RB4 represent control diet and diets supplemented with zinc oxide, 2% and 4% red beetroot respectively.

From the top genera (Table S1), *Megasphaera, Streptococcus, Anaerovibrio, Rumminococcaceae unclassified, Erysipelotrichaceae unclassified, Bacillus unclassified, Terrisporobacter* and *Clostridiaceae unclassified* abundance were significantly influenced by the diets ($P < 0.05$) and the gut locations ($P < 0.02$) examined. The mean relative abundance of eleven genera (e.g., *Megasphaera, Selenomonadaceae unclassified, Phascolarctobacterium, Firmicutes unclassified, Bacteria unclassified, Erysipelotrichaceae unclassified, Negativibacillus, Anaerovibrio*), functionally recognized as lactate utilizing bacteria (LUB) were higher ($P < 0.05$) in the caecum, but comparable in the jejunum and ileum. Likewise, lower ($P < 0.05$) *Streptococcus, Lactococcus, Lactobacillales unclassified, Streptococcaceae unclassified,* and *Bacilli unclassified* abundance (mostly lactic acid producing bacteria –LAB) was observed in the caecum compared to the jejunum and ileum.

3.2. Differential abundant genera modulated by the diets

Differential abundance at the genus level and distribution per diet was computed by Welch's *t*-test and FDR-corrected in STAMP. The result indicated an increased ($P < 0.05$) *Megasphaera, Selenomonadaceae_unclassified and Veillonellaceae_unclassified* abundance in pigs fed CON diet, *Erysipelotrichaceae_unclassified, Clostridiaceae_unclassified* and *Rumminococcaceae_unclassified* in ZNO pigs, while *Bacilli_unclassified* and *Anaerovibrio* increased in RB2 and RB4 pigs respectively. Further analyses of each gut location with inter-diet comparisons as shown in figure 4, presented decrease in *Veillonellaceae_unclassified and Selenomonadaceae_unclassified* abundance in the jejunum of RB2 and RB4 pigs, compared to CON and ZNO pigs, whereas in the ileum, only RB2 pigs showed an increased *Terrisporobacter* abundance relative to CON pigs. The caecum was enriched with nine genera (e.g., *Romboutsia, Clostridiaceae_unclassified, Terrisporobacter, Candidatus_Soleaferrea, Muribaculaceae_ge* and *Clostridium_sensu_stricto_1*) in ZNO pigs compared to CON but diminished in genus *Selenomonadaceae_unclassified*. Pigs fed red beetroot diets (RB2, RB4) had increased caecal *Selenomonadaceae_unclassified* and/or *Anaerovibrio* abundance relative to ZNO pigs.

3.3 Metabolite profile and association with gut microbial composition

Short chain fatty acid (SCFA) profile followed the expected pattern of increased levels in the lower gut (caecum and colon), including the fecal samples. Nonetheless, the experimental diets influenced SCFA levels observed in these locations (Table 3). Concomitant with the species richness in the gut locations, SCFA levels increased significantly in the jejunum of RB2 pigs, reduced in the ileum of ZNO pigs, but was comparable in the caecum across the diet groups. Overall, total SCFA levels reduced significantly ($P = 0.01$) in RB4 and ZNO pigs, as was for most SCFA (e.g., acetate, propionate, and butyrate).

Similarly, the trend of high jejunal bile acid concentration (approx. 1 to 3-fold) and levels observed in other locations examined is not biologically relevant (Table 4). Pigs fed RB2 diet had higher ($P < 0.05$) TCA, GCDCA, CA, GLTCA, CDCA and DCA compared to other diets, but equivalent total and unconjugated bile acids (CA, DCA, CDCA and LCA) levels with CON pigs. Conversely, bile acid concentration reduced in ZNO (TCDCA, TDCA, GCDCA, GDCA and CDCA) and RB4 (TCA, GLTCA, DCA, CA) pigs relative to CON, which cumulatively ensued lesser total and unconjugated bile acids levels.

Although, total SCFA in CON and RB2 pigs was higher, caecal SCFA levels were comparable across the diet, acetate and propionate levels correlate significantly to bacteria (e.g., *Firmicutes_unclassified, Mitsuokella, Megasphaera, Streptococcus, Streptococccceae_unclassified, Anaerovibrio, Lactobacillus* and *Selenomonadaceae unclassified*) abundance in the caecum for CON and RB pigs, but closely associated with the jejunum and ileum (e.g., *Phascolarctobacterium, Bacteria_unclassified*) genera abundance in ZNO and RB4 pigs. Across the gut locations, *Faecalibacterium, Blautia, Clostridia_unclassified, Clostridiaceae_unclassified, Dialister, Olsenella, Selenomonadaceae_unclassified, Veillonellaceae_unclassified,* and *Firmicutes*

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_unclassified are examples of genera significantly associated with butyrate in ZNO and RB4 pigs (Figure 5a and 5b), most of which were associated with ileal butyrate in RB2 pigs although not significant (Figure S1b).

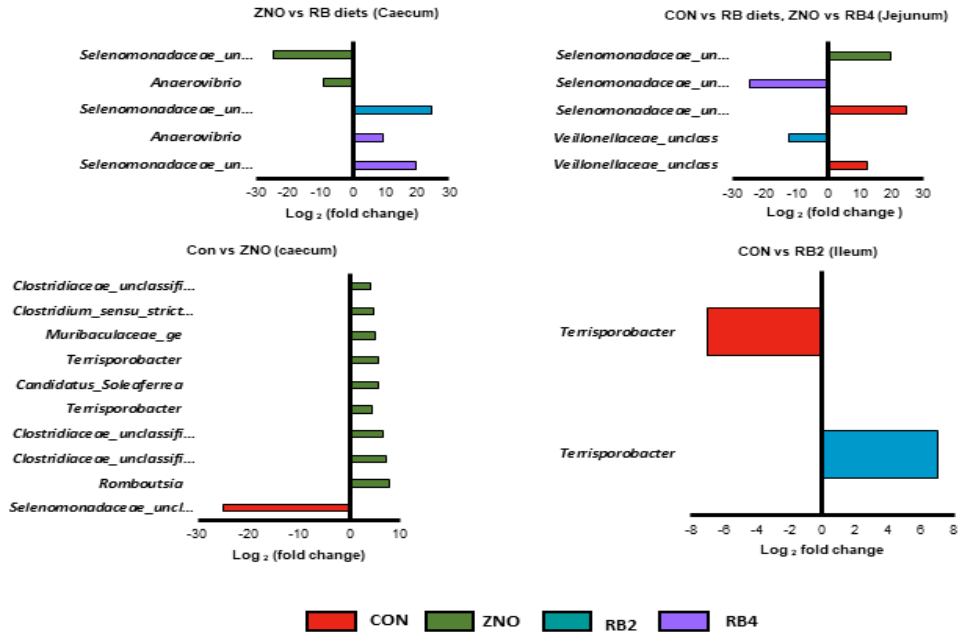

Figure 4. Differentially abundant genera, comparisons between diets in the gut locations. Only comparisons with significant ($P < 0.001$) log 2-fold changes are presented. RB diets; red beetroot diets. CON, ZNO, RB2 and RB4 represents; control diet and diets supplemented with zinc oxide, 2% and 4% red beetroot, respectively.

Table 3. Short chain fatty acid (mM) profile of diet groups and locations evaluated.

SFCA	Diets					*P* value		
	CON	ZNO	RB2	RB4	SEM	*L	#D	+L*D
Acetate	69.14^{a}	54.76^{b}	63.81^{ab}	56.64^{b}	3.32	< 0.01	< 0.05	> 0.05
Propionate	23.83^{a}	17.04^{d}	20.34^{b}	19.16^{c}	1.42	< 0.05	< 0.01	< 0.01
Isobutyrate	2.59^{a}	1.60^{b}	1.79^{b}	0.86^{c}	0.36	< 0.02	< 0.02	< 0.02
Butyrate	9.48^{a}	6.76^{b}	8.08^{ab}	7.09^{b}	0.61	< 0.05	< 0.05	> 0.05
Isovalerate	1.11^{a}	0.71^{b}	0.67^{b}	0.44^{c}	0.14	< 0.02	< 0.02	< 0.05
Valerate	1.55^{a}	0.82^{b}	0.58^{c}	0.63^{c}	0.22	< 0.02	< 0.02	< 0.05
Location								
[1]Plasma	1.99	1.87	2.31	1.61	0.15	< 0.01	> 0.05	< 0.05
[1]Jejunum	7.40^{b}	7.88^{b}	11.28^{a}	7.08^{b}	0.97	< 0.01	< 0.05	< 0.05
[1]Ileum	9.21^{a}	7.91^{b}	9.82^{a}	8.45^{a}	0.42	< 0.01	< 0.05	< 0.05
Caecum	24.91	22.36	23.71	22.12	0.65	< 0.05	> 0.05	< 0.05
Colon	24.57^{a}	19.25^{b}	17.32^{b}	22.29^{ab}	1.61	< 0.05	< 0.05	< 0.05
Feces	39.61^{a}	22.43^{c}	30.84^{b}	23.27^{c}	4.00	< 0.05	< 0.05	< 0.05
Total SCFA	107.70^{a}	81.69^{b}	95.28^{a}	84.82^{b}	5.876	< 0.05	< 0.05	< 0.05

Data represent mean SCFA for each diet group and gut location, significant differences were indicated by different superscripts across the rows. CON, ZNO, RB2 and RB4 represent; control diet, diets supplemented with zinc oxide, 2% and 4% red beetroot respectively. [1]SCFA levels in these locations were significantly different from levels in the caecum. *p value for effect of location on SCFA levels, #p value for significant effect of diets, +p value interaction between location and diet.

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Associations between the gut genera abundance and bile acid levels are presented in figure (S2). Focusing on the unconjugated (CA, CDCA, LCA) and conjugated (GCA, TCA, GDCA, GCDCA, TCDCA, TDCA, GLTCA, THCA, GHDCA) bile acids, in the jejunum, CA, CDCA and total bile acid levels were strongly associated with most genera in RB2, unlike ZNO and RB4 pigs (Figure 6). Conjugated bile acids were significantly associated with the ilea genera (e.g., *Bacteria_unclassified*, *Selenomonadaceae_unclassified* and *Veillonellaceae_unclassified*) abundance in the CON and RB pigs, however most bacteria (e.g., *Lactobacillus*, *Lactococcus*, *Streptococcaceae_unclassified*, *Firmicutes_unclassified*, *Terrisporobacter*, *RF39_ge*) were associated with, unconjugated, conjugated, and total bile acids in the ileum of ZNO pigs. A significant correlation was observed in the caecum between the bacteria genera (*Dialister*, *Streptococcus*, *Lactobacillaes_unclassified*, *Streptococcaceae_unclassified*, *Lactococcus*, and *Selenomonadaceae_unclassified)* and unconjugated bile acids (CA, CDCA) in CON, RB2 and RB4 pigs but not in ZNO pigs.

Table 4 Bile acid profile (nmol/g digesta/feces) from diet groups and locations evaluated

Bile acids	Diets					*P* value		
	CON	ZNO	RB2	RB4	SEM	*L	#D	+L*D
THCA	40.74^{a}	27.29^{b}	16.88^{c}	26.86^{b}	4.90	< 0.01	< 0.01	0.75
GHDCA	33.78^{b}	46.08^{a}	25.76^{c}	33.45^{b}	4.20	< 0.01	0.05	0.62
TCA	1.50^{b}	1.56^{b}	1.96^{a}	1.30^{b}	0.14	< 0.01	< 0.01	< 0.01
GCA	0.40^{b}	0.47^{b}	0.45^{b}	1.37^{a}	0.23	> 0.05	0.01	> 0.05
TCDCA	21.90^{a}	8.89^{c}	8.30^{c}	15.09^{b}	3.18	< 0.01	< 0.01	< 0.01
TDCA	5.11^{a}	3.42^{b}	4.07^{ab}	3.90^{b}	0.36	0.02	0.05	0.93
GCDCA	15.42^{c}	13.88^{c}	35.91^{a}	25.40^{b}	5.10	< 0.01	< 0.01	0.25
GDCA	3.57^{b}	4.91^{a}	2.95^{c}	3.26^{b}	0.43	< 0.01	< 0.01	0.05
GLTCA	2.11^{ab}	1.99^{b}	3.60^{a}	1.96^{b}	0.40	< 0.05	< 0.01	0.18
CA	3.80^{b}	2.59^{c}	4.23^{a}	1.63^{d}	0.59	< 0.01	< 0.01	< 0.01
CDCA	121.16^{b}	72.28^{c}	147.09^{a}	74.32^{c}	18.34	< 0.01	< 0.01	< 0.01
DCA	0.10^{b}	0.14^{b}	0.31^{a}	0.12^{b}	0.05	< 0.02	< 0.02	0.20
LCA	134.25^{a}	108.90^{b}	118.20^{b}	107.66^{b}	6.13	< 0.01	0.05	0.42
			Location					
[1]Jejunum	138.20^{b}	135.03^{b}	210.83^{a}	128.68^{c}	19.32	< 0.01	< 0.05	< 0.05
Ileum	95.20^{a}	40.21^{c}	29.11^{d}	50.09^{b}	14.40	< 0.01	< 0.01	< 0.05
Caecum	23.95^{a}	17.37^{b}	15.65^{b}	12.84^{c}	2.35	< 0.01	< 0.05	< 0.05
Colon	59.88^{a}	28.45^{c}	46.40^{b}	21.10^{d}	8.77	<0.01	< 0.05	< 0.05
Faeces	66.59^{c}	71.33^{b}	67.72^{c}	83.62^{a}	3.90	< 0.01	< 0.01	< 0.05
Total unconjugated	259.30^{a}	183.91^{b}	269.83^{a}	183.74^{b}	23.41	< 0.05	< 0.05	< 0.05
Total conjugated	124.52^{a}	108.48^{b}	99.88^{b}	112.60^{b}	5.12	< 0.05	< 0.05	< 0.05
Total bile acids	**383.82^{a}**	**292.39^{b}**	**369.71^{a}**	**296.33^{b}**	**23.98**	< 0.001	< 0.05	< 0.01

Data represents mean bile acid levels for each diet and location, significant differences indicated by superscripts across the table for the diet groups. CON, ZNO, RB2 and RB4 represent control diet and diets supplemented with zinc oxide, 2% and 4% red beetroot respectively. *p value for effect of location, #p value for significant effect of diets, +p value interaction between location and diet. Taurohyodeoxycholic acid (THCA), glycohyodeoxycholic acid (GHDCA), taurocholic acid (TCA), glycocholic acid (GCA), taurochenodeoxycholic acid (TCDCA), taurodeoxycholic acid (TDCA), glycochenodeoxycholic acid (GCDCA), glycodeoxycholic acid (GDCA), cholic acid (CA),

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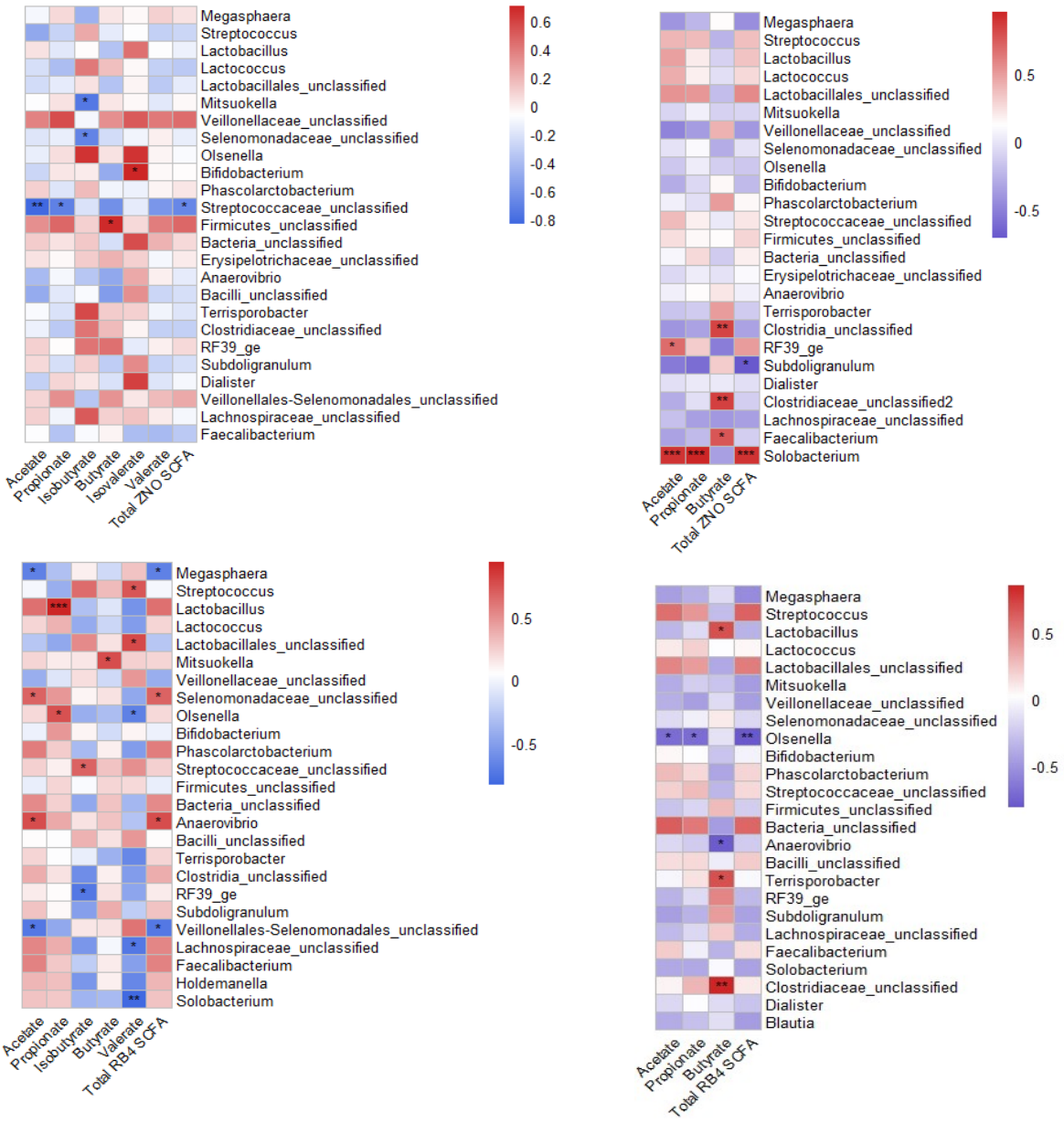
glycolithocholic acid (GLTHCA), chenodeoxycholic acid (CDCA), deoxycholic acid (DCA) and lithocholic acid.



(a) (b)

Figure 5. Spearman correlation analyses between top abundant bacteria genera and (a) caecal short chain fatty acids (b) ileal short chain fatty acids. Omitted fatty acids were not detected in the corresponding location hence not shown, color depth depicts correlation between genera and gut metabolite where red color denotes a positive correlation and blue color a negative correlation. The strength of association between the subjects is indicated by the color intensity, *** $P \leq 0.001$, ** $P \leq 0.01$, * $P \leq 0.05$). CON, ZNO, RB2 and RB4 represent control diet and diets supplemented with zinc oxide, 2% and 4% red beetroot respectively.

3.4 Predicted gut microbiota functions

Compared to ZNO pigs, pathways enabling bacteria response and adaptation to environmental changes (e.g., biofilm formation, flagella assembly and two-component

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system) were upregulated in the caecum of pigs on CON diet. Also, pigs fed RB4 diet had pathways influencing lipid metabolism (inositol phosphate, glycerol-phospholipid, fatty acid degradation, chloroalkane and chloroalkene degradation) enhanced. Aside from these, there were no variations between the diets in the functional pathway predictions from the jejunal and ilea microbiota.

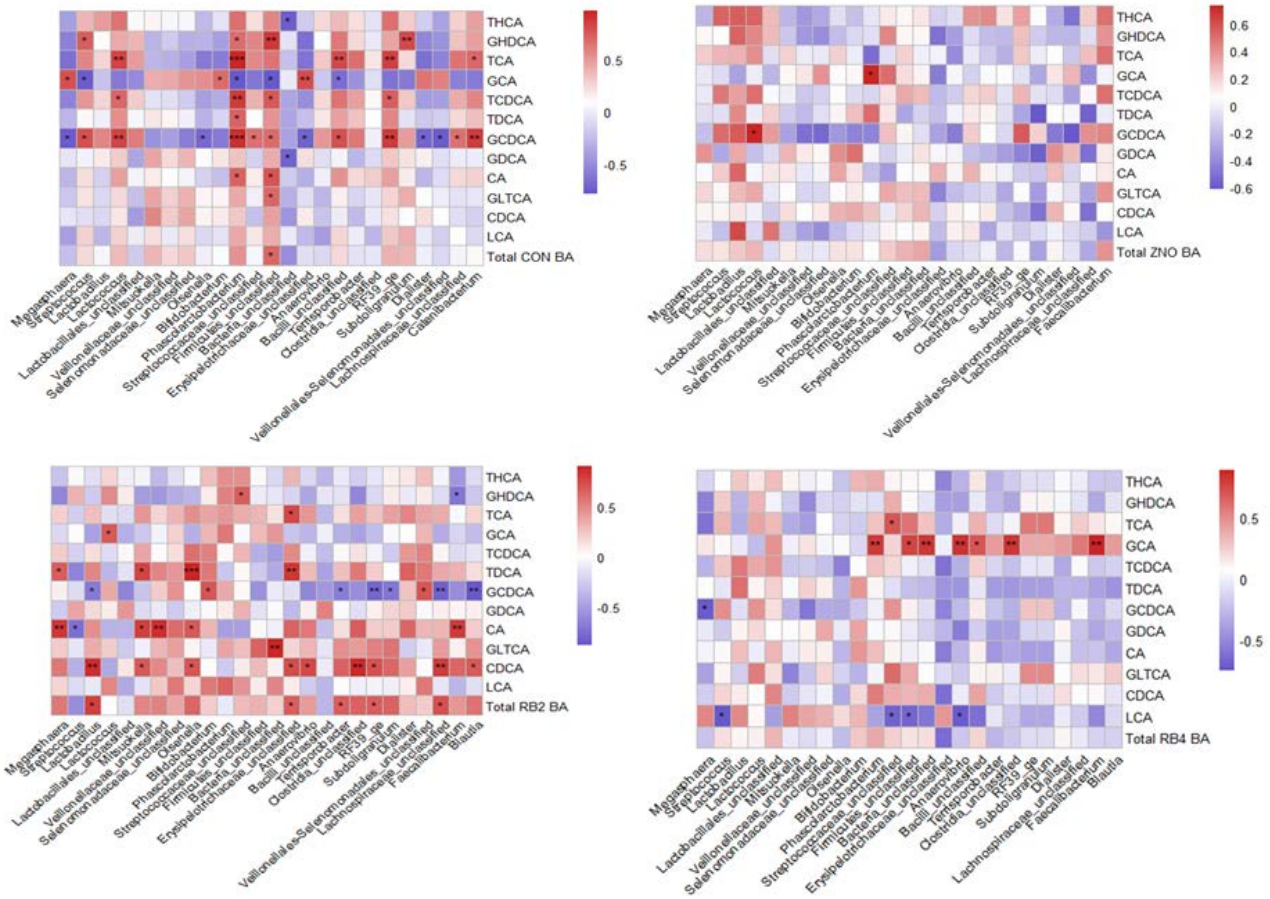

Figure 6. Spearman correlation matrices between jejunal genera abundance and bile acid levels. Omitted bile acid (deoxycholic acid - DCA) was not detected in the jejunum for the pigs hence not shown. Correlation depicted by color depth, where red color denotes a positive and blue color a negative correlation. The strength of association between the subjects is indicated by the color intensity and *** $P \leq 0.001$, ** $P \leq 0.01$, * $P \leq 0.05$). CON, ZNO, RB2 and RB4 represent control and diets supplemented with zinc oxide, 2% and 4% red beetroot respectively. Taurohyodeoxycholic acid (THCA), glycohyodeoxycholic acid (GHDCA), taurocholic acid (TCA), glycocholic acid (GCA), taurochenodeoxycholic acid (TCDCA), taurodeoxycholic acid (TDCA), glycochenodeoxycholic acid (GCDCA), glycodeoxycholic acid (GDCA), cholic acid (CA), glycolithocholic acid (GLTHCA), chenodeoxycholic acid (CDCA) and lithocholic acid.

4. Discussion

Current reports on the health potential of red beetroot have necessitated evaluations of its probable effect on the gut microbiota and as an alternative to zinc oxide in weaned pig diet. Red beetroot, a rich source of nutrient, fibre, and bioactive compounds is well recognized for its anti-inflammatory, antioxidant, antimicrobial properties, and prebiotic effect in the gut. Given these benefits, adding red beetroot to weaned pig diet could promote beneficial microbiota modulation of the gut, thus prevent gut dysbiosis and diarrhea post weaning.

 doi:10.20944/preprints202305.1372.v1

Diet, remains an uncontestable factor shaping and modulating the gut microbiota, towards the achievement of gut health and overall wellbeing [60]. Capper, *et al.* [61] in a controlled clinical trial with healthy humans consuming whole cooked red beetroot, showed gut microbiota modulation with reduced Bacteroidetes, increased alpha diversity and short chain fatty acid (SCFA) levels combined with a normal fecal score. The weaning phase in pig production is of focus here due to the attending economic impact, more so the recent ban on in-feed ZnO which has further exacerbated the health implication on weaned pigs.

Studies on pig gut microbiome have long established that early modulation of the gut microbiota of young pigs is vital to the prevention of post weaning diarrhoea, maturation of the immune system and improvement of growth performance [2,62,63]. Adaptation of the weaned pig gut to a new diet and achievement of a relatively stable gut microbiota 7 to 10 days post weaning is essential for early attainment of a rich-diverse gut microbial composition and gut health [64].

In this present study, 14-day supplementation of weaned pig diet with 2% red beetroot (RB2) influenced the alpha diversity, increasing the species richness of the jejunum compared to other diets. Although, a comparable number of species was observed in the caecum of all the pigs. Reduced bacteria species in the jejunum [65,66] or ilea digesta [67] of weaned pigs fed diet containing ZnO have been linked to the antimicrobial and growth-promoting ability of in-feed ZnO. According to Bonetti, Tugnoli, Piva and Grilli [25], in-feed ZnO reduces gut bacteria activity, making available more energy for growth and metabolism.

The caecum had a rich and more diverse bacteria as supposed, whereas the jejunal and ileal microbiota were closely related. This certifies the existence of more unique taxa in the caecum compared to the jejunum and the ileum [68]. Although, RB2 diet compares with ZNO, the latter clearly modulated a diverse caecal microbiota with more distinct bacteria than CON and RB4. This was possibly driven by decreased caecal Firmicutes abundance, causing increased relative mean abundance of other phyla (e.g., Bacteroidota). To the best of our knowledge, this is the first report on supplementation of red beetroot in weaned pig diet, however observations from ZNO diet resonate with previous findings on pharmacological dose of ZnO in weaned pig diet [66], while RB2 diet improved the species richness of the gut.

Meanwhile, increased RB levels did not translate to a diverse caecal microbiota despite increased fibre levels, depicting the gut microbiota acted differently toward the fibre. Besides, the functions of dietary fibre in the gut are largely determined by its source and physicochemical characteristics (e.g., solubility, viscosity, fermentability), which subsequently impacts the gut microbial composition and metabolite output [69]. In this study, pigs fed RB4 had a significant increase in Proteobacteria, reduced SCFA and secondary bile acids. Bacteria in this phylum tend to increase during weaning stress and in pigs on diet rich in protein, fat, and fibre, consequently depleting beneficial bacteria like; *Lactobacillus, Lactococcus* and *Bifidobacterium* [70].

Consistently, Firmicutes is the most dominant phylum, accounting for < 95% of all phyla observed in the gut [71-73]. However, reduced caecal Firmicutes abundance observed in this current study negate reports of higher populations in the pig caecum [68,74]. Similarly, Actinobacteriota was the second predominant phylum compared to Bacteroidota, reported in most studies. While these phyla are important commensal of the gut, differences in management, experimental diets, sampling age and location used in these studies may explain the observed disparity [4,75]. However, such gut microbiota alterations have recently been attributed to lactate accumulation in the gut.

Wang, *et al.* [76] confirmed gut microbiota variation from lactate accumulation, where phylum Bacteroidetes and Firmicutes were replaced by Actinobacteria and Proteobacteria, with concomitant reduction in butyrate and propionate production. Proteobacteria (e.g., *Campylobacter* and *Salmonella* species) utilize lactate under microaerophilic

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conditions, to produce carbon dioxide and water [77] and have predominantly been linked to gut perturbations mostly associated with diarrhea.

The small intestine is usually dominated by lactic acid bacteria -LAB (e.g., *Lactobacilli, Lactococcus, Streptococcus*, *Bifidobacterium* etc.) and is responsible for lactate production through various biochemical pathways [78]. Lactate prevents the growth of pathogenic organisms by lowering the gut pH, but increased levels can be harmful, causing alterations in gut microbiota, toxicity, and pathogenic colonization of the gut. To corroborate this claim, high ilea lactate levels (mM) were observed in CON (96.76) and RB4 (76.85) pigs, but levels in ZNO (44.72) and RB2 pigs (48.83) were similar (unpublished).

It is noteworthy that the gut microbiota employs lactate utilizing bacteria - LUB (from the phylum Firmicutes) with remarkable SCFA producing ability to avert the detrimental effect of lactate accumulation, thereby stabilizing the gut microbiota [79,80]. Hence, a balance between the LAB and LUB (functional groups) in terms of production and utilization of lactate has been found necessary for gut health [77]. Prominent LUB (e.g., *Megasphaera, Phascolarctobacterium, Negativibacillus and Veillonellaceae*) and LAB (e.g., *Streptococcus, Lactococcus, Lactobacillales unclassified* and *Streptococcaceae unclassified*) were identified in this study. Importantly, a higher caecal relative LUB abundance but reduced LAB was observed, while the abundance of both bacteria groups was comparable in the small intestine. These genera were associated with SCFA (acetate, propionate, and butyrate) levels across the gut locations (jejunum, ileum, and caecum) as shown by the correlation matrices.

Though the small intestine is not the major site for microbiota fermentation and SCFA production, significant SCFA levels, and correlations with the jejunal and ilea microbiota were observed in RB diets. The nutritional functions of the jejunum with capacity for energy metabolism and fibre fermentation have been confirmed in many studies, while the gut microbiota metabolite impact on the jejunal immune system, barrier function and cell proliferation [81,82]. In addition, the host immune system is regulated by continuous interaction between the gut microbiota and dietary metabolites hence, reduced gut microbiota association with butyrate levels observed in this study may partly be due to host immune responses as well as lactate accumulation on the gut [83]. Moreover, a decline in bacteria sensitivity to metabolite production may have doused a strong correlation between the gut microbiota and butyrate levels in RB2 pigs, unlike in ZNO pigs. Overall, inter-individual variability in response to diet as well as variations in gut microbial composition and function cannot be ruled out.

Bile acids have also been linked to host physiology and immunity via gut microbial metabolism. Diet influences the gut microbiota composition, and bile acid levels through bile salt hydrolyzing bacteria (BSHB) species (e.g., *Clostridium spp., Lactobacillus, Bifidobacterium, and Enterococcus*) that possess inducible genes responsible for the conversion of primary bile acids to secondary bile acids [84,85]. However, interactions between the bile acid and the gut microbiota can be severely impaired in the event of gut dysbiosis at weaning. Song, *et al.* [86] observed dietary supplementation with CDCA, a natural primary bile acid in animal bile, improved growth performance and reduced diarrheal incidence in weaned pigs.

Generally, RB2 diet increased individual primary and secondary (CA, CDCA and DCA) unconjugated bile acid levels compared to CON. DCA (deoxycholic acid) was mainly observed in the colon and feces, hence not correlated with the gut microbiota abundance. Another study by Tian, *et al.* [87] confirmed a higher and potent antibacterial activity in unconjugated bile acids compared to their other counterpart, and the sensitivity of bile acids to Gram-positive bacteria than Gram-negative was also demonstrated. The jejunal bile acid profile was strongly associated with the jejunal microbiota of RB2 pigs unlike ZNO and RB4 pigs, but same as observed in the ileum for ZNO pigs. Most bacteria in this region (small intestine) are usually resistant to bile acids, offering protection against pathogenic invasion [88]. Reduced bile acid levels in the gut have been implicated in bacterial overgrowth and inflammation [11]. Conversely, across the diet groups in the caecum, very

 doi:10.20944/preprints202305.1372.v1

few genera (e.g., *Streptococcus*, *Lactobacillales unclassified*, *Lactococcus*, *Selenomonadaceae_unclassified*, and *Erysipelotrichaceae_ unclassified*) were (associated) involved in bacterial metabolism of the unconjugated bile acids (CA and CDCA). The reasons for reduced bile acid levels with increased red beetroot is not clear. Usually, a high fat diet increases bile acid discharge, increasing circulating bile acid levels. Alteration of secondary bile acids by dietary fibre and an increased Proteobacteria abundance with RB4 are some possible causes of this trend.

Exploring differentially abundant genera in the pigs, lactate utilizers (e.g., *Veillonellaceae_ unclassified and Selenomonadaceae_unclassified*) increased in the jejunum of CON pigs relative to those fed RB diets, which signifies an increased abundance of lactic acid producing bacteria LAB, and a potential for lactate accumulation in CON pigs. T*errisporobacter*, an anaerobic Gram-positive bacterium in the family Peptostreptococcaceae, was differentially increased in the ileum of RB2 pigs compared to CON and associated with butyrate, GDCA and GLTCA. Other compositional differences observed in the cecum include increased gut fermenters (e.g., *Romboutsia, Muribauculaceae_ge, Terrisporobacter, Clostridiaceae_unclassified*) and decreased *Selenomonadaceae_unclassified* in ZNO pigs compared to the control. Except for an increased *Selenomonadaceae_unclassified*, RB2 was not different from ZNO, while RB4 had increased *Anaerovibrio* inclusive.

Generally, *Clostridiaceae_unclassified, Rumminococcaceae_unclassified* and *Erysipelotrichaceae_unclassified* were significantly higher in pigs fed ZNO diet, which coincides with results from [20] in pigs fed 2425 mg/kg dietary zinc. The presence of these strict anaerobes demonstrates a rapid transition of the pig gut microbiota from a (milk-based diet) suckling microbiota to a post weaning (solid-based diet containing complex compounds) microbiota. The preceding genera are linked to bile acid and SCFA production. However, increased *Erysipelotrichaceae* abundance has been implicated in dysbiosis-related disorders of the gut [89], and in mice post treated with broad spectrum antibiotics (e.g., gentamicin) [90].

Similarly, many studies have confirmed associations between bacteria belonging to this genus and host lipidemic profiles [91-93] and cholesterol metabolism [94,95]. This characteristic may be connected to the high systemic and hepatic lipid peroxidation observed in the plasma and liver tissue of pigs in this group (unpublished data), coupled with an increased tendencies for hepatic toxicity and oxidative stress in ZnO fed pigs. This additionally coincides with downregulation of pathways facilitating lipid metabolism for pigs fed ZNO diet compared to RB4.

Moreover, dietary supplementation with quercetin was reported to inhibit Erysipelotrichaceae [96]. Quercetin is a flavonoid (polyphenol) found in fruits and vegetables recognized for its health benefit and potential therapeutic effects. Polyphenol and bioactive pigments (betalains) in red beetroot may be responsible for a decreased abundance of *Erysipelotrichaceae_unclassified* in RB pigs.

Interestingly, red beetroot has been described effective in preventing lipid peroxidation in membrane decreasing oxidative damage [97], this aligns with observations of upregulated lipid metabolism pathways (i.e., inositol and glycerol-phospholipid metabolism) in the cecum of pigs in this group relative to ZNO pigs. An increased *Anaerovibrio* abundance; a strictly lipolytic bacteria known for hydrolysis of triglycerides to fatty acids, in pigs fed red beetroot supplemented diet further confirm these inferences. Overall, the predicted functional profile from the microbiota of each gut location (jejunum and ileum) did not differ from each diet, aside what has been earlier described, pathways in response to bacteria adaptation to environmental changes was enhanced by the caecal microbiota of CON pigs.

This study provides baseline outcomes of red beetroot supplementation of weaned pig diet; however, some limitations were identified. The absence of colon and fecal microbiota information hindered correlations with metabolite output. Also, whole red beetroot was used in this study, which represents a cost-effective way of providing red beetroot bioactive compounds (betalains, nitrate and polyphenols) to the pigs while also

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preserving its fibre content. Hence, the effect observed can only be inferred but not specific to any of these components. Future studies aimed at utilizing these components individually will improve outcomes and aid understanding of their roles in gut microbiota modulation and pig health.

5. Conclusions

Diet remains a viable strategy to modulate the gut microbiota of weaned pig, and red beetroot supplementation provides an avenue to explore its bioactives for pig gut health. From this study, weaned pig diet supplemented with red beetroot at 2% increased the species richness of the gut microbiota. However, inclinations of lactate accumulation were observed with increased RB to 4% (RB4), which was characterized by potential decline in butyrate and propionate and an increased Proteobacteria abundance. The jejunum and ileum microbial compositions were similar across the diet groups, but the cecum was diverse with ZNO diet, relative to RB2, while CON and RB4 were comparable. RB2 diet also increased the gut microbiota metabolites (SCFA and unconjugated bile acids) production in the jejunum and ileum, depicting fore gut fibre fermentation, but butyrate levels were not significantly associated with the gut microbiota as observed in ZNO and RB4. The functional pathway predictions from caecal microbiota were closely associated with the distinct bacteria present in the caecum of the pigs across the diets. Put together, red beetroot has the potential to modulate the gut microbiota of weaned pigs with increased species richness, enhanced lipid metabolism and metabolite production. Future work focused on purified red beetroot components and dosage in weaned pigs are warranted.

Supplementary Materials: Table S1: Comparison of mean relative abundance of top bacteria genera between diet groups and gut location. **Figure S1**: Spearman correlation matrices between top abundant bacteria genera and short chain fatty acids in the (a) jejunum (b) ileum. **Figure S2**: Spearman correlation matrices between top abundant bacteria genera and bile acid levels in the (a) ileum (b) caecum.

Author Contributions: Conceptualization, OOA, HM, CB and ARM.; validation, ARM, CB and KMcD.; formal analysis, OOA, KMcD and CB.; investigation, OOA.; resources, CB and ARM.; data curation OOA, KMcD and CB.; writing—original draft preparation, OOA.; writing—review and editing, OOA, CB, ARM, KMcD and HGH.; visualization, OOA and KMcD.; supervision, CB, ARM and KMcD.; project administration, OOA, HM, CB and ARM.; funding acquisition, OOA, CB and ARM. All authors have read and agreed to the published version of the manuscript.

Funding: This research was funded by the N8AgriFood consortium and a PhD scholarship (OA) from the Commonwealth Scholarship Commission.

Institutional Review Board Statement: The animal study was approved by the Animal Welfare and Ethical Review Committee (AWERC) of the University of Leeds, UK (Approval number: N0. 070510HM)."

Data Availability Statement: The 16S rRNA sequences (fastq files) generated from the study have been deposited with the NCBI Sequence Read Archive (with BioProject ID: PRJNA798387). All data analyzed have been described in the manuscript and other statistical outputs are available in the supplementary files.

Acknowledgments: The authors appreciate the contributions of the technical staff at the National Pig Centre, University of Leeds and members of the animal bioscience and food bioactive research group. We would also like to thank Amy Taylor and Steve Laird for their contributions.

Conflicts of Interest The authors declare no conflict of interest.

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