Dietary effects of red beetroot on health and gut health of weaned pigs

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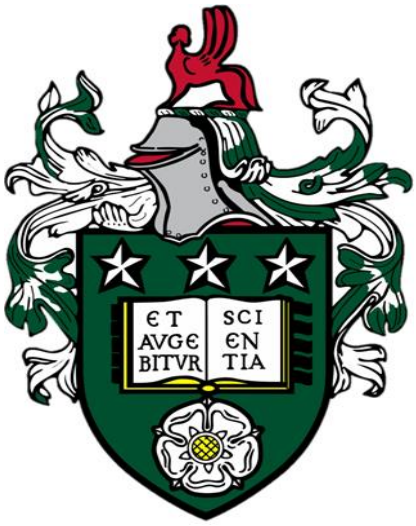
Submitted in accordance with the requirements for the degree of Doctor of Philosophy



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Declaration of Authorship

The candidate confirms that the work submitted is her own and that appropriate credit has been given where references has been made to the work of others.

Conference abstracts

Adekolurejo, O., McDermott, K., Miller, H., Mackie, A. and Boesch, C. 2021 '45. Impact of red beetroot supplemented diets on the gut bacterial composition of weaned pigs', *Animal - science proceedings*, 12, 35.

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Manuscripts in preparation for submission

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Unto, The King, Eternal, Invisible and the only wise God to Him be glory, honour, power and might, forever and ever, Amen.

Abstract

In the combat against antimicrobial resistance, weaned pigs' health and gut health has been impacted by reduction in antibiotics use and ban on in-feed zinc oxide (ZnO) in pig production. Fruits, vegetables or their waste are rich sources of bioactive compounds currently under investigation as animal feed resource to replace both antibiotics and ZnO. Red beetroot is an important example, with proven *in vitro* and *in vivo* anti-inflammatory, antioxidative, antimicrobial, anticarcinogenic, and hepatoprotective properties. However, empirical studies relating to its potential effect in weaned pigs are lacking. This thesis aimed to investigate the effects of red beetroot as a dietary supplement on health and gut health of weaned pigs, comparing outcomes with pigs fed a basal control diet (CON) and diet supplemented with pharmacological dose of zinc oxide (ZNO). Results showed that weaned pig diet supplemented with 2% red beetroot (RB2) preserved pig health, downregulated inflammatory cytokines and maintained gut microbiota metabolite production, compared to the control diet. RB2 diet also prevented exacerbation of weaning induced gastrointestinal changes and dysfunction. Diet RB2 behaved similar to ZNO, with improved gut microbiota diversity, functional pathways and reduced caecal *Escherichia coli* population. However, comparisons between these diets showed hepatoprotective ability of RB2 with increased hepatic antioxidant enzymes, while pigs fed ZNO diet presented predisposition towards hepatic oxidative stress and toxicity. Pigs fed an increased red beetroot level (4%) had high Proteobacteria abundance, with differentially abundant ileal *Enterobacteriaceae* and an upregulated pathway driving pathogenic *E. coli* and Shigellosis infection. A reduction in gut microbiota metabolite production (bile acids and short chain fatty acids) and an increased systemic pro-inflammatory cytokine interleukin-1B was also observed. Summarily, this thesis demonstrates *in vivo* anti-inflammatory, antioxidative and hepatoprotective characteristics of red beetroot, which may be linked to its betalain content and other bioactive components e.g., polyphenols. The similarities between RB2 and ZNO diets suggest, 2% red beetroot can potentially replace pharmacological doses of zinc oxide in weaned pig diet. Future research investigating the contributions of pure betalains and other components in red beetroot to health and gut health of weaned pigs are required.

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List of Abbreviation

ABTS	2, 2′-anizo-bis-(3-ethylbenzthiazoline-6-sulfonic acid)
ADFI	Average daily feed intake
ADG	Average daily gain
AMR	Antimicrobial resistance
ANOVA	Analysis of variance
BSA	Bovine serum albumin
BW	Body weight
CAT	Catalase
CD	Crypt depth
cDNA	Complimentary DNA
CLDN3	Claudin 3
CON	Control diet
Ct	Cycle threshold
DCP	Di-calcium phosphate
DNA	Deoxyribonucleic acid
EDTA	Ethylenediaminetetraacetic acid
ELISA	Enzyme linked immunoassay
ETEC	Enterotoxigenic *Escherichia coli*
EU	European union
FABP	Fatty acid binding protein
FAO	Food and agriculture organisation of the United Nations
FCR	Feed conversion ratio
FRAP	Ferric reducing antioxidant power
FVW	Fruit and vegetable waste
GC	Gas chromatography
GIT	Gastrointestinal tract
GPx	Glutathione peroxidase
GSH	Glutathione
HRP	Horseradish peroxidase conjugate
IFN	Interferon
IL	Interleukin
MDA	Malondialdehyde
mRNA	Messenger ribonucleic acid
MUC	Mucin

NCBI	National centre for biotechnology information
NF-κB	Nuclear factor-Kappa B
NGS	Next generation sequencing
NMDS	Non-metric multidimensional scaling
OCLN	Occludin
OUT	Operational taxonomic unit
PBS	Phosphate buffer saline
PERMANOVA	Permutational analyses of variation
PWD	Post weaning diarrhoea
qPCR	quantitative polymerase chain reaction
RB	Red beetroot
RB2	2% red beetroot diet
RB4	4% red beetroot diet
Rt	Room temperature
RNA	Ribonucleic acid
SB	Sugar beet
SBP	Sugar beet pulp
SCFA	Short chain fatty acid
SD	Standard deviation
SEM	Standard error of mean
SOD	Superoxide dismutase
TAC	Total antioxidant capacity
TJ	Tight junction
TNF	Tissue necrosis factor
VH	Villus height
ZNO	Zinc oxide supplemented diet
ZnO	Zinc oxide
ZO1	Zona occludens

Chapter 1 **: General introduction**

1.1 Background - Antimicrobial resistance; a silent epidemic

Antimicrobial resistance driven by the indiscriminate use of antibiotics in humans and food animals remain a serious threat to global public health (WHO, 2001; Gajdács and Albericio, 2019). Over the past decades, antibiotics use in animals have brought great health benefits. For instance, in animal production, antibiotics have been administered as therapeutics, prophylactic, metaphylactic, feed efficiency enhancers and growth promoters (Aarestrup, 2005). However, the routine and continuous use of antibiotics raises a public health concern of emerging antibiotic resistant genes in humans, animals and the environment (WHO, 2013; WHO, 2016; Van Boeckel et al., 2017; Boqvist et al., 2018).

Pigs are regarded as important source of antimicrobial resistant genes with inappropriate use of antimicrobials in pig production thought to drive AMR in humans. Although antibiotics are effective in reducing disease occurrences in pig production, an extensive application of in-feed antibiotics introduce a selective pressure that may cause lasting changes, or stable reservoirs of antibiotic resistant genes in animal associated microbiomes (Bengtsson and Greko, 2014; de Been et al., 2014). Consequently, the use of antibiotics in livestock production has been associated with the proliferations of multi-drug resistant pathogens and occurrence of antimicrobial resistant infections in humans (Marshall and Levy, 2011; Scott et al., 2018). There are reports of transmission of antibiotic resistant bacteria from animals to humans (McCrackin et al., 2016), and the identification of antibiotic resistant genes within microbiomes associated with the human-animal food chain and the environment (Forsberg et al., 2012; Hu et al., 2016).

Subsequently, pharmacological levels of zinc oxide (ZnO, 3000 mg/Kg) were permitted to replace in-feed antibiotics in pig production, especially in weaned pig diet, to alleviate post-weaning diarrhoea of young pigs. However, the use of ZnO and other metallic oxides as antimicrobials in livestock production co-selects for antimicrobial resistance associated with the proliferation of resistant bacteria species (Jensen et al., 2018) and serves as a major source of heavy metal pollution in the environment (from accumulation of metals e.g., zinc and copper). These have been linked to the persistence of methicillin-resistant

Staphylococcus aureus (MRSA) in food animals (Amachawadi et al., 2015; Malan et al., 2015; Duan et al., 2016).

Hölzel et al. (2012) observed correlations between Zn concentrations in pig manure and phenotypic antimicrobial resistance in *E. coli*. Similarly, in-feed zinc oxide (ZnO) used in pig production have been incriminated in the emergence of multi-drug resistant *E. coli* in food animals. An occurrence likely driven by bacteria resistance to zinc (metals) and antibiotics (co-selection) as well as enhanced plasmid uptake by conjugation during bacteria crosstalk in the host (co-regulation) (Bednorz et al., 2013; Yazdankhah et al., 2014).

Antimicrobial resistance is caused by deoxy-ribonucleic acid (DNA) mutations, horizontal gene transfer (HGT) via transformation of the cell membrane, transduction or conjugation of the nuclei content (plasmid) from antibiotic resistant bacteria to other bacteria cells (Munita and Arias, 2016). Transfer of antimicrobial resistant genes (ARG) conferred by transferrable plasmid may be influenced by the presence of metals (e.g., Zn, Cu) used in animal farming and aquaculture. Thus, Zn possess the potential to act as a selective pressure that forces and contributes to the proliferation of antibiotics resistance in the environment (Seiler and Berendonk, 2012; Slifierz et al., 2014). Consequently, antimicrobial growth promoters and pharmacological levels of in-feed ZnO have been prohibited many countries.

According to the World Health Organisation (WHO), an average of 10 million deaths from AMR is predicted by 2050, suggesting AMR is potentially a silent epidemic. Although AMR is not a leading cause of death and disability, it can complicate epidemic with rapid rise in cases. AMR is a chronic endemic condition requiring a long-term mitigation strategy (MacIntyre and Bui, 2017). Koch et al. (2017) proposed antibiotics need to be used more responsibly and findings from Bottery et al. (2017) indicated discontinuing antibiotics use inhibits resistance, whereas a continuous use promotes bacteria resistance.

At present, practical approaches to combat AMR, particularly in animal production are necessary. Considering the three main point of transfer, the human interface, animals and the environment (Figure 1.1), cutting-back on the indiscriminate use of antimicrobials in animal production will ultimately impede the spread of AMR. Many developed countries (UK, EU, USA) have formulated policies to curb this menace by a ban on antibiotics use as growth promoters in livestock production, including pigs.

In the EU, the use of antibiotics as growth promoters was prohibited in 2006 (Regulation (EC) No 1831/2003 of the European Parliament and the counsel on additives for use in animal nutrition 2003), and policies guiding the withdrawal of pharmacological doses of ZnO from pig production have now been actioned. While several countries have restricted antibiotics use in livestock, AMR remains a global concern considering the contribution of low and middle-income countries - LMIC (Darwish et al., 2013) and the health impact. Therefore, there is an urgent need for replacement, safer alternatives exploring the use of natural herbs, functional food, nutraceuticals, dietary manipulations, plants, vegetables with bioactive compounds and health promoting benefits.

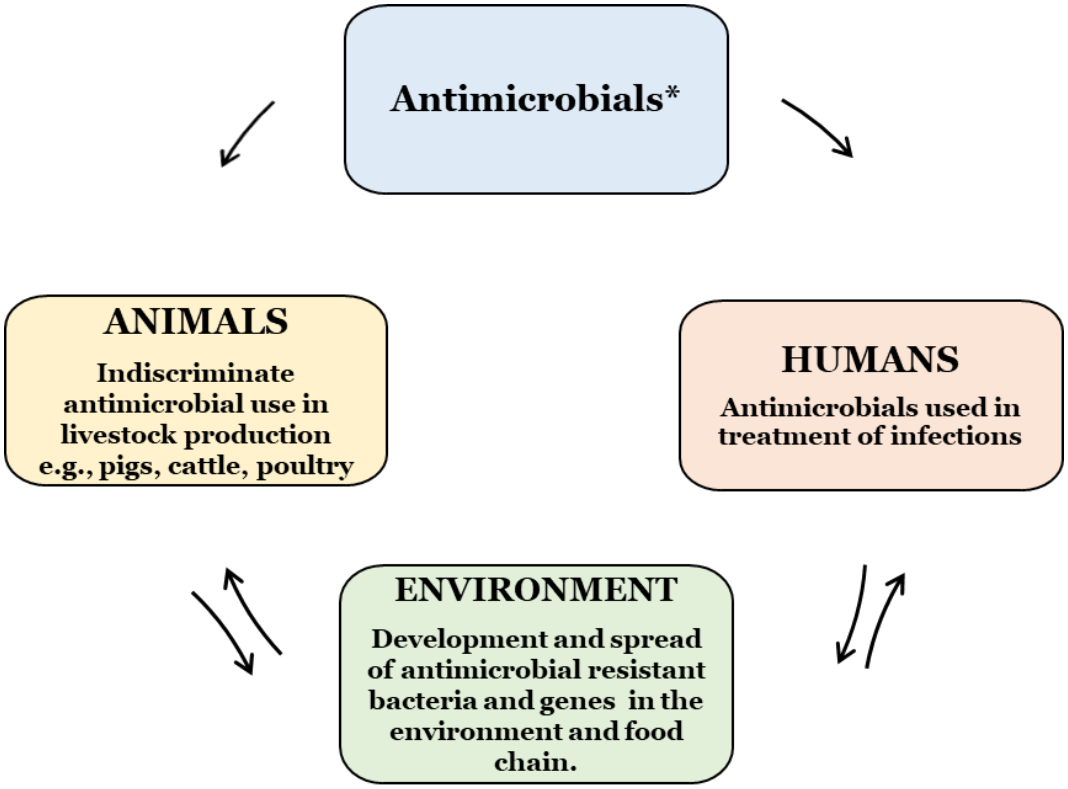


Figure 1.1: The main factors (humans, animals and environment) involved in antimicrobial resistance along the food chain. * Antibiotics (WHO 2001)

1.2 Alternatives to antibiotics use in animal production and their limitations

Several alternatives to antibiotic usage have been proposed in literature, including antibacterial vaccines, immunomodulatory agents, bacteriophages and their lysin, antimicrobial peptides (AMPs), probiotics, prebiotics, and symbiotic, phytogenic feed

additives and feed enzymes (Seal et al., 2013). Some other less traditional options such as clay minerals, egg yolk antibodies, essential oils, medium chain fatty acids, rare earth elements and recombinant enzymes were reviewed in Thacker (2013), each presenting different advantages and limitations.

Generally, anti-bacterial vaccines prevent bacterial infections, however in endemic situations vaccines compliment antimicrobial use, thus reduced antibiotic use and ZnO in pig production can be achieved by vaccination. Fricke et al. (2015) in their survey observed 60% reduction in colistin usage and 64% reduction in colistin combined with ZnO after vaccination against shigatoxin/oedema disease of young pigs. Truly, a few bacterial infections may be controlled by vaccines, however, vaccine development is very expensive and impracticable in low-medium income countries (Zhang and Sack, 2012).

Other examples such as immune-modulators and feed enzymes are neither bactericidal nor bacteriostatic; they preserve animal health (Cheng et al., 2014). Bacteriophages have also been used in food production, however with limited applications due to narrow host-strain specificity (i.e. strain dependent) and potential development of phage resistance following repeated exposure to the phage and emergence of unrelated members of the target bacteria genus /species (Endersen and Coffey, 2020; Vikram et al., 2021)

Similarly, there are reports of adverse reactions with the use of some plant extracts and enzyme preparations (Sarcia and Hebraud, 2005), as well as setbacks in the use of probiotics in livestock production. Particularly, reduced populations of gut commensals, inactivation during in-feed processing, inability to attain high number of viable cells to colonize the gut and lack of standard dosage have resulted in varying effect of probiotics and safety risks (Cheng et al., 2014). Biofilm inhibitors have also showed good results, yet only when used in combination with antibiotics and discontinued usage led to further production of bacterial biofilms (Lewis, 2001). Although AMPs have been shown to curb bacterial infections, their high cost and narrow action spectrum have restricted their wide use, they tend to induce bacterial resistance by altering the primary target of bacteriocins (Belfiore et al., 2007).

There is a long and growing list of alternatives under evaluation for their ability to replace in-feed antibiotics in livestock/pig production. Many studies have explored the potentials of these alternatives to replace antibiotic use in food animal production. However, existing evidence has demonstrated inconsistent contradictory findings regarding their potential use

as viable alternatives (Thacker, 2013). While combinations of some of these alternatives were thought to offer superior outcomes, Santana et al. (2015) observed similarities in the performance, pH of the digestive contents, organ morphometry or histology of the intestinal epithelium when weaned pigs were fed a combination of sodium butyrate, plant extracts and nucleotides. Hence, there is need for more research exploring other potential candidates.

1.2.1 Current trends in pig production and antibiotic use

Pigs are among the most important livestock species in the world and a major source of meat for human consumption (FAO, 2014). According to reports from the OECD-FAO 2019-2028 (OECD/FAO, 2019), meat from pig (pork) has been projected as a pivotal nutritional source of animal protein in many parts of the world. To meet the world's increasing demand for pork, there has been a surge in production. Hence, there is need to identify the critical phases in pig production and strategies for optimal pig performance in the face of a growing world demand and population.

Pig production is faced with varied challenges stemming from climate change to farm waste management and increased cost of production from the current global energy and economic crisis. For pigs raised in an outdoor production system, the spread of faeces and waste to surrounding neighbourhood can constitute a menace (environmental pollution). Consequently, an increased aerosol transmitted disease burden often characterises the intensive/indoor pig production systems (Park et al., 2017). Stärk (2000) observed that faecal excretions from infected animals, high stocking density, contact between susceptible animals, causative microorganisms and a shared airspace are factors required for disease transmission in pigs reared intensively. As a result, antibiotics were historically used to reduce disease burden and increase production.

Globally, pig production has become industrialised especially with rapid shift from outdoor, backyard production to intensive, indoor confined system. Asia (China) is responsible for more than 50% of the world's pig population (50 million metric tons of pork per year), followed by the European Union (EU) and United States of America (USA) (Statista, 2022). Given the importance of pig production on a global scale, understanding the trends in antibiotics use in pig production is crucial to addressing AMR. The pig microbiome has been implicated as an important repository for diverse resistance genes (Looft et al., 2012), which makes the control

of antimicrobial use in pig production critical to curbing the development of resistant species and genes in the environment and food chain.

Highlighting the extent of antibiotics use in various phases of pig production, Lekagul et al. (2019), in their review of antibiotics use pattern reported in scientific literature between 2000 and 2017, concluded that antibiotics were mostly administered as oral medication during suckling and post-weaning periods. Weaned pigs are often treated in groups during diarrhoea episodes, using medication in drinking water without any diagnostic test (Timmerman et al., 2006; Filippitzi et al., 2014). Hence, antibiotics administered (up to 93%) were mainly as prophylaxis (preventive measure) with barely 7% used as treatment of clinically healthy pigs (metaphylaxis), very few studies clearly distinguished between metaphylactic and prophylactic antibiotic use (Callens et al., 2012).

The farm size and type also played a significant role in the extent of antibiotic use; finishing and large farms used more antibiotics than small and farrow-to-finishing farms (Matheson et al., 2022). In a study conducted by Casal et al. (2007), 58% of farms surveyed used oral or in-feed antimicrobial prophylaxis and administered more than one compound, routine use of growth-promoters was significantly related to the production system, hence more frequently used in fattening units. Also, Kim et al. (2013) in a study conducted in Vietnam, observed comparable levels of antibiotics use across the various phases of pig production (piglets, fatteners and sows).

Further evidence from literature revealed that weaned pigs received more antibiotics than pigs in other production phases (Sjölund-Karlsson et al., 2011; Jensen et al., 2012; Fertner et al., 2015), especially as medicated feed for growth promotion. In Canada, 27% of antibiotic treatments were for multiple systemic infection (Glass-Kaastra et al., 2013). The lowest antibiotic use was reported in France and Sweden especially for fatteners with about 17% of total use for all the phases (Jensen et al., 2012). In a French pig veterinarian-based survey, 10% of antibiotics prescriptions were for treatment of disease conditions including post-weaning *E. coli* infection, however the study failed to specify the production phase in question (Chauvin et al., 2002; Sjölund et al., 2016). These findings highlight the increased use of antibiotic in pig production especially during the weaning and post-weaning period. Ultimately, finishing units were 84% to 94% more likely to use antibiotics than those in the farrow- to- finish units (43% - 92%) according to Merle et al. (2012). This suggests that although study environment, practises and policies may differ, AMR remains a global threat.

Given the current extent of antibiotics use in pig production and the emerging threat of AMR, there is need for continued global efforts to reduce antibiotics use in pig production and proffer viable alternatives.

1.3 Weaning, weaning transition and post-weaning diarrhoea (PWD) of pigs

Weaning is a transitory period in the course of a pig's life, where the young pig undergoes series of dietary, societal and environmental changes (Lallès et al., 2007). It is a crucial and stressful phase as piglets are withdrawn from the sow and solid feed replaces a milk-based diet. Weaning transition takes a longer process for pigs raised in their natural environment without human intervention, however for pigs raised intensively and for commercial purpose it is abrupt and ranges between 3 - 5 weeks, depending on the process adopted by the farm (Eriksen et al., 2021).

Generally, weaning is characterised by a decline in colostrogenic and lactogenic immunity from abrupt discontinuation in suckling, separation to a nursery environment and adaptation to solid feed, which induces stress and increases disease susceptibility of weaned pigs (Heo et al., 2013; Campbell et al., 2013; Poonsuk and Zimmerman, 2018). The change in diet to a more complex feed at weaning induces low intake in the first week post-weaning, causing anorexia, which has been strongly associated with the risk of disease occurrence. Weaning anorexia, contributes to lack of nutrients, gut dysbiosis that may upset the gut integrity leading to increased intestinal permeability and susceptibility to post-weaning diarrhoea. Therefore, weaned pigs are not only faced with adapting to a new environment and pen mates, but gastrointestinal changes (e.g., villus atrophy, crypt hyperplasia, microbiota shift) that may negatively affect digestive capacity and immunity (Barszcz and Skomial, 2011; Moeser et al., 2017; Guevarra, et al., 2018).

Additionally, the gastrointestinal tract, microbial community and mucosal immunity of the young pig is underdeveloped, hence the gut health and other gastrointestinal functions (e.g., absorption, barrier function and permeability) may be greatly compromised (Pluske 2016; Gresse et al., 2017; Moeser et al., 2017; Pluske et al., 2018). These stressful events accompanying weaning have been reported to cause disruption and alteration of the pig gut microbial composition, leading to post-weaning diarrhoea and other enteric infections (Lallès et al., 2007). Overall, the post-weaning period is characterised by poor piglet performance and health as young pigs become increasingly vulnerable to pathogenic invasion that often

require antibiotics protection and/or in-feed ZnO to weather through (Pluske et al., 2019; Campbell et al., 2019).

Post weaning diarrhoea (PWD) is a multifaceted condition that is alleged to evolve from the interactions between piglets and the sow before weaning, the environment and infectious agents e.g., bacteria, viruses. Its occurrence at weaning, the most pivotal phase of pig production, makes PWD one of the most economically relevant enteric disease with resultant financial loss globally (Hong et al., 2006; Rhouma et al., 2017; Eriksen et al., 2021). PWD affect pigs during the first 14 days post-weaning, it is characterised by sudden death, profuse diarrhoea, dehydration, significant mortality (up to 20% - 30%), weight loss and growth retardation in surviving piglets (Amezcua et al., 2002; Fairbrother et al., 2005). Consequently, increasing the potential of antibiotics use, cost of therapy and production.

Many pathogens and enterotoxigenic *Escherichia coli* (ETEC) can initiate PWD in pigs. ETEC can produce enterotoxins and adhesins that enhance microbial attachment and toxin release into the intestinal lumen. Predominantly described in literature is ETEC strain 0149 or combinations of other 0149 serotypes designated as heat-labile toxin (LT), heat-stable toxins: STa, STb and entero-aggregative heat-stable toxin (EAST1:F4ac) (Gyles and Fairbrother, 2010; Delisle et al., 2012). PWD can also be caused by protozoan parasites like coccidia, and *Cryptosporidium parvum,* and viruses (e.g., rotavirus, sapovirus) (Luppi et al., 2016; Eriksen et al., 2021), which may co-exist with bacterial infection, exacerbating PWD of young pigs and complicating disease prognosis. Although, these parasitic infections are still endemic in some LMIC, the importance of laboratory diagnosis prior to treatment cannot be underestimated in control and prophylactic measures against PWD, to avoid inappropriate antimicrobial use. A recent fall-out of the ban on ZnO in weaned pig diet is research efforts geared towards the development of point-of-care based test and rapid diagnostic techniques for identifying the aetiology of PWD (Malik et al., 2019; Jakobsen et al., 2022; Olech, 2022).

In recent times, non-ETEC post-weaning diarrhoea with or without haemolytic *E. coli* excretion have been increasingly reported (Luppi et al., 2016; Pluske 2016). Depicting a shift in the range of pathogens incriminated in PWD, with the gut microbial composition also deemed to contribute significantly to the occurrence and prevention of PWD (Gresse et al., 2019). To further complicate the occurrence of PWD, weaning induced intestinal dysbiosis predisposes weaned pigs to intestinal inflammation and diarrhoea (Gresse et al., 2017). Intestinal dysbiosis is characterised by gut microbiota alteration with remarkable decrease in

obligate anaerobic bacteria (e.g., *Prevotellaceae, Clostridiaceae*, *Actinobacteria*), and increase abundance of facultative anaerobes e.g., *Enterobacteriaceae* (Winter et al., 2013). These obligate anaerobes are important fibre fermenters responsible for gut metabolites production (e.g., short chain fatty acids - SCFA) found to increase with dietary supplementation of weaned pig feed with prebiotics, probiotics, dietary fibres, phytochemicals and plant bioactives. They modulate the gut microbiota to reverse/prevent adverse microbiota changes resulting from weaning transition (Guerra-Ordaz et al., 2014; Wang et al., 2018).

Therefore, weaning remains a critical hazard point in pig production, with potential threat to pig growth, health and performance. In addition, there exist a challenge to maintain gut health and functionality, prevent gut microbiota disruption in efforts to avoid economic losses associated with the weaning transition and post-weaning period. Nonetheless, in the likely occurrence of PWD, strategies focused at alleviating stress and improving gut health without promoting bacterial resistance or proliferation of resistant bacterial genes should be prioritized.

1.3.1 Weaning associated changes in the gastrointestinal tract

The gastrointestinal tract is the site for nutrient digestion and absorption, crucial to the gut health and immune functions of pigs. After weaning, the GIT undergoes morphological, immunological and microbial modification in adaption to changes induced by weaning and weaning stress. According to Burrin and Stoll (2003) and Montagne et al. (2007), weaning changes associated with the intestinal physiology were summarised into an acute phase characterised by deterioration of the gut structure and function, observed between 0 - 5/ 0 - 7 days post weaning, and an adaptive phase (5 - 15 / 7 – 14 days), where the gut adapt to the weaning diet which follows subsequently (Figure 1.2).

Although, this division was based primarily on changes in feed intake, each phase is characterised by several physiological, structural and clinical features that culminate to impair gut health and development. Changes in the gut morphology are characterised by decreased (atrophied) villus height, increased (hypertrophied) crypt depth and reduced villus height to crypt depth ratio (Pluske et al., 1997; Boudry et al., 2004). Hampson (1986) demonstrated rapid villi atrophy up to 25 - 35% within 24 hrs of weaning in 21-day old pigs. Impaired nutrient absorption, loss of matured enterocytes and reduced brush boarder digestive enzyme activity have been reported in many studies (Lallès 2010; Lackeyram et al., 2010). Changes in

hormonal zinc activity, reduced gastric motility and increased permeability to antigens and toxins were also emphasised in Heo et al. (2013) and Bomba et al. (2014).

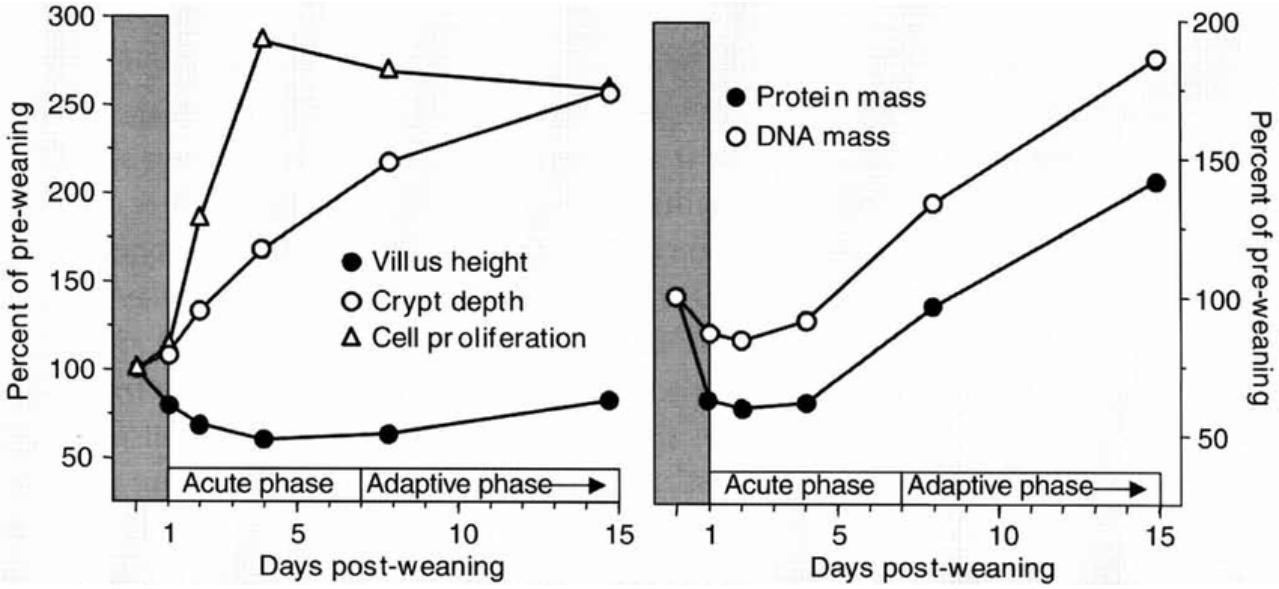


Figure 1.2: Acute and adaptive gastrointestinal development phase in weaned pigs *(Source: Burrin and Stoll, 2003).*

Weaning induces a structural breakdown in the intestinal barrier via disruption of the tight junction proteins (e.g., Zona occludens, Claudins and Occludin) expressed by the epithelial cells, which increases the intestinal permeability, promoting pathogen entry and inflammatory responses. The intestinal barrier is primarily responsible for preventing loss of electrolytes, salts and water, thus enabling host-gut homeostasis (Bischoff et al., 2014). An increased intestinal permeability (i.e., leaky gut) is the common pathway to diarrhoea occurrence in weaned pigs, although many other factors have been observed to contribute imperceptibly to the development of diarrhoea.

Importantly, the connection between the gut microbiota, intestinal barrier function and inflammation have been recognised, with improved growth performance and reduced diarrhoea occurrence found to be associated with increased SCFA production, especially butyrate/butyrate-producing bacteria (Ma et al., 2012; Grilli et al., 2016). The loss of gut microbiota diversity during the weaning transition has been linked to increased intestinal permeability with higher susceptibility of toxins and pathogens crossing through the intestinal epithelium.

Conversely, many studies have attributed improved feed intake, growth performance and reduced PWD to dietary supplementation with butyrate sources. The benefits of butyrate confirmed *in vitro* and *in vivo* are mediated by the impacts on the intestinal barrier function, via the regulation and repair of damaged intestinal epithelium, and as an energy source for mucosal cell development (Plöger et al., 2012; Furusawa et al., 2013; Han et al., 2020). Butyrate inhibits the expression of pro-inflammatory cytokines (interleukin 6, 17, and NF-kB signalling pathways), modulate inflammatory cytokines and tight junction proteins along the gut, thus it possesses anti-inflammatory potential that enhances intestinal permeability, barrier function and prevents oxidative stress (Wang et al., 2018; Wang et al., 2019). The ability of butyrate to mediate host appetite and immune function through the gut microbial composition (Zhong et al., 2019; Wu et al., 2023), inhibit enteric pathogens e.g., Enterobacteriaceae (Namkung et al., 2011) and promote overall gut health (Huang et al., 2015; Wang et al., 2020) have all been documented.

The gastrointestinal and systemic immune response to weaning involves alteration in the production of inflammatory cytokines and activation of inflammatory response pathways. Pro-inflammatory cytokines exert influence on the intestinal integrity and epithelial functions in relation to permeability and nutrient transport as previously alluded to. Tumour necrosis factor (*TNF*), a pro-inflammatory cytokine is a key mediator of intestinal inflammation, inducing increased tight junction permeability and reported to promote predisposition and exacerbation of gastrointestinal dysfunction (Ye et al., 2006). Pié et al. (2004) also demonstrated up-regulation of pro-inflammatory cytokines (e.g., interleukin (IL) - *IL1B*, *IL6* and *TNF*) in pigs weaned at 28-days old. An increased expression of pro-inflammatory genes has been linked to poor pig performance and responsible for the course of post-weaning diarrhoea (Campbell et al., 2013). More so, Zeng et al. (2016) and Wei et al. (2017) revealed that gut inflammation supports increased intestinal *Enterobacteriaceae* (*E. coli*) population and reactive oxygen species (ROS) 7 days after weaning.

Usually, the stressful effect of weaning is expected to abate within a short period (3 - 5 days) however if unresolved, gut inflammation and increased expression of pro-inflammatory cytokines can severely impact host metabolism. The GIT is the major site for amino acid oxidation and utilization of amino acids (nutrient utilisation) needed for protein synthesis (Burrin and Stoll, 2003; Bauchart-Thevret et al., 2009; Ren et al., 2015). Unavailability of nutrients via reduced feed intake, alters the GIT structure and amino acid metabolism during

the weaning transition, impeding protein synthesis and reducing lean tissue deposition (Feder and Hofmann, 1999; Campbell et al., 2013). According to figure 1.2, the change in DNA mass was associated with the change in protein mass, which may explain the growth check (about 50% reduction in pre- weaning weight) observed during the first few days post weaning (acute phase).

Aside the physiological and GIT changes associated with weaning, the abrupt change in diet and environment at weaning causes a shift in the gut microbial composition, mostly evidenced by reduced microbial richness and diversity (Bauer et al., 2006; Voreades et al., 2014). The gut microbiota interacts with the host to modulate biological processes essential for health. However, the gut microbiota of weaned pigs can also be modulated by dietary interventions aimed at promoting gut health via increased fermentation and production of gut signalling molecules (SCFA e.g., butyrate) that aid prevention of gut inflammation and enteric pathogen invasion (van der Beek et al., 2017; Xiong et al., 2019). This emphasizes the importance of evaluating the functional properties of dietary supplements and the benefits of supplementation of weaned pig diet to achieve optimal response.

Dietary and managemental interventions post-weaning is necessary to curb weaning induced deleterious effects. It is well established that diet is a major factor that shapes the gut microbiota, and numerous literature have reported on interventions using dietary fibres, proteins, feed additives and other feed ingredients focused at maintaining gut morphology, functionality (digestive and absorptive capacity, immunity) and health (Li et al., 2018; Choudhury et al., 2021). Modulation of the gut microbiota presents an attractive approach for increased gut butyrate production and other healthy microbiota metabolites. This will strengthen the immune system to fight pathogenic intrusion, enhance growth performance despite prevailing weaning changes and stress. Due to age-related changes in the gut microbiota and influence on growth performance and development, the timeliness of dietary interventions for weaned pigs is essential to shorten debilitating effects during the acute phase. This re-echoes the argument of providing creep feed for young pigs prior to weaning.

Dietary interventions should be accompanied with adequate managemental techniques, importantly ensuring optimum biosecurity measures are in place, and an all-in-all production to curtail disease spread. Intensively raised weaned pigs should be housed ideally in flat decks and well-constructed pens as against manure and soiled beddings, with easy access to feeding troughs and drinkers, ensuring that weaned pigs have opportunity to feed

immediately after weaning. It is necessary to provide adequate temperature in the nursery facility (26 - 28°C) to prevent heat stress or hypothermia, low temperature have been reported responsible for severe course of PWD (Rhouma et al., 2017).

1.3.1.1 Gastrointestinal microbiota; from birth to weaning

The microbiota of the GIT describes a dense, dynamic and highly complex community of microorganisms that colonize the gut, containing mostly bacteria, estimated to be over 100 billion (10^{14}) (Leser and Molbak, 2009). The gastrointestinal tract is in close interaction with the host and other members of the microbial community and has been reported to comprise over a 1000 bacterial species, with more than a 100-fold number of genes in the mammalian genome, responsible for numerous biological functions in the host (Bäckhed et al., 2005).

The gut microbiota serves as a functional organ that regulates many physiological functions in the host such as, digestion, growth modulation, immune system maturation (Rescigno, 2014), metabolism (Davila et al., 2013) and prevention of infections (Lee and Hase, 2014; Marchesi et al., 2016). It links the host immune system exerting control on many pathways that modulate the functionality of organs and systems (e.g., the brain and the immune system) thus contributes to the host homeostatic regulation (Dietert and Silbergeld, 2015; Celi et al., 2017).

The pig gut microbiota is a complex, dynamic ecosystem, undergoing compositional changes under the influenced of diet, environment and the host genome (Isaacson and Kim, 2012), additionally these factors significantly affect the functions and development of the intestinal microbiota. Early interventions and establishment of the gut microbiota provide stability to the gut and has been shown to enhance maturation of the intestinal immune system, barrier function and consequently the health and growth of pigs (Everaert et al., 2017; Schokker, Dirkjan et al., 2018; De Vries and Smidt, 2020). Therefore, early life establishment of a stable microbiota in crucial to the development of pigs. Depending on the environment, piglet gut microbiome modulation can be targeted by modulating the sow's gut microbiota and milk composition during gestation or lactation (Baker et al., 2013; Cheng et al., 2018).

Prior to birth, the gut of the neonate pig is believed to be germ-free, however at birth, piglets are exposed to a variety of bacteria from the birth canal, vagina, faeces and environment of the sow. The vaginal microbiota is usually influenced by the sow's faeces and has been found to contribute significantly to the early colonization of the intestinal microbiota of the piglets

(Nowland et al., 2019). Following birth and commencement of intestinal nutrition, the maternal milk becomes the main source of energy, nutrients (lactose, milk oligosaccharides, amino acids, fats) and substrate activating digestive functions which also alters the gut microbial composition greatly (Liu et al., 2019).

During the post-natal period, piglets show increased Bacteroides and *Rumminococcaceae* abundance with milk bioactive constituents (e.g., immunoglobulins, antimicrobial and anti-inflammatory factor) also contributing to the establishment and development of the pig's immature immune system and gut microbiota (Thai and Gregory, 2020). Additionally, Choudhury et al. (2021) reported increased *Ruminococcus*, *Lachnospiraceae*, *Roseburia* and *Prevotella* in the colon of suckling pigs on creep feed, a microbiota shift that was associated with post-weaning intestinal development.

However, at weaning, pigs undergo stress that may lead to gut microbiota disruption and dysbiosis which may impede the functions of the microbiota predisposing weaned pigs to gastrointestinal infections and dysfunctionality (Gresse et al., 2017). The microbiota shift (change) at this stage is attributed mainly to the abrupt transition from liquid to a solid (plant-based) diet, which affect the physicochemical conditions of the digesta and substrate availability for energy metabolism (Dunne-Castagna et al., 2020; Meng et al., 2020). As the pig progress in age, the gut microbiota undergoes microbial succession until a climax (stable) community is achieved, usually from start of solid diet intake till the finishing phase (Honda and Littman, 2016).

1.3.1.1.1 Weaning transition and gut microbiota changes

Weaning induces qualitative and quantitative changes in the gut microbiota, which has been linked to PWD in many studies (Costa et al., 2014; Pop et al., 2014). Understanding the pig gut microbiota dynamics during weaning transition contributes to the overall health, growth performance and well-being of the pigs. This will provide insights into efficient management of pig health and gut health during the weaning transition. There are evidence suggesting a decreased *Lactobacillus* population and increased population of pathogenic bacteria species (e.g., *Escherichia*), a hallmark difference between healthy and diarrheic pigs, consequently, exposing young pigs to increased risk of GIT infections (Fouhse et al., 2016; Dou et al., 2017; Yang et al., 2019).

Lactobacilli are core genera of a healthy pig gut microbiota alongside other fibre-fermenters, with members identified to promote overall health and growth performance. They influence the intestinal physiology, immune system, enable a balance intestinal ecology (Niu et al., 2015; Yang et al., 2019) and induce resistance to GIT infections, especially species with probiotic properties (Valeriano et al., 2017). Zhao and Kim (2015) observed improved gut microbial balance and diarrhoea reduction by high *lactobacilli* to *enterobacteria* ratio, therefore, abundant gut lactobacilli increase the population of other beneficial bacteria (e.g., *Bifidobacterium* and *Faecalibacterium*), competitively preventing colonisation of the gut by pathogenic bacteria species (Liu et al., 2014; Wang et al., 2019).

The host genome also contributes significantly to the gut microbiota shift observed in the early pig life and weaning, it determines the susceptibility or resistance of pigs to post weaning infections. Surprisingly, high abundance of Firmicutes and Bacteroidetes were observed as core gut phyla regardless of the breed, *Lactobacillus* was predominant at the genus level and *Prevotella* was prevalent in the caecum. However, Firmicutes was more abundant in Landrace pigs than in Duroc and Yorkshire (Pajarillo et al., 2014). Predisposition of weaned pigs to gut microbiota shifts leading to post weaning diarrhoea have also been linked to host genetic background with particular reference to the presence of intestinal receptor for ETEC Fimbriae; (F4 and F18) (Ren et al., 2012; Schroyen et al., 2012; Riis et al., 2018).

Similarly, antibiotic use during the weaning period can cause further disruption to the gut microbiota stability and diversity, which may favour intestinal inflammation. Especially, when broad-spectrum antibiotics are used, they possess the ability to destroy or prevent both pathogenic and beneficial microbes in the gut (Zhang et al., 2016; Dou et al., 2017). Notably, Bacillaceae and *Lactobacillaceae* bacteria families with species known to produced different antimicrobial compounds (e.g., bacteriocins, lantibiotics, non-ribosomal peptide synthetase) were found depleted in antibiotics treated animals according to (Lan et al., 2017). Consequently, antimicrobial compounds may exacerbate enteric infections and other adverse effects of weaning transition on the GIT (Gresse et al., 2017).

A striking increase in Proteobacteria (1 to 11%) was observed in control pigs against antibiotics treated pigs in Looft et al. (2012) on day 14, from which *E.* coli resistant genes were identified in 62% of the population. The same study also reported decreased Bacteroidetes (genus; *Prevotella*) against an increase that characterises a normal swine

microbiome. Similar changes in gut microbiota populations with antibiotics were demonstrated in many studies (Lamendella et al., 2011; Kim et al., 2012). Li et al. (2017), after a 28-day experimental period, observed slight increase in Firmicutes in the jejunum and ileum but reduction in the caecum and colon. Meanwhile, age dependent changes with antibiotics use, of increased Bacteroidetes (6.5% to 33%) from day-15 onwards and a decrease in Proteobacteria (13.8% to 1.7%) from day 0 to 37 post weaning was reported in (Soler et al., 2018). Antibiotics treatment significantly altered ileal and faecal SCFA concentrations in comparison to the control group in Pi et al. (2018) with reduced bacteria phyla Firmicutes and Bacteroidetes.

1.3.1.2 Gut microbiota metabolites

The gut microbiota wields its function on the host physiology through signalling molecules produced from the digestion of substrates in the gut. These products termed metabolites, play important role in host-microbiome crosstalk and are intermediate or end products of microbial metabolism of dietary substrates in the host, modified molecules produced by the host (e.g., bile acids), or directly by bacteria regulation (Gill et al., 2021). Some metabolites are produced exclusively by the gut microbiota (e.g., short chain fatty acids - SCFAs and secondary bile acids), while other like polyamines, indoles and phenols are produced by interaction between the gut microbiota and the host cells. Gut microbiota metabolites influence immune maturation, immune homeostasis, host energy metabolism and maintain the intestinal integrity (Arpaia et al., 2013; Fan and Pedersen, 2021).

The gut microbiota is a complex system with each bacterial species and strain capable of performing specific functions resulting in inter-individual variations in metabolic output. Alterations in the gut microbiota have been found to influence metabolic output and functions in the host and has been implicated in the aetiology and pathogenesis of many diseases (e.g., autoimmune, cardiovascular diseases, Crohn’s and ulcerative colitis in humans) (Ottman et al., 2012; Perez-Lopez et al., 2016; Zhou et al., 2021). Diseases can cause alteration in the gut microbiota metabolites, where the metabolite serves as a biomarker for diagnosing the disease. However, changes in the gut microbiota can incite diseases (metabolic disorder), depicted through deviations in the metabolite profile, hence a risk factor for that disease (Ma et al., 2022). Accordingly, the gut microbiota metabolite profile can be used to uncover differences in the gut microbiota of healthy and diseased subjects and are

detectable in a wide range of biological tissues including digesta, faeces, blood, tissue homogenates and liver (Holmes et al., 2011; Krishnan et al., 2018).

The composition and diversity of the gut microbiota of weaned pigs is highly impacted by diet, importantly, the level and sources of dietary protein and fibre offered to pigs post weaning (Huting et al., 2021; Gao et al., 2022). Varying the level of crude protein in the diet influence by-products of amino acid fermentation. Dietary composition and supplementation of weaned pig diet also influence the gut microbiota composition causing increases or depleted production of metabolites and corresponding biological response, owing to the growth or inhibition of certain groups of enteric microorganisms (Vasquez et al., 2022). In-feed antibiotic growth promoters and antibiotics treatment can alter the gut microbiota metabolism of lipids, bile acids, amino acids and amino acid related substances in the gut (Becattini et al., 2016).

Notably, dietary fibre has been described responsible for the production of gut microbiota metabolites especially short chain fatty acids (SCFA), due to its fermentability in the lower segment of the gut (Heinritz et al., 2016). The potential benefit of dietary fibres on the gut health and as therapeutic agent in gastrointestinal disorders is attributed mainly to its ability to regulate digestion, improve glycaemic and lipidemic responses, limit bile acid resorption by plasma cholesterol regulation, influence digesta transit, and enhance microbiota composition and fermentation (Holscher, 2017; Gill et al., 2021). The role of dietary fibre in the control of PWD of pigs may have stemmed from these health promoting functions and metabolites production.

Similarly, plant polyphenols have been reported to act as substrate for microbial production of phenolic acids and SCFAs, the gut microbiota transformation of polyphenols into these metabolites and subsequent modulation of the gut microbiota composition and metabolism yields beneficial health outcome in the host (Tuohy et al., 2012; Ozdal et al., 2016). Two main metabolites produced by the gut microbiota (short chain fatty acids and bile acids) are discussed further.

1.3.1.2.1 Short chain fatty acids (SCFAs)

Short chain fatty acids (SCFAs) are microbial metabolite produced from the fermentation of indigestible polysaccharides (e.g., fibre and resistant starch) (Louis and Flint, 2017; Nakatani et al., 2018). In cases of low dietary fibre diet, branched chain fatty acids (mainly isobutyrate and isovalerate) are produce by fermentation of amino acids to provide energy for intestinal

cells. SCFA have been shown to possess antimicrobial effects against pathogenic bacteria species by decreasing the gut pH (Trefflich et al., 2021), an important function of SCFAs in maintaining gut health is mainly attributed to butyrate. Their production depends critically on the availability of substrate (fibre) for fermentation and the gut microbial composition (essentially, fibre fermenters in the phylum Firmicutes). SCFAs perform key mediatory role, bridging interactions between the gut microbiota and the immune system, regulating host metabolism and cell proliferation. They influence the adaptive immune response of the host by T-cell regulation (Smith et al., 2013). Likewise, in many studies, SCFAs (e.g., butyrate) have been credited with maintaining the intestinal barrier, regulating appetite by stimulating gluconeogenesis in the liver and have been widely used as feed additives (Tan et al., 2014; Diao et al., 2019).

SCFA comprise mainly acetate, propionate and butyrate, found in high concentrations in the caecum and colon. Acetate is the main SCFA essential for the growth of many gut bacteria, and an energy source for muscle and adipose tissues as well as substrate for cholesterol metabolism, lipogenesis and satiety regulation (Jocken et al., 2018). Acetate reduces inflammation by suppressing activation of inflammatory cytokines via G protein-coupled receptors 43 (GPR43) a key receptor for SCFAs (Xu et al., 2019). Propionate is absorbed through the portal vein to the liver and converted to glucose through hepatic gluconeogenesis. It also contributes to the regulation of inflammation and metabolic disease (Nishitsuji et al., 2017). Whereas butyrate is the main source of energy and growth for the enterocytes. It is a cellular mediator, regulating many functions of the gut cells including gene expression, differentiation, gut tissue development, immune modulation, oxidative stress reduction and diarrhoea control (Bedford and Gong, 2018).

Butyrate producing bacteria represents an abundant phylogenetically diverse group of bacteria in the colon e.g., *Faecalibacterium prausnitzii*, *Eubacterium rectale* and *Roseburia intestinalis* within the phylum Firmicutes. While bacteria belonging to the phylum Bacteroidetes are known for their ability to produced acetate and propionate, lactic acid bacteria (e.g., *Lactobacillus*, Streptococcus) produces lactate and succinate which can be converted to propionate by the gut microbiota (Brestenský et al., 2017). Accumulated evidence from past studies have shown improved growth performance, intestinal integrity and pig health with supplementation of weaned pig diet with butyrate (Huang et al., 2015; Zhong et al., 2019). Butyric acid and its derivatives (e.g., sodium butyrate and butyrate

glycerides) are reported to show strong antimicrobial activity against gram-positive and negative pathogenic bacteria *in vitro* and *in vivo* (Namkung et al., 2011). The ability of butyric acid to protect host against intestinal injury and leaky gut by promoting tight-junction formation as well as regulate T-cell inflammatory response (gene expression) by inhibition of histone deacetylase have been highlighted in literature (Kim et al., 2014; Grilli et al., 2016).

1.3.1.2.2 Bile acids

Bile acids are hydroxylated steric acids, produced in the liver from cholesterol metabolism, and stored in the bile sac pending released into the gut during digestion. They play integral role in digestion, lipid absorption, cholesterol metabolism and uptake of fat-soluble vitamins (Wahlström et al., 2016). Primary bile acids (cholic and chenodeoxycholic acid) synthesised in the hepatocytes are released into the small intestine, majority of which are reabsorbed in the distal ileum via enterohepatic circulation and conjugated (with taurine or glycine) in the liver (conjugated primary bile acids e.g., taurocholic, glycocholic acids). However, about 25% of the primary bile acids can seep further down the intestine where they are deconjugated (mainly in the small intestine) and dehydroxylated (large intestine) into secondary bile acids (deoxycholic and lithocholic acid) by the gut microbiota.

Gut microorganisms (e.g., *Lactobacillus, Clostridium, Bacteriodes and Bifidobacterium*) deconjugate bile acids via bile salt hydrolases (BSH) enzymes to produce free primary acids, these are then transformed into secondary bile acid by 7α-hydroxylation. Secondary bile acids are absorbed into the blood to modulate hepatic and systemic lipid and glucose metabolism through the nuclear (Farnesoid X receptor) - FXR (Li and Chiang, 2015). Meanwhile, both primary and secondary bile acids undergo enterohepatic circulation, conjugated bile acids in the small intestine can bind to fibre substrate in the gut before they are deconjugated. Deconjugation is a pre-requisite for further biotransformation of primary bile acids to secondary bile acids, which ensures a continuous and bi-directional interaction between bile acids and the gut microbiota. In turn, the secondary bile acids regulate the gut microbiota composition by their antimicrobial activity (Molinero et al., 2019; Zhan et al., 2020). For instance, Devkota and Chang (2015) reported gut microbiota modulation of bile acids in an animal model of inflammatory bowel disease, with taurocholate increasing the relative abundance of bacteria associated with inflammation.

Similarly, secondary bile acids (lithocholic acid and deoxycholic acid) are reported to decrease cell proliferation and gene expression of tight junction proteins (Lin et al., 2019), depicting their contributions to gut inflammation and barrier integrity. Lin et al. (2020) demonstrated downregulation of integral antioxidant genes; catalase and superoxide dismutase by lithocholic acid. Bile acid metabolism in the gut microbiota could serve as a potential target to boost gut health. However, interactions between bile acids and the gut microbiota can be impaired in the event of gut dysbiosis, especially at weaning with reduced feed intake, changes in diet or antibiotics usage. Undernutrition in weaning piglets increased secondary bile acids, which also led to altered gut microbiota with decreased lactobacillus and enhanced nuclear FXR signalling (Lin et al., 2019). It is important to understudy how dietary supplementation of weaned pig diet impact on bile acid profile of weaned pigs and its relationship with other biomarkers of gut health.

1.3.2 Alleviation of post-weaning diarrhoea of pigs

Dietary and non-dietary strategies have been employed in alleviating post weaning diarrhoea of pigs with the aim to improve their immune response and health. Non-dietary measures will involve ensuring adequate pen and personnel hygiene as well as preventing transfer of pathogens between the pens and the environment. Past efforts have been to reduce gut microbial load and limit dietary microbial growth stimulants, which often times result in the loss of healthy bacteria (e.g., probiotic bacteria), microbial metabolites, and reduced growth performance.

Several antimicrobial agents have been investigated as a replacement for in-feed antibiotics and as summarised in Seal et al. (2013) and Thacker (2013). Notably, the importance of zinc oxide (ZnO) in mitigating PWD of pigs have been established in scientific literature (Starke et al., 2014; Trckova et al., 2015). During the first 14 days post-weaning when pigs are highly predisposed to digestive disturbances, gastrointestinal changes, and stress, pharmacological doses of ZnO (≥ 2000 mg/kg) was effective in the prevention of post-weaning diarrhoea and other pig-associated digestive disorders (Zhang and Guo, 2009; Li et al., 2010).

Furthermore, due to reduction and deterioration of the gut absorptive surface and structure from post-weaning anorexia, nutrient digestion can be enhanced in the proximal small intestine of pigs by providing diets with more digestible grains (e.g., replacing wheat with rice bran) and smaller grain particle size (Pluske et al., 2002; Vicente et al., 2008). Additionally,

modifying diet composition with respect to the protein level and source, alongside the level of dietary fibre (e.g., dried sugar beet pulp with highly fermentable fibre) will support the gut microbiota fermentation and metabolites production, thus prevent PWD and enhance the growth performance of weaned pigs.

More recently, the successfully treatment of *Clostridium difficile* infection in humans with faecal microbiota transplant (FMT), is now being explored in alleviating PWD of young pigs (Geng et al., 2018; Nowland and Kirkwood, 2020). The past decade witnessed the use of encapsulated frozen stool for FMT with high efficacy in patients with intestinal dysbiosis and related disease (Kao et al., 2017; Cheminet et al., 2018). The FMT method aimed at re-establishing a healthy microbiota by transferring faeces from a healthy donor to a sick recipient, presents a huge potential for use in pig/animal production, due to its ability to restore and stabilise the gut microbiota (Cheng et al., 2018; Xiang et al., 2020).

Compared to probiotics bacteria, a healthy donor faeces provides better effects of a complete microbiota structure and metabolites. Tang et al. (2020) reported capsulized faecal microbiota transplant, not only ameliorated PWD by modulating the gut microbiota but also improved other gut health parameters (e.g., gut morphology and integrity). Interestingly, the application of FMT in the early life (first 14-days) of pigs was found to decrease diarrhoea, improve gut morphology and downregulate pro-inflammatory cytokines (Su et al., 2021; Ma et al., 2022).

Also, crucial to alleviating post-weaning diarrhoea of pigs are strategies that modulate the immune response. The immune system is exceptionally sensitive to nutrient excesses and deficiencies in the diet (Haase and Rink, 2009). Nutrients that affect the immune system established in animals and human studies included essential amino acids (e.g., lysine, methionine, tryptophan), fatty acids – linoleic acids, vitamins (e.g., A, E and B) and minerals (zinc, copper, iron and selenium). Deficiencies (undernutrition) due to insufficient energy intake and macronutrients, and/or inadequate intake of specific micronutrients or in rare cases a deficiency of both will impair maturation of the immune system and increase host susceptibility to infection (De Rosa et al., 2015).

Typically, during the post weaning period, feed intake reduces sharply due to weaning stress and change in diet, hence a balanced diet and nutrient pool is necessary for renewal and proliferation of intestinal cells and a stable gut microbiota. Over-nutrition from increased

intake of energy dense diet disrupt the immune balance and increase host inflammatory responses. A high protein diet increases the catabolic activity of bacteria enzymes and generation of proteolytic fermentation metabolites (biogenic amines) that activate inflammatory responses, creating a conductive environment for the growth of pathogenic bacteria (Zhang et al., 2020).

Other supportive ways to alleviate PWD are prevention of physical and psychological stressors that initiate pro-inflammatory cytokines release from immune cells. Adequate managemental measures will ensure pigs are in thermally safe environment and comfortable pens, considering that fluctuating temperatures, draught, low or temperatures above pig comfort zones increases stress and reduces the immune response to disease challenge (Ross et al., 2015; Rhouma et al., 2017). Put together, a holistic strategy, combining good management practises to feeding strategies, and adjusting diet composition will mitigate PWD.

1.3.3 Roles and mechanisms of action of ZnO in the alleviation of PWD of pigs

While the mechanism of action of ZnO remains poorly understood, several host regulatory pathways are suggested to be involved (see Figure 1.3). Primarily, ZnO has a broad spectrum of action along the GIT, evident by improvement in pig gut architecture, nutrient digestion and absorption, immune response and growth performance (Bonetti et al., 2021). However, the effect observe is determined by the form of ZnO utilized, if readily availability in the GIT or bound to other compounds (e.g., ZnO nanoparticles/microencapsulated) (Baholet et al., 2022).

Weaning induces zinc deficiency that impair enzyme activity, nuclei acid metabolism, appetite and growth, hence low level ZnO (100ppm to 150 ppm) is permitted in weaned pig diet to maintain host function. However, owing to gastrointestinal dysfunctionality and increased susceptibility to PWD, a pharmacological dose (2000 ppm to 3000 ppm) is recommended as a prophylactic measure (Sloup et al., 2017; Shannon and Hill, 2019). Dietary zinc (Zn) supplementation in form of in-feed ZnO, stimulates ghrelin secretion enhancing feed intake and digestion, thus ZnO has the ability to prevent weaning anorexia, reduce PWD and mortality. Stensland et al. (2015) and Kaevska et al. (2016) observed 83% and 50% reduction in PWD symptoms following supplementation of weaned pig diet with 2500-3000 mg ZnO/kg.

Indeed, many studies confirmed improved performance response of weaned pigs fed ZnO supplemented diet by enhancing the gut structure and absorptive surface (villus height and crypt depth). ZnO decreases intestinal permeability, inhibiting translocation of pathogenic bacteria through the intestinal epithelium, with an apparent increased expression of tight junction proteins (e.g., Occludin), reported in many studies (Zhang and Guo, 2009; Grilli et al., 2015; Zhu et al., 2017). This prevents a leaky gut and ensures proper functioning of the intestinal barrier framework. Also, ZnO reduces the secretion of pro-inflammatory histamine by inhibiting the activation of intestinal mast cells thereby alleviating stress and modulating gut inflammation (Kim et al., 2012; Gammoh and Rink, 2019).

The antioxidative characteristics of ZnO on the gut mucosa is one of its means of preventing intestinal oxidative stress at weaning. Zn is an important metal in the catalytic action of superoxide dismutase - an antioxidant found to increase in ZnO supplemented pigs (Zhu et al., 2017), as well as expression of metallothioneins, an antioxidant preventing oxidative stress from heavy metal toxicity (Lee 2018). ZnO causes oxidative stress in microbial cells, which explain its antimicrobial activity, however other humoral cells can be significantly affected leading to tissue toxicity and heavy metal contamination (Bonetti et al., 2021). Despite this, ZnO has been reported to exert a moderate antimicrobial effect against *Escherichia coli* F4 (K88), the most common strain causing PWD (Dubreuil et al., 2016; 2017)

The effect of ZnO on the gut microbiota of weaned pigs has been largely inconsistent, differences in diet composition, environment, sampling time and sample size, bacterial adaptation mechanisms to Zn (free Zn, nano Zn) as well as experimental design/methods (e.g., genomic sequencing – 16S rRNA and quantitative polymerase chain reaction - qPCR) may partly be responsible. Starke et al. (2014), identified reduced *Enterobacteriaceae* and *Lactobacillus spp*. in weaned pigs fed ZnO diet for 5 weeks. After a 15-day experiment period, high ZnO dose reduced coliforms and *Escherichia coli* in the distal small intestine compared to conventional ZnO levels (110 mg/kg) without any effects on the *Lactobacilli* and *Clostridium XIVa* with qPCR (Wang et al., 2019).

Similarly, Mukhopadhya et al. (2019) recorded increased caecal Bacteroidetes in weaned pigs fed diets supplemented with ZnO and milk hydrolysate, and decreased phylum Actinobacteria with ZnO and yeast β-glycan. Wei et al. (2020) in their study observed 11% and 1.9% reduction in the relative abundance of faecal *Lactobacillus* and *Megasphaera* in pigs on high zinc diet. The same study also reported a significant increase in the relative

abundance of *Streptococcus*. However, Hung et al. (2020), detected no changes in gut microbial metabolites (SCFA and secondary bile acids) with pharmacological dose of ZnO, but increased acetate and total SCFA was reported in Zhang et al. (2022) with ZnO and the tetrabasic form (zinc chloride). While Oh et al. (2021) observed variable effects of different forms of ZnO (chelated, nanoparticle sized) on diarrhoea incidence and the gut microbiota of weaned pigs.

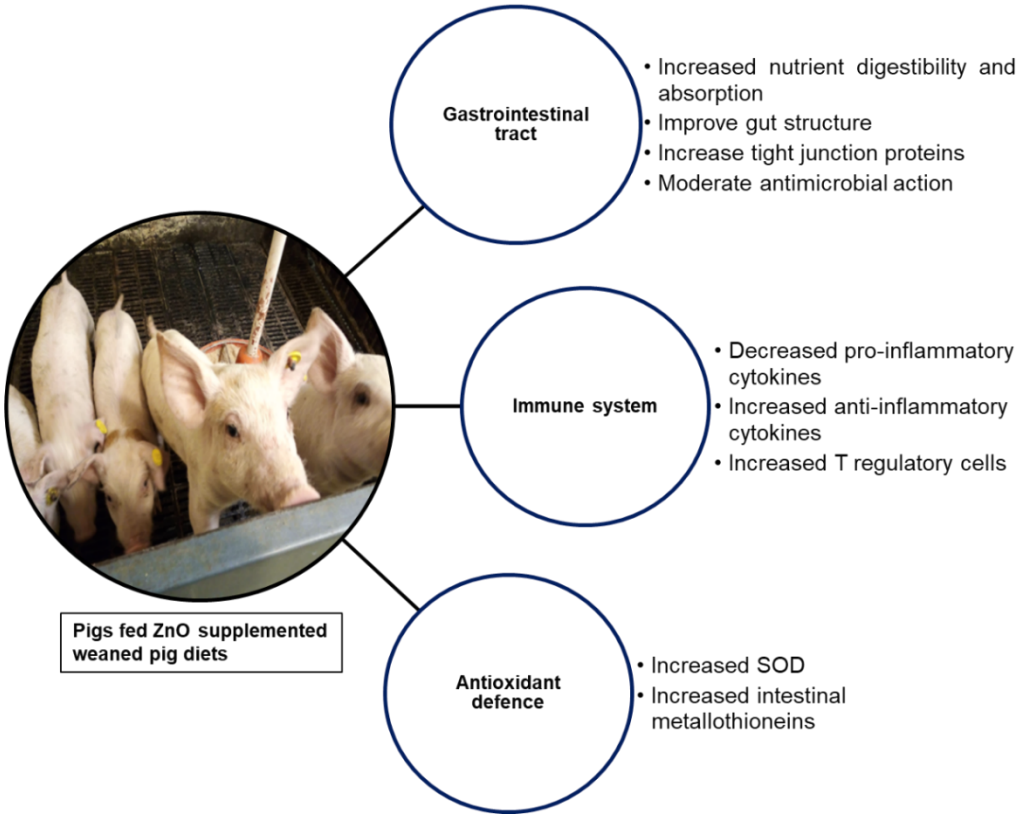


Figure 1.3: Roles and mechanism of action of zinc oxide in weaned pigs
adapted from (Bonetti et al., 2021)

1.3.4. Risk associated with zinc oxide in weaned pig diet

In-feed zinc oxide affords the benefit of mitigating post-weaning diarrhoea by improving growth response and supressing bacterial growth. However, prolonged use of ZnO can override these benefits, resulting in toxic effects (Burrough et al., 2019; Bonetti et al., 2021), of excessive accumulations of Zn in tissues (e.g., kidney, liver and pancreas) and farming environments (Martin et al., 2013; Komatsu et al., 2020). A major risk associated with in-feed ZnO is the environmental pollution that ensues from the application of Zn-rich manure from pig farms to farmlands. Bonetti et al. (2021) on the risk of ZnO, reported an increase in the

application of Zn-rich pig slurry between the year 1986 and 2014, that increased soil Zn concentration by 2% - 5%. This constitutes a significant risk to aquatic life with Zn leaching from fertilised farmlands to ground and fishing waters.

Numerous evidence from the literature highlights the contributions of pharmacological doses of ZnO in weaned pig diet to the acquisition of antibiotic resistance genes (Yazdankhah et al., 2014). Vahjen et al. (2015) reported on the diffusion of resistant genes among intestinal *E. coli* in high ZnO fed weaned pigs and a tendency for the emergence of a heavy metal tolerant strain (Slifierz et al., 2014; Johanns et al., 2019) that indicates the antibiotic-like activity of ZnO. This has necessitated the complete ban on pharmacological doses of ZnO in weaned pig diet and the urgent search for alternatives.

Although the positive and negative impact of ZnO on the gut microbiota remain debated, a transient to minor modification, as well as significant increase in *Enterobacteriaceae* abundance have been emphasised in many studies (Li et al., 2001; Vahjen et al., 2015; Yu et al., 2017). ZnO exerts a moderate antimicrobial effect on the gut commensals, suppressing Gram positive species without affecting Gram negative species. This attribute has been linked to its potential to act as a growth promoting antibiotic, bringing about lower bacterial activity in the gut and increase available energy to the host, however, not without the loss of some beneficial commensal bacteria e.g., *Lactobacillus*.

1.3.5 Alternatives to zinc oxide and current trends towards zero zinc oxide diet

From June 2022 onwards, administration of pharmacological doses of ZnO in pig production ends in the EU, including the UK (Commission, 2005; EFSA, 2020; Bonetti et al., 2021). While this bold step has not been taken by other high pork producers in the world e.g., China and Brazil, there is a strong global demand to stop indiscriminate use of antibiotics and ZnO in pig production (Maron et al., 2013). In achieving a zero-zinc oxide weaned pig diet, novel alternatives, nutritional strategies and approaches are currently under investigation. Some essential managemental steps suggested are, improved farm biosecurity, ensuring optimum animal health, adequate vaccination against infectious diseases and preventing heat stress.

In addition, a gradual introduction of solid feed to weaned pigs in form of creep feed will permit smooth transitioning from lactation to nursery environment. Weaned pigs should have easy access to feed and water. Feed intake can be enhanced by providing good quality, palatable and digestible feed. Essentially, diet should be formulated to prevent pathogenic bacterial

intake, support digestion, improve gut barrier function, and modulate the gut microbiota and immune system. Dietary supplementation with feed ingredients or additives (e.g., plants, SCFAs, antimicrobial peptides and phytochemicals) recognised to boost gut health of young pigs have also been emphasized in literature (Pluske et al., 2018; Xiong et al., 2019; Kim 2019).

In a recent summit on zero zinc oxide diet (Ploegmarkers, 2022), it was agreed that feeding piglets with diet supplemented with additives pre-weaning can support the development and establishment of a healthy gut microbiota post weaning. The gut health was identified as a major contributor to alleviating post weaning diarrhoea (PWD) of pigs, with the gut microbial composition and metabolite produced recognise to contribute significantly to a healthy gut, supporting the proliferation of a beneficial and balanced microbial population. Therefore, it is essential that young pigs develop a healthy gut microbiome in their early life, which will ultimately influence their wellbeing.

Again, diet can be adapted to steer a healthy gut microbiota as an alternative way to improve gut health without dependence on zinc oxide. Zhao et al. (2021) demonstrated high dietary lactose due to its sweetness and palatability, improved the growth performance of piglets by stimulating the proliferation of beneficial bacteria (*Lactobacillus*) and SCFA in the first week after weaning. This suggests the probiotic effect of lactose, and its contribution to pig nutrition and gut health after the lactation phase (post weaning), however at excess levels, increased fermentation in the hind gut can ensue, tipping pigs into PWD. Accumulated evidence from past studies have shown that weaned pig diet low in dietary protein and high in fibre favours a healthy gut microbiota (Millet et al., 2018; Batson et al., 2021), hence a promising alternative to in-feed ZnO. Congruently, reduced dietary crude protein may be optimized via provision of essential and nonessential amino acids to improve growth while reducing the risk of diarrhoea (Sun et al., 2020).

The dietary addition of pre-fermented feeds with probiotic bacteria fermented feed have recently gained attention, due to its ability to improve growth performance and gut health, by selective inhibition of intestinal pathogens, improvement of nutrient digestibility and neutralization of anti-nutritional component in feed (Wang et al., 2018; Sugiharto and Ranjitkar, 2019). Satessa et al. (2020) showed that piglet fed fermented rapeseed meal improve feed intake hence a promising alternative to in-feed ZnO of weaned pigs. To conclude, an integrated approach maximising the absorption of nutrients with improved

managerial and pig welfare that commences early at farrowing and continues post-weaning will contribute immensely to gut health and optimal piglet performance (Gaillard et al., 2020; Huting et al., 2021).

1.4 Fruits and vegetable waste as animal feed supplement with health benefits

With the increasing world population, demand and consumption of animal products are expected to increase drastically by 2050, with a 73% and 58% increase in demand for meat and milk, respectively (FAO, 2011). However, the ability to meet the feed requirement of livestock in a sustainable way remains a challenge, especially in the face of climate change, urbanization, increased farming and anthropogenic land use, water shortages and loss of biodiversity. Sustainability in livestock production requires efficient use of available feed resources via reduction of wastages and increased feed sources.

Fruit and vegetable production is on the increase, with over 500 million metric tonnes produced yearly according to FAO (2017), making available waste and by-products from agro-allied processing industries worldwide (Esteban et al., 2007; Katongole et al., 2008; Gracia and Gómez, 2020). On average, 55 million tonnes of waste are produced yearly from these industries globally, with a large proportion dumped into landfills and water bodies, resulting in environmental pollution (Wadhwa and Bakshi, 2013). Processing of fruits and vegetables generate a significant amount of waste at different stages such as collection, handling, shipping and processing, with about 25% - 30% of the waste product mostly from pomace, peels, rind and seeds (Chang et al., 2006; USEPA, 2012; Panda et al., 2016). Interestingly, fruit and vegetable waste (FVW) can be recycled as livestock feed sources and value-added products after undergoing further processing such as drying, ensiling and fermentation (Wadhwa et al., 2016; Bakshi et al., 2016).

The use of FVW as animal feed is an excellent source of nutrients for livestock that will enhance livestock feed sustainability, contribute to food security and mitigate environmental challenges associated with waste disposal (Gertenbach and Dugmore, 2004; Katongole et al., 2011; Nwafor et al., 2017). FVW are important sources of dietary fibre, especially the agro-industrial by-products after extraction of juice and coproducts of distillers and milling industries. They serve as animal feed supplements, functional ingredients rich in nutritional value and helpful in alleviation of PWD (Ayala-Zavala et al., 2011; Jha and Berrocoso, 2015; Regassa and Nyachoti, 2018).

In addition, FVW contain pigments (e.g., carotenoids from tomato peels and carrot pomace, anthocyanin from banana bract and betalains from red beetroot pulp) and a plethora of bioactive phytochemicals essential for industrial development of edible antimicrobial films, prebiotics and pharmaceutical applications (Kumar et al., 2020). These bioactive phytochemicals are mainly secondary plant metabolites like polyphenols, alkaloids, and carotenoids, along with tannins, terpenes and saponins. They play important role in reducing disease-causing agents such as bacteria and viruses as well as free-radical associated disease. Antioxidant compounds from FVW can prevent oxidative damage and lipid peroxidation by scavenging reactive oxygen species (ROS) when consumed or used as ingredients for other products (Viuda-Martos et al., 2010). Hence, FVW are reported to exhibit antioxidant, antimicrobial, antifungal, anti-inflammatory, anti-parasitic and antiprotozoal properties (Kathirvelan et al., 2015). Other biochemical constituents of FVW include carbohydrates, proteins, nucleic acids, enzymes, oils, vitamins, fatty acids and other compounds (Galanakis, 2012).

Indeed, many studies have shown that nutrients and phytochemicals are abundantly present in peels, seed and other constituent of vegetable and fruits (Rudra et al., 2015). For instance, fruit peel from avocado, grapes, lemon and seeds of jackfruits and mangoes contains more phenolic concentration (up to 15%) than their pulp (Gorinstein et al., 2001; Soong and Barlow, 2004). Also, fruit and peel of pomegranate, citrus and mangoes are abundant in functional ingredients like antioxidants, fibre and oligosaccharides that can serve as prebiotics (Cerezal and Duarte, 2005; de Moraes Crizel et al., 2013; Orayaga et al., 2015; Chan et al., 2018) that aid digestion and promotion of a healthy microbiota.

Moreover, there are increasing evidence on the importance of FVW as feed arsenal (Table 1.1), providing nutrients, dietary fibre and supplements to improve animal wellbeing, thereby reducing the cost of feeding /production (Guil-Guerrero et al., 2016; Pérez-Jiménez and Saura-Calixto, 2018). Constituent bioactive compounds in FVW have also been described to act by modifying the gut microbiota and stimulating immune response, which consequently enhances feed digestion and increases the resistance of animals to infectious diseases (Achilonu et al., 2018). With these, FVW can be incorporated into animal feed as supplements to provide specific nutrients (Martínez et al., 2012) and as a replacement for in-feed antibiotics, to provide health and production benefits.

Table 1.1: Nutrients and potential health benefits of livestock feed resources from plants fruits, vegetables and their waste

Plant/ Fruit/ vegetable	Part utilised	Animals fed	Potential health benefits / effects observed	Nutrient composition	References
Carrot *(Daucus carota)*	Dried Carrot tops 25% and 50%	Rabbits	No negative effect on growth performance	Rich source of carotene and vitamins C (300 – 700 mg/kg) depending on the variety.	(Klinger et al., 2017)
Apple *(Malus domestic)*	Fermented apple pomace (5%)	Weaned pigs	Improved growth, plasma biochemistry, immune indicators and gut microbiota	High in dietary fibre, (442 to 495 g/kg in vacuum-dried pomace) polyphenols and minerals.	(Ao et al., 2022)
	Dried apple pomace 2%, and 4%	Weaned pigs	Beneficial effect on feed conversion ratio, increased faecal consistency, pathogen excretion and increased microbiota richness		(Dufourny et al., 2021)
Banana *(Musa acuminate)*	Dried ripe banana peels, banana meal	Growing pigs (20%), replaced maize (75-100%) in weaned rabbit, Broilers (10%)	No depression in pig growth. No adverse effect on performance	Contain 8% polyphenols, few condensed tannins.	(Renaudeau et al., 2014; Bakshi et al., 2016)
	Fermented and unfermented banana stem	Growing pigs	Improved intestinal morphology (villus height		(Arjin et al., 2021)
	Banana leaves	30% in diet of young rabbits. 10% in standard broiler diets	Increased rabbit weight gain. No adverse effect on feed conversion efficacy.		(Rohilla and Bujarbaruah, 2000; Wadhwa and Bakshi, 2013)

Citrus *(Citrus limetta)*	Dried citrus pulp	2% in pigs (5-10%) in Poultry and pig diet.	2% promoted growth of beneficial bacteria communities in the gut and modulated colonic fermentation. Above 10% can adversely affect growth rates, feed conversion efficacy and carcass yield.	Essential oil (0.5 -3.0 kg oil/tonne of fruit), source of prebiotic fibre	(Almeida et al., 2017; Uerlings et al., 2021; de Araujo et al., 2022)
	Citrus pulp (as dietary fibre source) at 9%	Pigs	Low feed intake and daily weight gain		(Pascoal et al., 2015)
	Citrus pulp silage	Pigs	Reduced faecal *Enterobacteriaceae* and similar lactobacilli counts compared to the control pigs.		(Cerisuelo et al., 2010)
Mango *(Mangifera indica)*	Mango pulp	Pigs	Impact gastric emptying, SCFA production (propionate) along the GIT	Rich in gallotannis used in pharmaceuticals as antimicrobials, antioxidants and hepatoprotective.	(Low et al., 2021)
	Waste	Poultry	Improved growth and development in chickens.		(Engels et al., 2010; Orayaga et al., 2015)
Grapes *(Vitis vinfera)*	grape seed or grape skin fibre, dietary grape pomace	Pigs, Poultry	No adverse effect on performance, maintained plasma antioxidant capacity.	Grape skin is rich in pro-anthocyanidins and other polyphenolic compounds.	(Li et al., 2020; Erinle et al., 2022)
	grape seed procyanidins (150mg/kg)	Weaned pigs	Increased brush-border enzyme activity and intestinal barrier function, suppressed weaning stress, improved antioxidant enzyme activity		(Hao et al., 2015; Fang et al., 2020; Costa et al., 2022)

1.4.1 Beets

Beet (*Beta vulgaris*) originates from the family Amarantheceae, subfamily; Betoideae and order Caryophyllales. It is a root vegetable, with an earthy sweet taste and several varieties grown all over the world. Other synonyms for beetroot/beets are table beet, garden beet, red beet, dinner beet or golden beet (Gledhill, 2008). Beets are economically relevant plant, classified based on their betalain constituent (a water-soluble pigment, red to violet - betacyanin and yellow to orange - betaxanthin) present in most plants in the order Caryophyllales (Francis, 1999; Strack et al., 2003). Several cultivated varieties classified as; *Beta vulgaris subsp*. *Vulgaris Conditiva* group, are grown for their edible roots and leaves (beet green), examples of which are sugar beet used in production of table sugar, the root vegetable (red beetroot or garden beet), the leaf vegetable known as Swiss chard (*Beta vulgaris L. spp*) or spinach beet and mangel-wurzel which is a fodder crop.

Beets contain a substantial amount of betaine responsible for changes in body composition and fat loss when ingested (Cholewa et al., 2018; Gao et al., 2019; Schwarz and McKinley-Barnard, 2020). Red beetroot (*Beta vulgaris L.*) has the highest betalain content, with varying proportions of red (betacyanin) and yellow (betaxanthin) depending on the cultivar, plant part, maturation stage, climatic factors and farming practice (Celli and Brooks, 2017). Beets appear in different colours ranging from deep red to bright purple, and red to yellow as in cream candy cane stripped Chioggia beets and bright zesty yellow in golden beets. Utilization of beet plant in animal nutrition have been with sugar beet pulp, obtained after sugar extraction from sugar beet.

1.4.1.1 Sugar beets

Sugar beet (*Beta vulgaris var. altissima*) is one of the cultivars of *Beta vulgaris* with sucrose concentrated roots used for sugar production. The root contains 75% water, 20% sugar and 5% pulp (Dohm et al., 2014), the by-product of processed sugar beets (SB) is used as pulp and molasses (Punda et al., 2009). Sugar beet pulp can be dried and sold as dried sugar beet pulp or mixed with molasses for animal use. In New Zealand, SB is widely cultivated and harvested to feed dairy cows. Russia, USA, Germany, France and Turkey are top sugar beet (SB) producers globally.

Sugar beet pulp (SBP) is the by-product of sugar extraction from sugar beets, with high dietary fibre up to 77.7%, 52% of which is made up of soluble fibre like; pectin, glycan and highly branched arabinoxylan (Knudsen, 1997; Fadel et al., 2000). SBP has been studied extensively in animal nutrition as a source of dietary fibre in animal feed, due to its high fermentable properties and ability to stimulate the production of beneficial microbes against GIT pathogens (Laitat et al., 2015; Amarakoon, 2017). Contrariwise, SBP has high water retention capacity that may increase its laxative effect in digestive system.

Supplementation of pig diet with SBP is alleged to promote the growth of beneficial gut microbes (e.g., *Lactobacillus* and *Bifidobacterium*) and prevent pathogenic colonization of the gut but reports from most studies have been largely inconsistent. Yan et al. (2017) and Laitat et al. (2015) observed similarities in growth performance and diarrhea incidence in pigs fed 12% and 23% SBP respectively, due to energy dilution from fibrous diet, faster intestinal transit time and reduced ingestion from increased satiety. Similarly, Zhang et al. (2013) reported a decrease in the apparent tract total digestibility with an increasing percentage of SBP in the diet.

Although, pigs may digest SB fibre, feeding beet-top is avoided due to the presence of oxalic acid that could depress feed intake and performance (Wadhwa and Bakshi, 2013). SBP is bulky and relished when moisten or soaked, however young pigs do not particularly thrive on the SBP due to the risk of scour from high water retention of the soluble fibre. In addition, Anguita et al. (2007) also reported reduced the voluntary feed intake with dietary inclusion of SBP compared to coarse corn and wheat bran. SBP is usually fed with another source of insoluble dietary fibre (e.g., wheat bran), to maximise its benefit in pigs (Table 1.2).

Table 1.2: Sugar beet pulp (SBP) as animal feed source; effects on pig growth performance and gut health

Percentage inclusion of Sugar beet pulp and diet characteristics	Duration of trial (days)	Effects on growth, performance, digestibility diarrhoea occurrence and gut microbiota	References
6% SBP in 2% soybean hulls	28-42 days	Decreased ADG, elevated ADFI	(Montagne et al., 2012)
5% SBP in 8% wheat bran	20-23 days after weaning	Decreased ileal digestibility of organic matter but not crude protein or starch	(Pieper et al., 2012)
3, 6,9 and 12%	21-35 days	No significant difference between treatments on growth performance.	(Amarakoon, 2017)
2.5 or 5%	21 days	Increased apparent total tract digestibility of all nutrients except crude protein.	(Berrocoso et al., 2015)
23% in fattening pig	7 days	No significant reduction in diarrhoea incidence (high faecal moisture due to presence of undigested fibre) low digestibility of nutrients.	(Laitat et al., 2015)
6% in corn and soybean-based diet	0-14 and 14 - 28 days	Reduced apparent total tract digestibility of dry matter on day 14 and 28, reduced villus height crypt sept compared to wheat bran diet. Increased abundance of Lachnospiraceae and acetate and total SCFA compared to control.	(Shang et al., 2020)
3,6,9 or 12% in a corn-based diet	5 weeks	No significant differences in growth performance and diarrheal incidence	(Yan et al., 2017)
10% in 15% corn distillers dried grains	7 days	Decreased ileal *Escherichia-Shigella* compared to ETEC challenged control. Increased abundance of *Streptococcus, Roseburia* and colonic *Prevotella.*	(Li et al., 2020)
10% in 15% wheat bran (gestation); 5%in 7.5% wheat bran (lactation)	Day 85 of gestation to weaning. Day 21 of lactation	Enhanced intestinal barrier function increased ileal Occludin level and improve gut microbiota in weaned piglets	(Shang et al., 2021)

ADG (Average daily gain); FCR (Feed conversion ratio); ADFI (Average daily feed intake); ETEC (Enterotoxigenic E. coli); SCFA (Short chain fatty acids)

1.4.1.2 Red beetroot

Red beetroot (*Beta vulgaris L. var conditiva*) is a variety of beet, with high betalain content responsible for its red colour (Lee et al., 2005; Vulić et al., 2014). It is a root vegetable, grown in many countries of the world all year round and consumed as part of a normal diet (Zielińska-Przyjemska et al., 2009; Georgiev et al., 2010). In 2016, red beetroot production was estimated at 690,000 tonnes, however the global market is projected to increase significantly to about 11 million tonnes in the next decade, 2027 (Insights, 2017). Unlike sugar beet, red beetroot or waste generated from processed beet has not been explored as animal feed ingredient.

Importantly, red beetroot has been reported to contain many bioactive compounds other than betalains, whose health benefits have not been considered in the alleviation of weaning stress and gut dysfunctionality of weaned pigs. Red beetroot (RB) contains polyphenols, betaine, fibre, nitrate, ascorbic acid and carotenoids which are important source of health promoting phytochemicals (Clifford et al., 2015; Kathirvelan et al., 2015). It is considered one of the top 10 vegetables with several functional constituents responsible for its numerous health promoting properties (Azeredo, 2009; PAnghAl et al., 2017), some of which have been summarised in Table 1.3. The nutritional constituent of red beetroot depends on the cultivar and the conditions in which it is grown. The processing and drying technique also contributes to the quality, nutritional composition and betalain profile of the final red beetroot product (Nemzer et al., 2011).

Red beetroot is the richest source of betalains, a group of bioactive pigment imparting a red to yellow colour. Betalains are water soluble indole derived pigment found in the plants belonging to the family Caryophyllales. They contain two predominant forms, determined by the range of redness to yellowness of the beet. The betacyanins which depicts the red colour and the betaxanthins indicating the yellow colour (Szopińska and Gawęda, 2013). Other derivatives of these broad groups have also been investigated and reported in literature; betaxanthin (vulgaxanthin-I and vulgaxanthin – II (Ravichandran et al., 2013) and betacyanins are (betanin, prebetanin, isobetanin and neobetanin) (Nemzer et al., 2011).

Table 1.3: Bioactive compounds present in red beetroot and potential health benefits

Bioactive Compounds	**Plant parts/sources**	**Uses and health benefits**	**References**
Phenolic compounds	Peel	Resistance to stress, antimicrobial and anti-inflammatory activity.	(Zeb and Zeb, 2021)
Flavonoids *(Betagarin, betavulgarin, quercertin, astragalin, kaempferol, rhammocitrin, rhamnetin)*	Root, leaf and peal	Antioxidant activity, hepatoprotective, antiviral and anti-inflammatory activity. Inhibit the synthesis of microbial cell membranes	(Slavov et al., 2013; Vulić et al., 2014)
Carotenoids	Root, leaf	Prevention of chronic disease, antioxidant function.	(Rebecca et al., 2014)
Betalains: *Betacyanins* *(Betanin, prebetanin, isobetanin and neobetanin)* *Betaxanthin (vulgaxanthin-I, vulgaxanthin-II and indicaxanthin)*	Root	Regulate vascular homeostasis, chemo-preventive role. Strong antioxidant activity, therapeutic effects on metabolic syndrome.	(Madadi et al., 2020; Hadipour et al., 2020)
Betanin, betanidin	Red beetroot	Inhibit lipid peroxidation, strong antioxidant activity. Anti-inflammatory.	(Kapadia and Rao, 2013; Clifford et al., 2015)
Others; *saponins, ferulic acid, taurine, sesquiterpenoids, alkaloid, nitrate*	Root and leaf	Antimicrobial, antifungal, virucidal, hypo-lipidemic activity, antioxidant activity, treatment of diarrhoea, flu and burns, anti-inflammatory, analgesic, anxiolytic neuroprotective, probiotic effects.	(Lim and Lim, 2016; PAnghAl et al., 2017; Lorizola et al., 2018)

Many *in vivo* and *in vitro* models have been employed to demonstrate the health potentials of red beetroot, especially its antioxidant and anti-inflammatory capabilities (Tesoriere et al., 2004b; Pavlov et al., 2005; Lee et al., 2009). These properties have been linked to the high nitrate content, phytochemicals (including ascorbic acid, carotenoids, phenolic acids and flavonoids) and bioactive pigments (betalains) in red beetroot (Ormsbee et al., 2013; Vidal et al., 2014; Masih et al., 2019). According to Wootton-Beard et al. (2011) and Slavov et al. (2013), red beetroot has a higher antioxidative capacity compared to fruits and vegetable juices like tomato, carrot, oranges and pineapple. Predominantly, betanin with its aglycone betanidin have been found to have great antioxidant activity and effective in preventing lipid peroxidation (Kathiravan et al., 2014). Regular consumption of diet containing red beetroot have been found to provide protection against oxidative stress-related health issues and improve food digestion (Akan et al., 2021).

These findings have generated increased interest and investigations into the potential use of red beetroot as a chemo-preventive or therapeutic resource for a range of diseases associated with infectious agents, oxidative stress and inflammation, especially in livestock (e.g., weaned pigs). Red beetroot is therefore being considered in the development of functional foods and nutraceuticals (Mirmiran et al., 2020) and as a potential alternative to synthetic non-steroidal anti-inflammatory drugs (Calixto et al., 2004).

1.4.1.2.1 Bioavailability of red beetroot betalains

The bioactive compounds in red beetroot must be released to the host in sufficient quantities after ingestion, for absorption and circulation to important body organs for metabolism, thus making it available to the cells. The bioavailability of betalains have been reported to be very low in many studies, due to the involvement of several pathways in the elimination of betalains (e.g., renal, gastrointestinal tract and hepatic). Baião et al. (2020) and da Silva et al. (2019), reported betanin bioavailability as low as 2.7% of total oral intake with excretion in human urine and faeces. Clifford et al. (2017) in their study failed to detect betanin in the plasma after intake of 300g whole beetroot (with 66 mg betanin). Again, 0.13% - 0.93% betanin was found in the urine after 3, 8 and 24 hours with 50 mg betalain/betacyanin supplementation (Rahimi et al., 2019). Moreover, native betalains and their deglucosylated, decarboxylated and

dehydrogenated metabolites were observed in human physiological fluid after consumption of fermented juice of red beetroot by Wiczkowski et al. (2018).

Tesoriere et al. (2013) in their study demonstrated absorption of un-metabolised betalains (betanin and indicaxanthin) via the paracellular transport, in a simulated model of the small intestine. Betalains are also absorbed into the systemic circulation in their unchanged form, allowing them to retain their molecular structure and biological activity (Ting et al., 2014). However, orally administered betanin is reported to be poorly absorbed in the small intestine but most mostly metabolised in the large intestine (Khan, 2016).

Red beetroot has other constituent (phenolic e.g., epicatechin, rutin and caffeic acid), whose bioavailability have not been evaluated. Netzel et al. (2005), measured the total phenolic compound present in beetroot, connoting phenolic content in red beetroot are well absorbed, thus acting synergistically with betalains to increasing the *in vivo* antioxidant response of red beetroot after ingestion. Many factors are considered to affect the bioavailability of betalains, the GIT role in the bioavailability of betalains is however dependent on the food matrix which may prevent bio-accessibility of the bioactive compound and influence its stability in the presence of increased gut pH, and digestive enzymes, as it transits the gut. Although, the bioavailability of red beetroot and its constituent was not a major focus of this thesis, it was briefly mentioned.

1.4.1.2.2 Bioactivity and other health benefits of red beetroot

Re beetroot exert a variety of beneficial effects on the host, mediated by the constituent bioactive compounds mostly betalains, phenols, fibre and nitrate (Chhikara et al., 2019; Martínez-Rodríguez et al., 2022). Some retrieved studies on the effects of red beetroot on the host and gut microbiota modulations have been summarised in Table 1.4. In an *in vivo* cellular attack with exposure to oxidative insult, beetroot extract maintained endogenous antioxidant activity, reduced glutathione, and glutathione peroxidase and catalase enzymes at normal cellular concentrations (Vulić et al., 2014).

Consistently in literature, the endogenous antioxidant capacity of beetroot *in vivo* has been reported. The high antioxidant ability of betalains stem from its electron donating

capacity to defuse highly reactive radicals. It also holds the ability to mediate the transcription of antioxidant genes, thus beetroot supplementation may serve as a useful strategy to strengthen endogenous antioxidant defences, protecting cellular components from oxidative damage. Besides, red beetroot contains several other bioactives e.g., phenolics (rutin, epicatchin and caffeic acids) with excellent antioxidant properties (Georgiev et al., 2010; Clifford et al., 2015;).

Measuring the antioxidant capacity of red beetroot using ferric reducing antioxidant power (FRAP), Wootton-Beard et al. (2011) observed increased antioxidant capacity following simulated digestion, due to alteration in the structure of these compounds with the production of secondary metabolites that possess more antioxidant functions. These compounds and their secondary metabolites work synergistically to active the nuclear factor (erythroid derived 2) - like 2 (*Nrf2*) and the antioxidant response element pathway that mediates an increase in endogenous antioxidant activity.

Notably, the antiviral, antimicrobial effect and radical activity of betalain have been described in many scientific literature (Ninfali and Angelino, 2013; Kumar and Brooks, 2018). Red beetroot provides phytochemicals that stimulate the immune system, protecting the kidney and liver. Similarly, El Gamal et al. (2014), demonstrated the anti-inflammatory effect of ethanoic extract of red beetroot on gentamicin induced nephrotoxicity by reduced concentration of inflammatory cytokines and immune cells (TNF, IL6 and signs of oxidative damage) in rats.

Aside the antioxidative capabilities attributed to red beetroot, betalains have emerged as potent anti-inflammatory agents. The anti-inflammatory effect of red beetroot is mediated by its ability to interfere with pro-inflammatory signalling cascade via the nuclear factor- kappa B (NF-κB), which activates and regulate the inflammatory response (Chen et al., 2021). Betalains and betaine can reduce the levels of pro-inflammatory cytokines (TNF, IL6, IL8 and IL1B), reactive oxygen and nitrogen species. They have also been reported to suppress cyclooxygenase2 (*COX2*) expression an important precursor molecule for pro-inflammatory arachidonic acid metabolites (Lechner and Stoner, 2019). Emerging evidence showed that betanin treatment (25 and 100 mg/kg/bm for 5 days) significantly inhibits NF-kB activity in rats induced with acute renal damage (Tan et al., 2015). Betalain extracts from red beetroot may also supress chronic inflammation implicated in the development of malignant

tumours by inhibiting cell proliferation, angiogenesis and tumorigenesis (Lechner et al., 2010).

There has been a growing interest on the positive effects of red beetroot on gastrointestinal health. Some studies have shown that soluble fibre present in sugar beet (pectin) could influence the gastric emptying, nutrient absorption and availability in the small intestine as well as enhance fermentation in the large intestine (Tian et al., 2016; Prandi et al., 2018). Sugar beet pectin modulated increased abundance of *Prevotella copri* and *Ruminococcus spp*. with elevated levels of propionic acid. Many other genera in the phylum Bacteroidetes were also increased, enhancing gut fermentation of sugar beet pectin.

Red beetroot contains polysaccharides and oligosaccharides that can modulate the gut microbiota positively, showing resistance to enzymes in the fore gut and ability to reach the large intestine where they are fermented (prebiotic effect) by microorganisms. This leads to the production of metabolites (SCFA) that impact on host metabolism, physiology nutrition and immune functions (Leijdekkers et al., 2014; Danneskiold-Samsøe et al., 2019). Conversely, some studies have investigated into the prebiotic-like property of beet consumption on the gut microbiota. In which it acts as substrates (prebiotics) that are selectively utilised by microorganisms in the gut conferring a variety of health benefits to the host (Gibson et al., 2017), essentially by the proliferation of healthy bacteria species in the gut.

1.4.1.2.3 Red beetroot as livestock feed resource

Most studies involving red beetroot were conducted in humans and rodent with few representative studies in rainbow trout. Red beetroot as livestock feed resource has been insufficiently explored, unlike sugar beet. Though, de Oliveira et al. (2021) reviewed the benefits of beet on gastrointestinal health, the impact of red beetroot in weaned pig diet is grossly lacking. Red beetroot supplementation, as a substitute for in-feed zinc oxide has neither been explored, nor investigated in post weaning diarrhoea or associated GIT issues. Utilization of red beetroot as animal feed resource, presents a cost-effective way of providing bioactive compounds (betalains, nitrate and polyphenols) in red beetroot to the pigs while also preserving its fibre content.

Table 1.4. Retrieved studies on the bioactivity of red beetroot and gut microbiota modulatory effects; *adapted from (de Oliveira et al., 2021; Clifford et al., 2015)*

Experiment model/ clinical parameters	Tested beet part /product	Duration	Observations	References
Beetroot derivatives on oxidative stress in Albino rats after intraperitoneal injection of gentamicin for 8 days	Fresh beetroot extract (250 and 500 mg/kg/body mass (bm) /day	28 days	Attenuated NF-kB DNA binding activity. Reduced pro-inflammatory cytokines and oxidative damage related to the blunting of the NF-KB pathway.	(El Gamal et al., 2014)
Osteoarthritis patients	Betalain rich oral capsules from beetroot extracts (100, 70 or 35 mg per day)	10 days	Alleviated inflammation and pain. Decreased TNF-α and interleukin 6 (IL-6) to baseline levels	(Pietrzkowski et al., 2010)
Hepatotoxicity in rats treated with toxic chemical N-nitrososiethylamine NDEA	Beetroot juice 8 mL/kg/bm/day	28 days	significant hepatic protection observed against inflammatory markers induced by the toxicant	(Krajka-Kuźniak et al., 2012)
Evaluation of hepatoprotective and anticarcinogenic effect of betanin in non-tumour hepatocytes and hepatocellular carcinoma cells	Beetroot betanin (2, 10, 20 µM)		Betanin induced the expression of detoxifying enzymes, indicating that it is partly responsible for the beet hepatoprotective ability	(Krajka-Kuźniak et al., 2013)
Exposure to cobalt via gamma radiation (80 male rats)	Betalains from beetroot (0,5, 20 and 80 mg/kg/bm/day	30 days	Decreased malondialdehyde levels with increased antioxidant enzyme activities	(Lu et al., 2009)
Assessment of beet and beet bioactive compound on intestinal microbiota	Fermented Beet juice with *Lactobacillus brevis* and *L. paracasei* (0.7 L/ kg)		Anti-mutagenic activity	(Klewicka and Czyzowska, 2011)
Determination of faecal microbiota composition in healthy adults	Beetroot	4 days	Weight loss, increased plasma nitric oxide and decreased lipid peroxidation. Effects primarily attributed to high contents of fibre and nitrate in beetroot pulp.	(Henning et al., 2017)
Wistar rats fed a basic casein diet	6 ml daily of fermented beetroot juice		Modulation of Caecal microbiota and metabolic parameters of rats with decreased Enterobacteriaceae	(Klewicka et al., 2009)

It is necessary to investigate if the health benefits attributed to red beetroot can be leverage towards alleviating weaning stress and PWD in pigs, thereby improving pig immunity and health. While investigations on the health benefits of red beetroot have continued over the years, there is a need to explore the potentials of RB as supplement in replacement for pharmacological doses of ZnO in weaned pigs focusing on gut microbiota modulation and gut health. Therefore, the aim of this present study exploring the role of red beetroot as in-feed anti-inflammatory supplements against gut dysbiosis and inflammation post weaning.

1.4.2 Dietary factors, health and gut health

Diet of weaned pigs is crucial to the prevention and control of PWD and deterioration of gut health. Nutritional stress at weaning, from change in diet and drastic reduction in feed intake, constitutes a major risk to pig health, performance and gastrointestinal functionality. Appropriate and adequate dietary composition at weaning is imperative to maximise feed intake and prevent deterioration of the gut structure (Pettigrew, 2006; Kil and Stein, 2010; Arrieta et al., 2014). Diet also aids modulation of the GIT microbial composition and function towards optimal health. Analyses of previous studies revealed that the gut microbiota is greatly influenced by various dietary components including probiotics, prebiotics, organic acid, phytochemicals, dietary fibre and crude protein levels (Walker et al., 2011; Macfarlane and Macfarlane, 2012; Knudsen, 2014). In this research the control diet comprised of a standard wheat (28%) based diet with protein sourced mainly from soya (18%) with other premixes added.

As summarised in Pluske et al. (2018), important factors to consider in the composition of weaned pig diet are palatability, increased digestibility with processed fibre source, high quality moderate protein content and low anti-nutritional factors (Heo et al., 2013; Park et al., 2016; Flis et al., 2017). Considering the importance of dietary fibre and bioactive phytochemicals in weaned pig diet towards the mitigation of post-weaning diarrhoea and promotion of gut health, fruits, vegetables and their by-products are suitable and sustainable sources of these components (Ruberto et al., 2007). Many studies have highlighted the potentials of feed supplement, functional foods and nutraceutical strategies from fruits and vegetables (e.g., rapeseed, grapefruit, carrot peel, tomato pomace, beets, orange peel and pulp) as dietary interventions to support

the immune system, modulate redox balance and inflammatory response towards improving animal health (Celi and Gabai, 2015; Rebezov et al., 2020).

As indicated previously, phytochemicals are naturally occurring plant metabolites with diverse biological functions that qualify them as potential alternatives to in-feed antibiotics. They exhibit a wide spectrum of antibacterial activities (Hammer et al., 1999; Wong et al., 2008) and their immune-regulatory activities have been demonstrated in both human and animal models (Sokmen et al., 2004; Liu et al., 2012; 2013). Previous studies on dietary supplementation of animals with phytochemicals reported reduced diarrhoea incidence, improved growth performance and enhanced disease resistance (Cicero and Colletti, 2016; Lillehoj et al., 2018). Thus, phytochemicals enable an improved gut health and barrier integrity (Yin et al., 2019; Dingeo et al., 2020; Xiao et al., 2022).

Dietary fibre enhances digestion, nutrient availability, gut microbial composition, gut fermentation and the production and utilisation of metabolites. They are important anti-inflammatory and anti-carcinogenic agents in the digestive system. Following the ban on in-feed antibiotics and ZnO in pig production, inclusion of FVW or plants containing bioactive compounds to weaned pig diet provides a viable source of essential dietary components and potential nutritional strategy to optimize gut health (Jha and Berrocoso, 2015; 2016; Klurfeld et al., 2018; Fang et al., 2022).

1.4.2.1 Gut health and biomarkers of gut health

Due to the multifactorial and complex characteristics of a healthy gut, gut health is considered as the ability of the GIT to maintain homeostasis with respect to its overall structure and function, in spite of the challenges (e.g., pathogenic, chemotactic agents, biochemical changes) it may encounter (Pluske et al., 2018). There is a continuous interaction between the nutrition (diet), the GIT mucosa (the barrier function), and the microbiota (microbial composition) within the GIT that may significantly impact the gut health (Niewold, 2015).

Gut health encompasses several physiological functions, summarised by Bischoff (2011) as, effective food digestion and absorption, absence of GIT infections, normal and stable microbiome, effective immune status and general wellbeing. Whilst these describe the functions of the GIT, the GIT also regulates the epithelial and immune

functions of other vital organs essential for the body homeostasis. For instance, the gut-brain connection axis (comprising; the enteric nervous system, the parasympathetic nervous system and the endocrine system) is crucial to animal wellbeing, health and proper functioning of the GIT (Celi et al., 2017; Moeser et al., 2017).

With the numerous studies involving dietary supplementation of animal diets with plants containing dietary fibres and bioactive phytochemicals, there is need to assess the impact of these interventions on the gut health. Several biomarkers (*in vitro* and *in vivo*) measuring GIT functionality and health as a mark of successful dietary interventions have been established in the literature. This was summarised in a review by Celi et al. (2019) and here presented in Table 1.5. Recently, the emergence of "omics" approach (e.g., metagenomics, transcriptomic, proteomics, metabolomics etc.) have further enhanced understanding the impact of diet on gut health. The mechanisms and impact of dietary supplementation on health as well as interactions between the gut microbiota, microbiota metabolites and functional pathways can be studied using these novel approaches (Xiong et al., 2019; Rostagno, 2022).

Table 1.5: Biomarkers of gut functionality and health in animal nutrition; *adapted from Celi et al. (2019)*

Features/Biomarkers	Test site	Biological samples	Method	Remarks and References
Digestion and absorption				
Total carotenoids	Duodenum and Jejunum	Blood	Spectrophotometry, High performance liquid chromatography (HPLC)	(Vieira et al., 2008; Raila et al., 2017)
Short chain fatty acids and branched chain fatty acids	Ileum and colon	faeces	Gas chromatography; HPLC; capillary electrophoresis	(Windey et al., 2012; Apajalahti and Vienola, 2016;)
Gut microbiota				
Lactate	Whole intestine	Blood; digesta content	Colorimetry, Fluorometry	Indirect measurement of intestinal permeability (Kurundkar et al., 2010)
Succinate	Whole intestines	Digesta content; faeces, blood		(Tsukahara et al., 2001; Tretter et al., 2016)
Immune status				
Pancreatitis-associated proteins or Regenerating islet derived III proteins	Small intestine	blood; faeces	Immunoassay	Lectins produce, stored and secreted in the intestine and in the pancreases. (Niewold, 2015; Soler et al., 2015)
Calprotectin	Whole intestine	Faeces; blood	Immunoassay	Marker of neutrophil activity, and loss of enterocytes (Šplíchal et al., 2005; Alibrahim et al., 2015)
Lipopolysaccharide	Whole intestine	faeces; blood	Immunoassay	Endotoxin presents on Gram-negative bacteria. (Mani et al., 2012; Metzler-Zebeli et al., 2013)
Gut barrier function				
Mucin 2	Whole intestine	Faeces; tissue	Fluorescence, qPCR	(Chen et al., 2015)
Tight junction proteins/ Intestinal fatty acid binding proteins	Whole intestine; small intestine	Tissue; blood; plasma; faeces	qPCR, western blot; immunoassay	(Suzuki, Takuya 2020; Chen et al., 2020)

1.5 Thesis aims, objectives and hypothesis

The need for alternatives to in-feed antibiotics and zinc oxide in weaned pig diet, have warranted a significant increase in studies exploring the supplementation of weaned pig diet with fruits, vegetables and their waste (FVW) or plant extracts. Many plants containing bioactives and fibre are currently under investigation for their probable ability to mitigate declining health and gut health of pigs post weaning. Red beetroot (RB) is one of such plants that have gained much attention recently from its prominent anti-inflammatory, anti-oxidative, hepatoprotective and other health benefits reported in the literature (Clifford et al., 2015; Mirmiran et al., 2020). These health benefits have been verified in humans, rodents (Wootton-Beard et al., 2014; da Silva et al., 2019) and rainbow trout (Pinedo-Gil et al., 2017; 2018). However, the effect of red beetroot supplementation in weaned pigs have not been considered, and studies demonstrating the potential of red beetroot supplementation on the gut health is lacking.

1.5.1 Hypothesis

This research is based on the premise that red beetroot through its health promoting constituents will modulate the gut microbial composition and diversity of weaned pigs, promoting the growth of beneficial gut microorganisms that will facilitate increase production of gut metabolites (short chain fatty acids and bile acids). Thus, improve the health and gut health of weaned pigs by alleviating gastrointestinal changes and dysfunctionality that ensues post weaning.

1.5.2 Thesis aim

The general aim of this thesis is to evaluate the impact of supplemental red beetroot on the gut health of weaned pigs through evaluation of gut microbiota, gut immunity, gut integrity and barrier functions, and overall implication on the health of weaned pigs.

1.5.3 Objectives

1. To investigate the effect of red beetroot supplemented diet on the gut microbial composition, diversity and metabolite production of weaned pigs, in comparison to a control diet and zinc oxide supplemented diet.

2. To extrapolate, the gut microbiota mediated functions, growth performance, diarrhoea occurrence and health implications of diet supplemented with red beetroot in weaned pigs.
3. To assess the structural and functional changes associated with the gastrointestinal tract (gut structure, integrity and barrier functions) with red beetroot supplemented diets, post-weaning.
4. To evaluate the anti-inflammatory and antioxidative response of weaned pigs to diet supplemented with red beetroot.

1.5.4 Thesis outline

This thesis compares the effect of red beetroot supplemented weaned pig diet to a control – basal diet and diet supplemented with pharmacological dose of ZnO. Chapter 2 explores the impact of these diets on the gut microbial composition, metabolite production and the functional pathways enhanced by the gut microbiota. The chapter also reports on performance response and diarrhoea occurrence in the weaned pigs. Following this, chapter 3 investigates changes associated with the GIT, in response to the dietary treatments, especially the structural and functional changes. Then, chapter 4 evaluates the immune response of the pigs to the diets, measured by their anti-inflammatory and antioxidative status. In conclusion, chapter 5 summarises the benefit of the diets, industrial application of the study, as well as limitations and future research focus.

Chapter 2 : **Impact of red beetroot supplement on performance, gut microbiota diversity and metabolite profile of weaned pigs**

2.1 Abstract

A healthy and well-developed gut microbiota is essential to the production of healthy pigs with zero in-feed zinc oxide. With the current prohibition on pharmacological doses of zinc oxide in weaned pig diet, alternative ways to improve gut health and lower the risk of post weaning diarrhoea are still being considered. This study evaluated red beetroot, a natural source of bioactive compounds with anti-inflammatory and antimicrobial properties, as a potential alternative to zinc oxide with focus gut microbiota and metabolite changes. Forty-eight pigs weaned at 28 days old were randomly allocated (*n* = 12) to one of four diets which comprised; a control diet, diet supplemented with zinc oxide (3,000 mg/kg), or 2% and 4% red beetroot (CON, ZNO, RB2 and RB4; respectively) for a 14-day feeding experiment. The pigs were housed in a temperature-controlled flat deck with access to feed and water. After the experimental period, pigs were euthanized, blood and gut samples were collected for microbial composition and metabolite analyses. The results showed diet supplemented with red beetroot at 2% (RB2) increased the gut species richness compared to other diets, but marginally influenced the caecal diversity compared to diet supplemented with zinc oxide (ZNO). Total bile acids and short chain fatty acid levels in RB2 pigs were comparable with the CON, while these metabolites reduced in pigs fed ZNO and RB4 diet. Increased red beetroot levels (4%, RB4) led to loss of caecal diversity and increased Proteobacteria abundance. In conclusion, RB2 showed potential benefits on gut microbiota modulation and metabolite profile. However, red beetroot contains several constituents considered to exert different effect that impacted the outcome of this study. Future research investigating individual components in red beetroot are warranted for better elucidation of their contributions to gut microbiota modulation and pig health.

2.2 Introduction

Weaning is a stressful phase in pig production characterised by reduced feed intake, poor growth rate and diarrhoea that ensues from separation of young pigs to an environment different from the sow (Williams 2003; Lallès et al., 2004; Callesen et al., 2007). Due to a change in diet from milk to complex plant-based solid feed and the pigs' underdeveloped immuno-digestive system, the gut structure, function and microbial balance is disrupted (Kim and Isaacson, 2015; Guevarra et al., 2019). Diet remains a strategy to modulate a healthy gut microbial composition towards alleviating post weaning diarrhoea, with marked changes observed after two weeks of providing weaned pigs with supplemented diets (Leser et al., 2000; Cheng et al., 2018).

Several dietary supplements such as in-feed antibiotics, metallic oxides (ZnO, $CuSO_4$), organic acids, dietary fibre and proteins through their impacts on the gut microbiota have been reported to reduce piglet mortality during weaning (Miller et al., 2009; Slade et al., 2011; Seal et al., 2013; Thacker, 2013). For instance, a fibre rich diet sourced from alfalfa was reported to improve gut health and increase the abundance of beneficial microorganisms in weaned pigs (Liu et al., 2018)). Similarly, probiotics (e.g., *Lactobacillus spp.*) and prebiotics beneficially modify the gut microbiota (Guerra-Ordaz et al., 2014; Dowarah et al., 2017; Liao and Nyachoti, 2017).

Many studies have demonstrated the potentials of dietary supplements including in-feed antibiotics and zinc oxide to reduce pig mortality and enhance overall health (Kim et al., 2012). However, they have been implicated in gut dysbiosis and current emergence of antibiotic-resistant bacterial strains and genes that are harmful to human health (Holman and Chénier, 2015; Schokker et al., 2015; Zhao et al., 2018). Consequently, the use of in-feed antibiotics as a growth promoter around weaning has been banned in pig production in the (EU) European Union (January 2006; regulation (EC) No 1831/2003) and many developed countries of the world (Commission, 2003; Anadón, 2006).

Weaned pig diet supplemented with pharmacological levels of zinc oxide (ZnO; ranged 2,000 - 4,000 mg/kg) are used in many countries including the UK, until recently. ZnO has been found to supress the incidence of post weaning diarrhoea, by alleviating weaning-induced intestinal dysfunction and inflammation (Heo et al., 2013; Hu et al.,

2013; Dong et al., 2019). However, accumulated evidence from literature have shown unhealthy effects of ZnO on the gut microbiota. Which includes an increased population of coliforms (Højberg et al., 2005; Pieper et al., 2012), reduced anaerobic and lactic acid bacteria (Broom et al., 2006; Vahjen et al., 2011; Ciesinski et al., 2018) as well as reduced population of commensal bacteria (Starke et al., 2014; Bonetti et al., 2021).

Moreover, there are concerns about severe environmental pollutions from high faecal excretions of zinc (Jondreville et al., 2003; Jensen et al., 2018) and strong links between animals fed pharmacological levels of ZnO, with increases in multidrug resistant *E. coli* (Bednorz et al., 2013; Yazdankhah et al., 2014). This has raised the potential for zoonotic transmission of drug-resistant organisms from food animals reported by Cuny et al. (2015) and Lazarus et al. (2015) and cumulated in the decision of the EU to adopt a zero-zinc diet policy by June 2022 (EC regulation 2016/1095) (EFSA, 2017; EFSA, 2020). Consequently, there is an increased interest exploring other alternatives to ZnO in weaned pig diets, employing diet supplemented with plants containing bioactive compounds and health promoting properties. This study therefore explored the effect of red beetroot as a supplement in weaned pig diet.

Red beetroot (*Beta vulgaris subsp. vulgaris conditiva*) is a rich source of bioactive compounds such as betalains, polyphenols, saponins, inorganic nitrate (NO_3), fibre (Chhikara et al., 2019) and minerals like potassium, sodium, phosphorus, calcium, magnesium, copper, iron, zinc, and manganese (Mirmiran et al., 2020). It is classified as one of the top ten plants with high antioxidant, anti-inflammatory, antimicrobial, anticarcinogenic and hepatoprotective characteristics (Georgiev et al., 2010; Singh and Hathan, 2014). Red beetroot is considered a therapeutic ingredient in the development of functional foods to address health challenges. Although many studies have focused on the potential health benefits of red beetroot *in vitro* and *in vivo* in humans, murine species, (Clifford et al., 2015; da Silva et al., 2019) and rainbow trout (Pinedo-Gil et al., 2017; 2018), effect in pigs and evidence on gut microbiota modulation is deficient. This study reports on the effect and health implications of red beetroot supplement on gut microbiota composition, short chain fatty acid (SCFA) and bile acid levels in weaned pigs.

2.3 Materials and methods

2.3.1 Materials

The basal diet used for this study was provided by Primary diet, UK, and whole red beetroot powder (200 mg/g fibre) was purchased from (Buy Wholefoods online Ltd, Ramsgate, UK). All bile salts used as reference (taurohyodeoxycholate, glycohyodeoxycholate, taurocholate, glycocholate, taurochenodeoxycholate, taurodeoxycholate, glycochenodeoxycholate, glycodeoxycholate, cholate, glycolithocholate, chenodeoxycholate, deoxycholate and lithocholate acid), were either purchased from Sigma-Aldrich (Steinheim, Germany) or Cayman (Cambridge, UK). Also, a mixed volatile short chain fatty acid standard containing (acetate, propionate, butyrate, valerate, isobutyrate and isovalerate) was obtained from (Supelco – Merck life science Ltd, Dorset, UK). The solvents used, acetonitrile (99.9%), methanol (99.9%), phosphoric acid (85%) and formic acid (98 -100%) were obtained from (Sigma-Aldrich, Germany) and (Fischer Scientific, Loughborough, UK). Chemical reagents, ammonium acetate was purchased from (Fischer Scientific, Loughborough, UK). DNA extraction kit QIAamp Power faecal DNA was from Qiagen (Hilden, Germany), and Strata-X 33µm polymer-based cartridges from Phenomenex, Cheshire, UK. NEBNext Q5 Hot start HiFi PCR master mix was from (New England BioLabs, Ltd, UK) and AMPure XP beads was purchased from (Beckman Coulter, United Kingdom).

2.3.2 Methods

2.3.2.1 General overview and ethical statement

The animal trial presented in this thesis was conducted at the National Pig Centre, University of Leeds, UK. The experiment was conducted under an ethical approval granted by the University of Leeds Animal Welfare and Ethical Review Board (AWERB) designated N0. 070510HM (Appendix A). All husbandry practises were set by the farm in accordance with the Welfare of Farmed Animals, England Regulation 2007. All procedures were conducted following the Animals (Scientific Procedures) Act 1986, amended regulation by the EU directive 2012/3039/EU and were undertaken by personnel holding a Home Office Personal Licence.

2.3.2.2 Experimental design and sample collection

Piglets were farrowed and sourced from the National Pig Centre, University of Leeds, UK. Forty-eight piglets (Large White X Landrace X Duroc) weaned at 28 days old (average weight: 7.81±0.178 kg) were allocated to one of four dietary treatments balanced for body weight, sex and litter origin (*n* = 12) for a 14-day feeding trial. The pigs were housed in pens in a temperature-controlled flat deck, with open feed troughs and nipple drinkers, allowing access to feed and water ad libitum. Each pig represented an experimental unit and samples were collected per pig. As the main focus of this study was to determine the effect of red beetroot supplementation on gut microbiota modulation the sample size was estimated based on previous studies with similar objectives (Tian et al., 2017; Li et al., 2019; Gu et al., 2019).

The experimental diets comprised a control (CON) and diet supplemented with 3000 mg/kg zinc oxide (ZNO), both formulated to meet nutritional piglet requirements set by the National Research Council (2012). While the red beetroot supplemented diets (RB2 and RB4) were mixed on-farm by adding 2% (20 g/kg) and 4% (40 g/kg) whole red beetroot powder to the control diet respectively (Table 6.1). Feed samples from each diet group were analysed for ash, ether extract, crude protein, crude fibre and zinc, at Sciantec Analytical Services Ltd (Cawood, UK).

During the trial, pigs were weighed individually on days 0, 7 and 14, feed intake was estimated per diet group daily, for calculations of average daily feed intake (ADFI), average daily weight gain (ADG) and feed conversion ratio (FCR). Faecal score was assessed by visual observation of faecal droppings on the pen floor, by the same personnel and scored on the scale of 1 to 5 (where 1 = firm faeces, 2 = soft faeces, 3 = mild diarrhoea, 4 = severe diarrhoea and 5 = scour). At the end of the experiment, one mortality was recorded for the CON group and nine animals (2 pigs each from CON, ZNO, RB2 and 3 pigs from RB4) were taken off trial due to weekly weight loss from inability to adjust to post weaning diet, changes and stress. Thirty- eight pigs were euthanatized by captive bolt and exsanguination, blood samples were collected from the jugular vein into heparinized tubes, and from the portal vein into plain tubes. Plasma was separated from blood samples after centrifugation at 2000 x *g*, 4°C for 10 mins. Faecal samples were collected from the rectum into designated samples tubes. The abdominal cavity was opened, each intestinal segment (duodenum, jejunum,

ileum, caecum and colon) was separately tied, cut and emptied into a sterile beaker. The digesta was immediately mixed, aliquoted into sterile 2 mL tubes. All samples were immediately snapped frozen in liquid nitrogen and later stored at -80°C.

2.3.2.3 Quantification of short chain fatty acids (SCFA) and bile acids

Short chain fatty acid (acetate, propionate, butyrate, valerate, isobutyrate and isovalerate) concentrations in the plasma, digesta from the jejunum, ileum, caecum, colon and faeces was determined using gas chromatography (Varian 3400; Varian Ltd., Oxford, UK). As described by Taylor et al. (2018), 1 mL digesta sample was mixed with an equal volume of distilled water in an Eppendorf tube and centrifuged at 12,000 x *g*, 4°C for 10 min. Faecal samples were also treated in the same way (0.6 g in 0.6 mL of distilled water). Then phosphoric acid (50 µL, 85% v/v) and 0.15 mL of caproic acid (150 mM/L) was added as internal standard to the supernatant (0.5 mL) collected. The mixture was made up to 1mL with distilled water, centrifuged at 14,000 x *g* for 20 min and the supernatant was collected for SCFA analysis.

Bile acid in digesta samples was determined using method described by Zhang et al. (2018) with slight modifications. Approximately 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*, 4°C for 20 min. The supernatant collected was passed through 33µm polymer based solid phase extraction cartridges, after they had been conditioned with methanol and water. Extracted 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 phases: 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 to 0.1mM) 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.

2.3.2.4 DNA extraction, amplification and sequencing

Digesta from the jejunum, ileum and caecum (*n* = 8 pigs per diet) was used for evaluation of the gut microbial composition, selecting about equal number of pigs from each pen per diet group. DNA was extracted from (~ 1.0 g) digesta sample with QIAamp Power faecal DNA kit (Qiagen, Hilden, Germany) according to manufacturer's instructions. The concentration and purity of each DNA extract was measured spectrophotometrically with Nano Drop® ND-1000 (Nano Drop Technologies Inc., Dover, USA) and absorbance ratio at 260/280nm observed was within the range 1.8 - 2.0. Samples were submitted to the University of Leeds Next Generation Sequencing facility, St. James' Hospital, Leeds, UK, for 16S rRNA gene library preparation and genomic sequencing.

The V4 region of the bacteria 16S rRNA gene was amplified using a two-step polymerase chain reaction (PCR) with Illumina adaptor overhang and primers (564F, 806R) described in Illumina 16S Metagenomics sequencing library preparation protocol. First, a mixture of NEBNext Q5 Hot start HiFi PCR master mix (12.5 µL), 10µM of each primer (0.5 µL) and 5µL of DNA extract was combined for each sample, the volume was made up to 25 µL with nuclease free water. The program for amplification was set at 95°C for 3 min followed by 28 cycles of 95°C for 30 sec, 50°C for 30 sec, 72°C for 30 sec, a final extension step at 72°C for 5 min then a holding step at 4°C. Amplification was confirmed on a Bio-analyser 1000 chip (Agilent, Santa Clara, CA, USA), then amplicons were purified away from the primers and primer dimer species using AMPure XP beads (Beckman Coulter, United Kingdom). A dual index and Illumina sequencing adapter was attached to the PCR product using the Nextera XT index kit (FC-131-1001) (Illumina, San Diego, CA, USA) for a further 8 cycles of PCR. The final library was cleaned up, quantified with Quant-iT^{TM} (Thermofisher scientific, Waltham, Massachusetts, USA) and pooled. The pooled libraries were pair-end sequenced on Illumina MiSeq platform using 2 x 250 base pairs MiSeq reagent kit v3 (Illumina, San Diego, CA, USA).

2.3.2.5 Sequence processing and bioinformatics

Sequence reads were processed in Mothur v.1.43.0 (Schloss et al., 2009) according to the MiSeq standard operation procedure developed by the Schloss group (Kozich et al., 2013). The forward and reverse sequence reads were combined to form contigs,

ambiguous bases were removed and contigs with 250 to 275 base-pairs long were processed further for genus level identification (McDermott et al., 2020). Unique sequences were identified and aligned to the SILVA reference database (v.138), a maximum homopolymer length of 8 was set then chimeras along with sequences identified from the 16S rRNA for archaea, chloroplasts and mitochondria were identified and removed. The sequences were pre-clustered allowing for 1 difference in every 100-base pair of sequence. Sequences with 97% similarity were clustered into operational taxonomic unit. A “Biome” file was generated to transfer the OTU table, associated taxonomy and metadata for use in R environment.

The “Biome” file was processed statistically using R (v 3.6.2 and 4.0.0) with the statistical packages Phyloseq (v. 1.31.0) (McMurdie and Holmes, 2013) (McMurdie and Holmes 2013), ggplot2 (v. 3.3.0) (Wickham, 2011) and vegan (v. 2.5-6) (Oksanen et al., 2013). Alpha diversity (Chao 1, Shannon and Simpson indices) measure of the gut microbial composition was performed, a general linear model of the three indices was conducted to evaluate the effect of diet and gut location on the gut microbial diversity. The models were reduced with lmer Test (Analysis of Deviance, AOD) using the nlme package (v. 4.1.1) in R (Lindstrom and Bates, 1988).

For the Beta diversity, a PERMANOVA (Permutational multivariate analysis of variance, 999 permutations) was used to identify significant differences in the Bray Curtis distances between the two factors (diet and gut location) and interactions between the samples. While a non-metric multidimensional scaling (NMDS) plot of the Bray Curtis distance was used to visualize the non-phylogenetic distance in the bacterial communities across the gut locations and between the diets. A multilevel pairwise comparison between diets and gut locations was also conducted using pairwise Adonis function from vegan package in R (v. 4.0.0). Differentially abundant genera between gut locations per diet was identified using a two-sided Welch’s *t*- test and graphically presented using STAMP (Statistical Analysis of Metagenomics and other Profiles) software (Parks et al., 2014). Further analysis employed DESeq2 (v. 1.27.32) in R (Love et al., 2014) with Wald hypothesis testing for distinct genera in each gut location comparing multiple diet groups. Differences were estimated as fold change (Log 2- fold change) between diets, corrected by Benjamini-Hochberg, false

discovery ratio (FDR) and statistically significant results were presented as probability (*P)* values ≤ to 0.05.

2.3.2.6 Predicted gut microbiota functions

To predict the functions of the gene sequenced from the samples, the OTU abundance table and their representative sequences generated from Mothur were sent to Piphillin (https://piphillin.secondgenome.com/). The representative sequences were screened against KEGG (Kyoto Encyclopaedia of Genes and Genomes) database (2018) as described in Iwai et al. (2016) using USEARCH version 8.0.1623 with global alignment setting for sequences identification fixed to 97% cut-off. However, at 97% cut off no OTU matched the 2018 version of the KEGG database. Hence, following the recommendations in Iwai et al. (2016), a 90% cut off described to have a significantly high correlation with Taxa4fun and PICRUSt (Phylogenetic Investigation of Communities by Reconstruction of Unobserved States) (Langille et al., 2013) was adopted. This method allows for prediction of functional genes present in the representative sample sequence from the microbial community, based on a marker-gene survey, however, unlike a full metagenomic sequencing, it does not provide direct information about the functional genes in the genome.

From the 90% cut-off, 95 OTUs with greater identity above the cut off matched the database and 130 genomes were inferred from the samples. The output was normalized with the 16S rRNA copy number of each genome, a metagenome was inferred using the gene contents of each genome in the database and the result table was exported to R environment with a metafile describing each sample to complete the analyses. Functional differences among the diets were compared in STAMP using two-sided Welch's *t*-test, differences were considered significant at $P \leq 0.05$ and after values were corrected for multiple-group comparisons by Benjamini-Hochberg FDR correction. Additionally, pathways differentially mediated by diet in the different gut locations were computed with DESeq2 in R with Wald hypothesis testing and *p* values adjusted for multiple inter-diet comparisons.

2.4 Statistical analyses

Bile acids levels in sample was computed in Lab Solutions 5.98 SP1 (Shimadzu Limited, UK). Short chain fatty acid (SCFA) and bile acid concentrations were analysed

in R (v. 4.2.2). Zero inflated data were analysed using negative binomial with square root link function, and 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 also performed between SCFA levels, bile acids and the mean relative abundance of top twenty-five genera in each gut location per diet using the "Psych" (Revelle, 2016) and "Pheatmap" (Gu et al., 2016) packages in R (v. 3.31). Pig growth and performance were computed in Excel 2016 and differences between diet group analysed in SPSS (v. 26.0) using ANOVA.

2.5 Results

2.5.1 Effect of diets on growth performance and diarrhoea incidence

Overall, there was no significant difference observed in the BW, ADG, ADFI and FCR of the pigs across the experimental period. The growth performance, feed intake and faecal score of weaned pigs are presented in Table 2.1. A reduction in BW was observed for pigs fed diets CON and ZNO during the first week of the trial. The BW of each diet group increased from day-8 to day-14 of the trial, albeit no significant difference was observed between the groups. The ADFI was significantly different for the pigs fed the different diets ($P = 0.05$) during the second week of the trial, with higher feed intake associated with the red beetroot supplemented diet when compared with zinc oxide. Also, the faecal score was not different from one pig to the other (Figure 2.1).

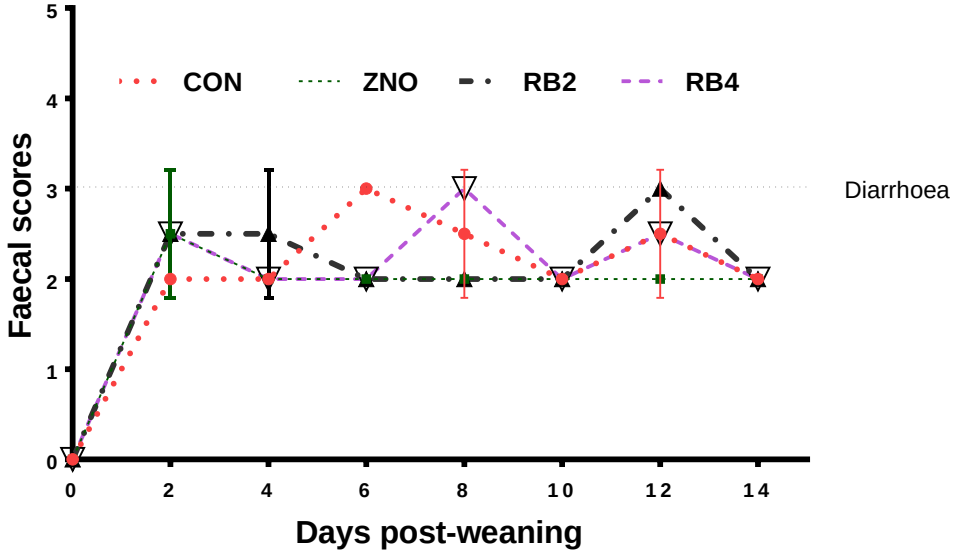


Figure 2.1: Effect of diet on faecal scores (day 0 to day 14) post weaning.

Values plotted are means and their standard error. Faecal scores on a scale of 1 to 5; firm faeces, soft faeces, mild diarrhoea, severe diarrhoea and scour. Diet: CON, ZNO, RB2 and RB4 represents; control, and diet supplemented with zinc oxide, 2% and 4% red beetroot respectively.

Table 2.1: Growth response and feed intake of weaned pigs

Period	Diets				*P* value
	CON	ZNO	RB2	RB4	
Pig weight (kg)					
Day 0	7.90±0.34	7.64±0.46	7.69±0.41	8.02±0.27	0.883
Day 7	7.88±0.27	7.49±0.44	7.94±0.40	8.45±0.43	0.416
Day 14	9.11±0.36	8.67±0.55	9.37±0.59	9.59±0.56	0.640
ADG (kg/day)					
0-7 days	-0.003±0.028	-0.021±0.039	0.035±0.037	0.060±0.030	0.268
8-14 days	0.191±0.037	0.185±0.048	0.222±0.036	0.175±0.036	0.449
0-14days	0.090±0.031	0.078±0.039	0.126±0.030	0.116±0.028	0.684
ADFI (kg/day)					
0-7 days	0.107±0.009	0.112±0.013	0.168±0.039	0.169±0.019	0.137
8-14 days	0.283±0.003^{ab}	0.244±0.021^{b}	0.351±0.033^{a}	0.379±0.267^{a}	0.049
0-14days	0.184±0.001	0.171±0.014	0.251±0.039	0.257±0.014	0.104

Values presented in mean±SE - standard error. ADFI; Average daily feed intake, ADG; Average daily gain. Values with different alphabets are significantly different from each other. P values ≤ 0.05 are statistically significant for the subject and period evaluated. CON, ZNO, RB2 and RB4 represents; Control diet, diet supplemented with zinc oxide, 2% and 4% red beetroot, respectively.

2.5.2 Effect of diets on short chain fatty acid and bile acid profile

The gut locations assessed influenced the SCFA levels, with reduced levels (1 to 10-fold) observed in the jejunum and ileum ($P < 0.001$) compared to the caecum, as expected. The caecum, colon and faecal SCFA levels were similar to each other, nonetheless SCFA levels in the faecal sample from the pigs was higher, ($P = 0.003$) compared to levels in the caecum (Table 2.2). The experimental diets ($P < 0.05$) influenced the SCFA levels observed in the different gut locations. SCFA levels was high in the jejunum of RB2 pigs, lower in the ileum of ZNO pigs, but comparable in the caecum across the diet groups. Also, pigs on diet RB2 ($P = 0.03$) and ZNO had higher ($P < 0.001$) butyrate levels in the ileum and jejunum respectively, whereas pigs fed diet RB4 ($P = 0.02$) and ZNO ($P = 0.03$) had lower faecal SCFA level. Overall, total SCFA levels reduced ($P = 0.01$) in RB4 and ZNO pigs, as was most SCFA (e.g., acetate, propionate, and butyrate), but levels in CON and RB2 pigs were comparable (see Table 6.2a, Figure 6.2a).

Table 2.2: 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
Faeces	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 represents mean SCFA for each diet group and gut location, significant differences 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.*

Similarly, the gut locations influenced the concentration of bile acids observed with high jejunal bile acid levels (approx. 1 to 3-fold) compared to other locations, which is a normal biological trend. However, pigs fed RB2 diet had higher (P < 0.05) TCA, GCDCA, CA, GLTCA, CDCA and DCA compared to other diets, but an equivalent, total and unconjugated bile acids (CA, DCA, CDCA and LCA) level with CON pigs (Table 2.3). 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. (Table 6.3; Figure 6.2b). RB2 fed pigs had the highest total unconjugated (CA, DCA, CDCA, and LCA) bile acids (RB2 > CON > RB4 ≤ ZNO). While pigs fed CON had the highest total conjugated (GCDCA, GDCA, TCDCA, TDCA, GHDCA, THCA, GLTCA, GCA, TCA) bile acids (CON > RB4 > ZNO > RB2).

Table 2.3. Bile acid profile (nmol/g digesta/faeces) from experimental diet groups

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

2.5.3 Effect of diets on alpha and beta gut microbial diversity

The alpha diversity measure of the gut microbiota of weaned pigs fed the experimental diets is shown in Table 6.4. There was a significant effect of the experimental diets (*P* = 0.01) on the species richness and diversity of the gut microbiota with respect to the gut locations (*P* < 0.001; jejunum, ileum, and caecum) examined. From the Chao1 index, diet RB2 increased the jejunal species richness (*P* = 0.02) compared to other diets. The caecum was comparatively species rich for all the diets (Figure 2.2a) and in addition more diverse (*P* < 0.001) than the jejunum and ileum.

While the Shannon index of alpha diversity measure, showed the gut microbial community was diverse, but not generally due to the diets (*P* = 0.07), howbeit a pairwise diet comparison showed ZNO was different from CON and RB4 (Figure 2.2b) but related to RB2. No significant species abundance, divergence, or evenness (i.e., dominance) was observed between the diets and in the gut locations for the Simpson index.

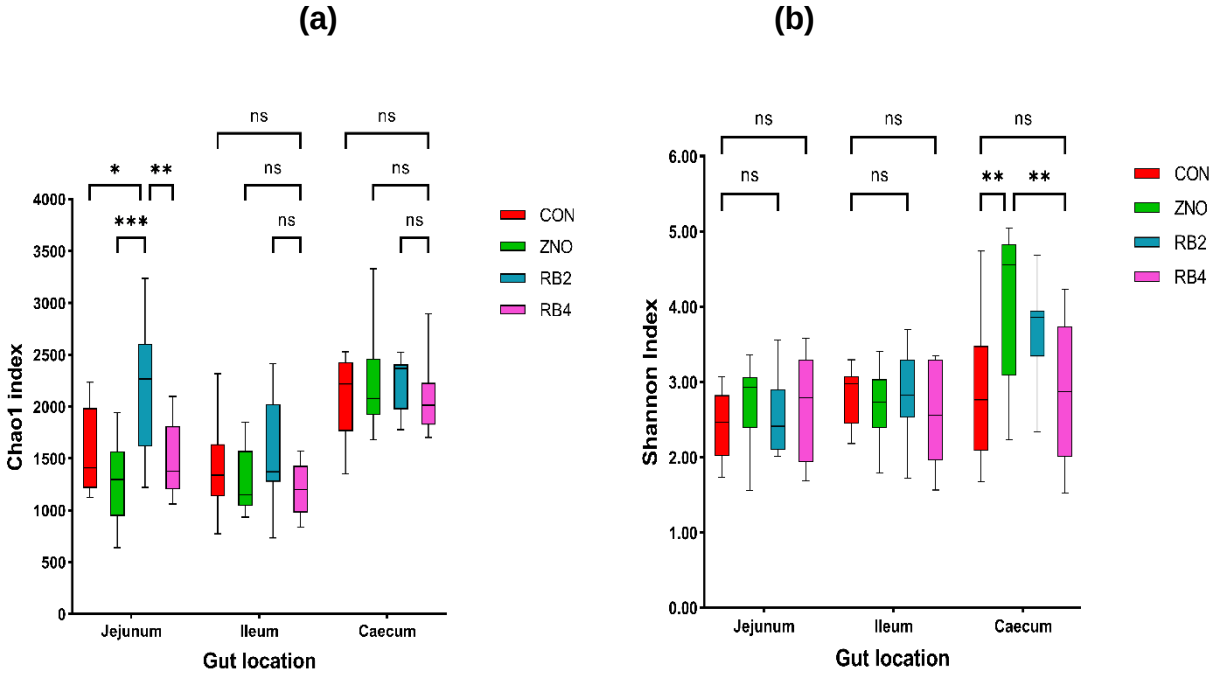


Figure 2.2: Alpha diversity indices of pig gut microbiota; (a) Chao1 index (b) Shannon index, showing diet effect on gut species richness and/or diversity. Boxplot represents mean (minimum to maximum) species richness and or evenness from each diet in the gut locations evaluated. Significant difference between diet 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.

The beta diversity of the pig gut samples is as shown in the non-metric multi-dimensional scaling (NMDS) plots (Figure 2.3). Again, the experimental diet influenced (PERMANOVA, R^2 = 0.067, *P* = 0.001) the bacteria communities between the samples, however most changes were observed in the caecum. Samples from the caecum clustered distinctively away from the ileum and jejunum, depicting the caecum had a different (PERMANOVA, R^2 = 0.177, *P* = 0.013) microbial composition from the ileum and jejunum (Figure 2.3a). The ileum and jejunum microbial communities were marginally different (PERMANOVA, R^2 = 0.041, *P* = 0.051). A subset analysis of the caecal biome (Figure 2.3b) comparing the diets indicated CON pigs had more (similar) closely related species in the caecum than ZNO (*P* = 0.03) and RB2 (*P* = 0.05) pigs, but comparable to caecal bacteria composition in RB4 pigs (*P* = 0.35). Hence, ZNO pigs contained more (diverse) dissimilar species (*P* = 0.03) than RB4 pigs, whereas RB2 and RB4 pigs were not different (*P* = 0.09).

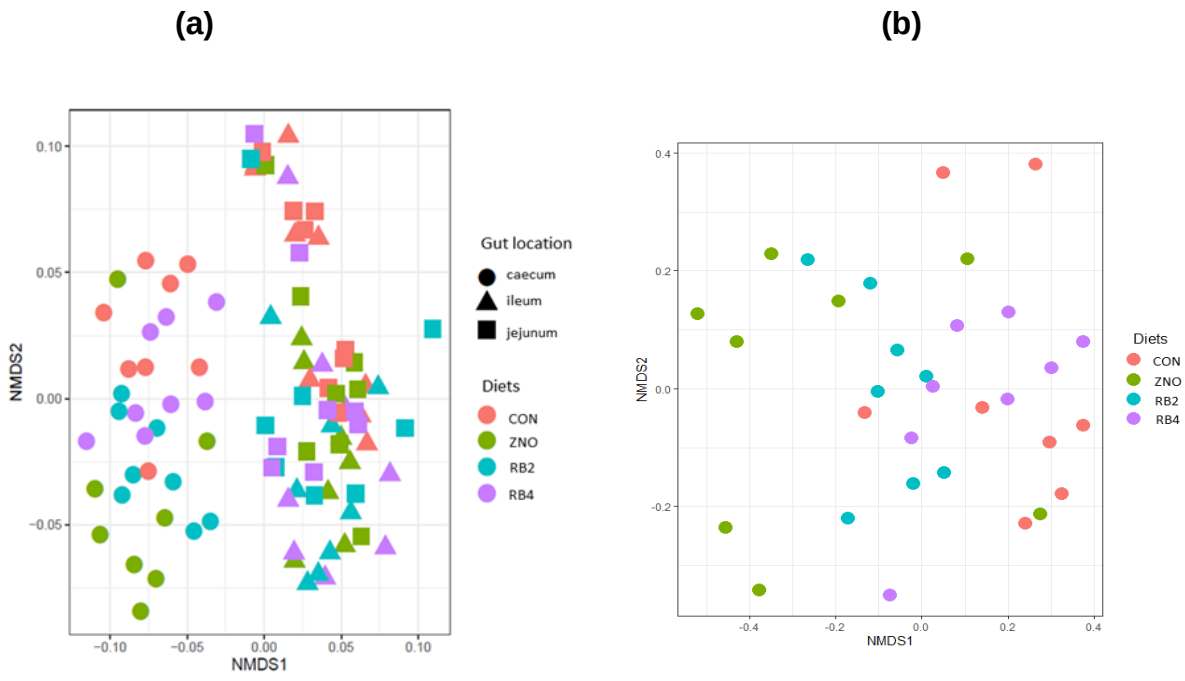


Figure 2.3: Non-metric multidimensional scaling (NMDS) plots of Bray Curtis non-phylogenetic distance matrices of gut microbial community of weaned pigs fed different diets (a) distribution of samples by diet and gut location (b) distribution of samples from the caecum. Diets: CON, ZNO, RB2, and RB4 represent; control diet, diet supplemented with zinc oxide, 2% and 4% red beetroot supplemented diets respectively.

2.5.4 Effects of diets on gut microbial taxonomic composition

Notably, 15 phyla and 310 genera were observed from the digesta samples analysed with approximately 99% of total sequences (17,573,278) assigned. Dominant bacteria phyla with mean relative abundance >1% were Firmicutes, Actinobacteriota, Bacteria unclassified, and Bacteroidota. Some other phyla observed but with low abundance included Proteobacteria, Verrucomicrobiota, Campilobacterota, Desulfobacterota and Spirochaetota (Figure 2.4a). Pigs fed CON and RB4 diets had a significantly increased mean relative abundance of Actinobacteriota and Proteobacteria ($P < 0.05$) respectively. There was no significant difference in the mean relative abundance of Actinobacteriota and Verrucomicrobiota in the gut locations, however, the relative abundance of Firmicutes was decreased in the caecum. Significant compositional differences in the phyla and genera across the diet groups and gut locations are presented in Table 2.4 and 2.5 respectively.

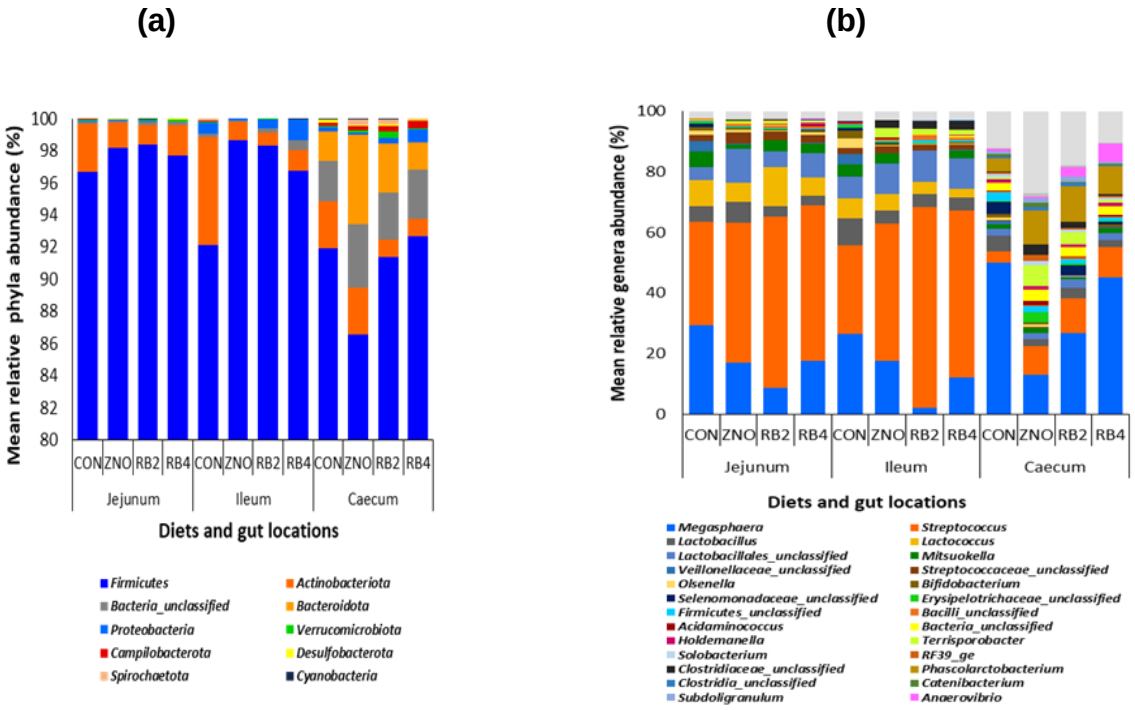


Figure 2.4: Mean relative abundance of (a) phyla and (b) genera in pig gut locations with respective diets. CON, ZNO, RB2 and RB4 represents control diet, diets supplemented with zinc oxide, 2% and 4% red beetroot respectively

At the genus level, *Megasphaera, Streptococcus, Lactococcus, Terrisporobacter, Clostridiaceae unclassified, Mitsuokella, Lactobacillus, Veillonellaceae unclassified, Lactobacillales unclassified, Phascolarctobacterium, Olsenella* and *Bifidobacterium* were among the top genera observed (Figure 2.4b). The mean relative abundance of eight genera (M*egasphaera, Streptococcus, Anaerovibrio, Rumminococcaceae unclassified, Erysipelotrichaceae unclassified, Bacillus unclassified, Terrisporobacter* and *Clostridiaceae unclassified*) were influenced by the experimental diets ($P < 0.05$) in the gut locations ($P < 0.02$) evaluated. Eleven (11) genera were abundant ($P < 0.05$) in the caecum than in the jejunum and ileum (e.g., *Megasphaera, Selenomonadaceae unclassified, Phascolarctobacterium, Firmicutes unclassified, Bacteria unclassified, Erysipelotrichaceae unclassified, Negativibacillus, Anaerovibrio* and others). *Streptococcus, Lactococcus, Lactobacillales unclassified, Streptococcaceae unclassified*, and *Bacilli unclassified* were significantly reduced ($P < 0.05$) in the caecum but high in the jejunum and ileum. *Clostridiaceae unclassified* was significantly reduced in the jejunum.

Table 2.4: Comparison of mean relative phyla abundance between diet groups and gut locations

Phylum	Diets				Gut locations			[d]SEM	*P* value		
	CON	ZNO	RB2	RB4	Jejunum	Ileum	Caecum		[e]L	[f]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 was expressed in means for each diet and gut location, significant differences were indicated by different letters within groups for the diets and gut location. ANOVA was conducted, and Tukey's HSD test was used to evaluate significant differences between the means. CON, ZNO, RB2 and RB4 represents control diet, diets supplemented with zinc oxide, 2% and 4% red beetroot, respectively.
[d] Standard error of mean, [e] *P value* for significant effect of gut locations, [f] *P value* for significant effect of diets, [g] *P value* of interaction between gut location and diets.

Table 2.5: Comparison of mean relative abundance of top bacteria genera between diet groups and gut locations

Genera	Diets				Gut locations			[d]SEM	P value		
	CON	ZNO	RB2	RB4	Jejunum	Ileum	Caecum		[e]L	[f]D	[g]L* D
Megasphaera	35.21[a]	15.90[b]	12.49[b]	24.95[a]	18.18[b]	14.57[b]	33.66[a]	2.78	0.007	0.011	0.441
Streptococcus	22.43[b]	33.72[ab]	44.77[a]	38.81[a]	47.03[a]	48.97[a]	8.79[b]	2.95	0.000	0.004	0.661
Lactobacillus	6.30[a]	4.36[a]	3.73[a]	3.17[a]	4.57[a]	5.40[a]	3.20[a]	0.48	0.161	0.104	0.594
Lactococcus	5.20[a]	4.04[a]	5.68[a]	3.03[a]	8.53[a]	4.84[b]	0.9[c]	0.75	0.000	0.511	0.605
Lactobacillales unclassified	4.55[b]	7.62[a]	6.08[a]	6.75[a]	7.14[a]	9.27[a]	2.33[b]	0.58	0.000	0.166	0.376
Mitsuokella	3.51[a]	2.23[a]	1.38[a]	2.49[a]	3.35[a]	2.54[a]	1.31[a]	0.53	0.303	0.575	0.846
Veillonellaceae unclassified	2.73[a]	0.12[b]	0.07[b]	0.25[b]	0.94[a]	0.96[a]	0.49[a]	0.30	0.750	0.003	0.895
Selenomonadaceae unclassified	1.95[a]	0.12[a]	1.19[a]	0.35[a]	0.36[b]	0.24[b]	2.11[a]	0.32	0.024	0.139	0.518
Olsenella	1.89[a]	0.68[ab]	0.24[b]	0.36[b]	0.82[a]	1.03[a]	0.53[a]	0.18	0.494	0.004	0.171
Bifidobacterium	1.63[a]	0.46[ab]	0.12[b]	0.60[ab]	0.60[a]	0.99[a]	0.52[a]	0.17	0.468	0.011	0.778
Phascolarctobacterium	1.42[a]	3.75[a]	3.98[a]	3.06[a]	0.03[b]	0.04[b]	9.09[a]	0.66	0.000	0.245	0.222
Streptococcaceae unclassified	1.36[a]	2.14[a]	1.75[a]	1.58[a]	2.94[a]	1.97[b]	0.22[c]	0.19	0.000	0.322	0.829
Firmicutes unclassified	1.08[a]	0.83[a]	0.92[a]	0.67[a]	0.30[b]	0.46[b]	1.86[a]	0.10	0.000	0.238	0.004
Bacteria unclassified	0.95[a]	1.37[a]	1.13[a]	1.26[a]	0.17[b]	0.28[b]	3.09[a]	0.21	0.000	0.796	0.800
Erysipelotrichaceae unclassified	0.64[ab]	1.35[a]	0.23[b]	0.15[b]	0.29[b]	0.36[ab]	1.12[a]	0.15	0.018	0.006	0.001
Negativibacillus	0.44[b]	1.91[a]	1.29[ab]	0.30[b]	0.009[b]	0.004[b]	2.94[a]	0.29	0.000	0.075	0.038
Anaerovibrio	0.30[b]	0.26[b]	1.09[a]	2.03[a]	0.027[b]	0.014[b]	2.72[a]	0.29	0.000	0.036	0.012
Bacilli unclassified	0.29[b]	0.64[ab]	0.99[a]	0.67[ab]	0.68[ab]	0.89[a]	0.38[b]	0.07	0.001	0.000	0.003
Rumminococcaceae unclassified	0.29[b]	0.97[a]	0.43[ab]	0.23[b]	0.01[b]	0.01[b]	1.42[a]	0.12	0.000	0.013	0.002
Terrisporobacter	0.19[c]	3.41[a]	2.16[ab]	1.10[bc]	0.48[b]	1.66[ab]	3.00[a]	0.31	0.001	0.000	0.015
Clostridiaceae unclassified	0.17[b]	2.15[a]	1.50[ab]	1.14[ab]	0.16[b]	1.97[a]	1.59[a]	0.24	0.002	0.013	0.111

Data was expressed as mean for each diet and each gut location, significant differences were indicated by different letters within groups for the diets and gut location. ANOVA was conducted, and Tukey's HSD test was used to check for significant differences between the means. CON, ZNO, RB2 and RB4 represents control diet, diets supplemented with zinc oxide, 2% and 4% red beetroot respectively. [d] overall mean for each phylum, [e]Standard error of mean, [f] P value for significant effect of diets, [g] P value for significant effect of gut locations, [h] P value interaction between gut location and diets.

2.5.5 Differential abundance analyses of genera from the diets

Differentially abundant genera per diet was computed by Welch's t-test and FDR-corrected in STAMP (See; Figures 6.1 to 6.5). 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 comparison presented decreased *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 fed 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 (Figure 2.5).

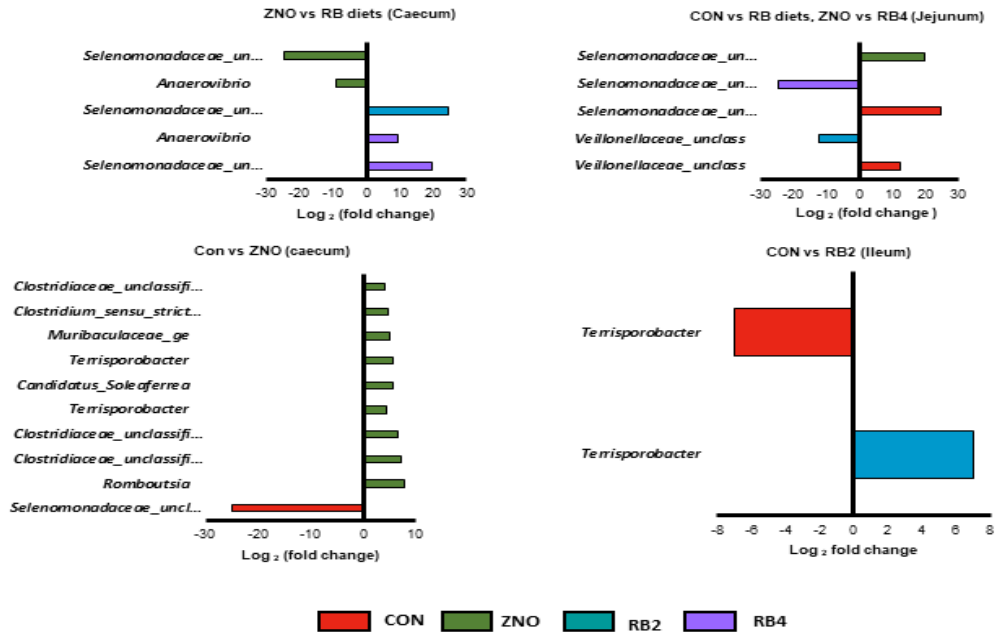


Figure 2.5: Differentially abundant genera, comparisons between diets in the gut locations. Only comparisons with significant (P < 0.001) log 2-fold changes are presented. CON, ZNO, RB2 and RB4 represents; control diet and diets supplemented with zinc oxide, 2% and 4% red beetroot, respectively.

2.5.6 Correlation between gut microbiota and gut metabolites (SCFA and bile acids)

Correlation analyses between the gut microbiota, SCFA and bile acid levels are presented in Figures 2.6 and 2.7 respectively. Although, total SCFA in CON and RB2 pigs was higher, caecal SCFA levels were comparable across the diet. Acetate and propionate levels were closely associated with *Firmicutes unclassified, Mitsuokella, Megasphaera, Streptococcus, Streptococcaceae unclassified, Anaerovibrio, Lactobacillus* and *Selenomonadaceae unclassified* in the caecum for CON and RB pigs, but closely associated with the jejunum and ileum *Phascolarctobacterium* and *Bacteria unclassified* abundance in ZNO and RB4 pigs. However, across the gut locations, *Faecalibacterium, Blautia, Clostridia unclassified, Clostridiaceae unclassified, Dialister, Olsenella, Selenomonadaceae unclassified, Veillonellaceae unclassified,* and *Firmicutes unclassified* are examples of genera significantly associated with butyrate in ZNO and RB4 pigs, most of which were associated with ileal butyrate in RB2 pigs but not significant (Figure 6.6a and 6.6b).

Associations between the gut genera abundance and bile acid levels are presented in Figure (6.7). 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. 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* and *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; *Dialister, Streptococcus, Lactobacillales unclassified, Streptococcaceae unclassified, Lactococcus,* and *Selenomonadaceae unclassified* and unconjugated bile acids (CA, CDCA) in CON, RB2 and RB4 pigs but not in ZNO pigs (Figure 2.7).

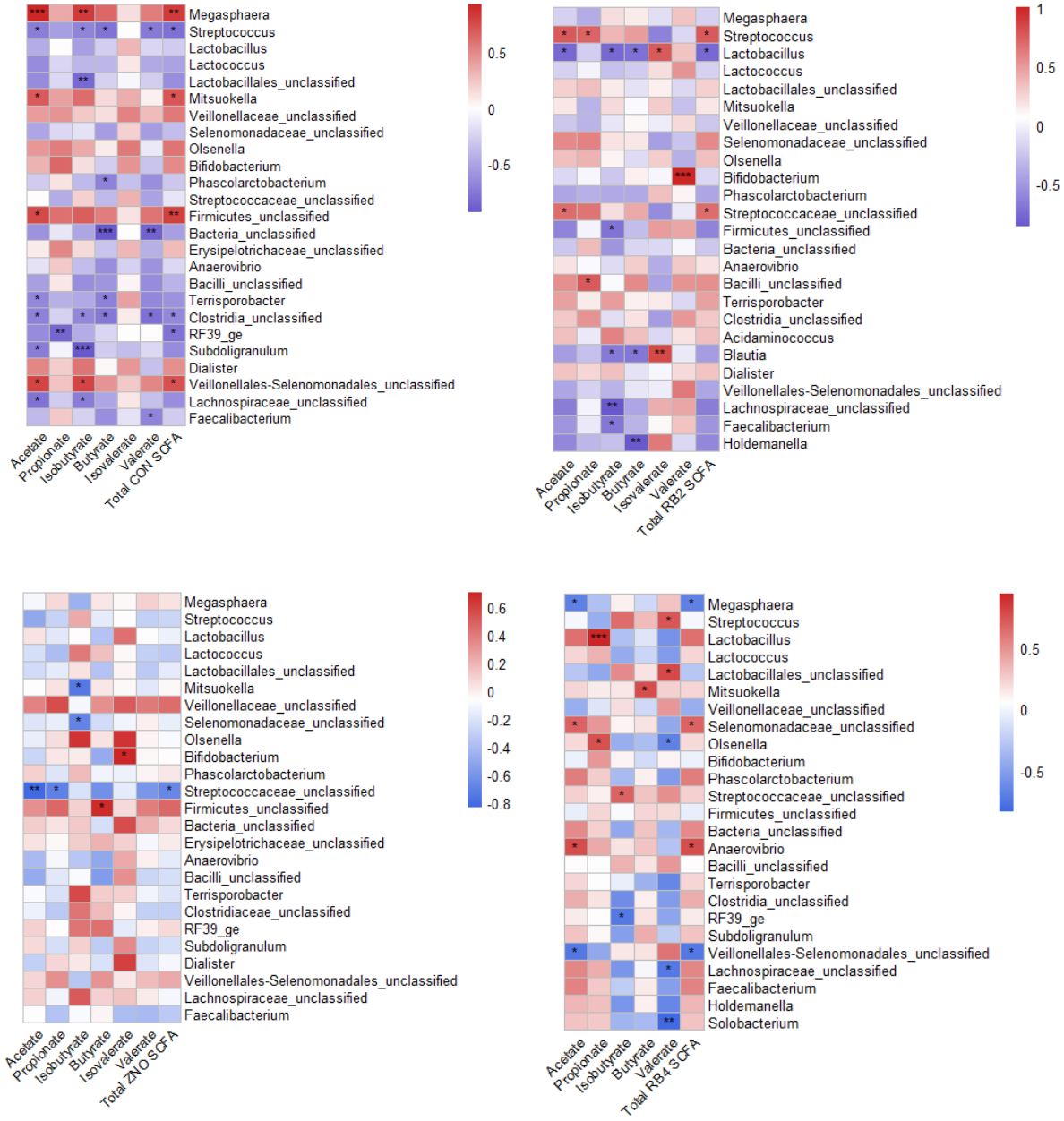


Figure 2.6: Spearman correlation matrices between top abundant bacteria genera and caecal short chain fatty acids. Fatty acids omitted were undetected in the corresponding location hence not shown, colour depth depicts correlation between genera and gut metabolite where red colour denotes a positive correlation and blue colour a negative correlation. The strength of association between the subjects is indicated by the colour intensity, *** P ≤ 0.001, **P ≤ 0.01, *P ≤ 0.05). CON, ZNO, RB2 and RB4 represent control diet and diets supplemented with zinc oxide, 2% and 4% red beetroot respectively.

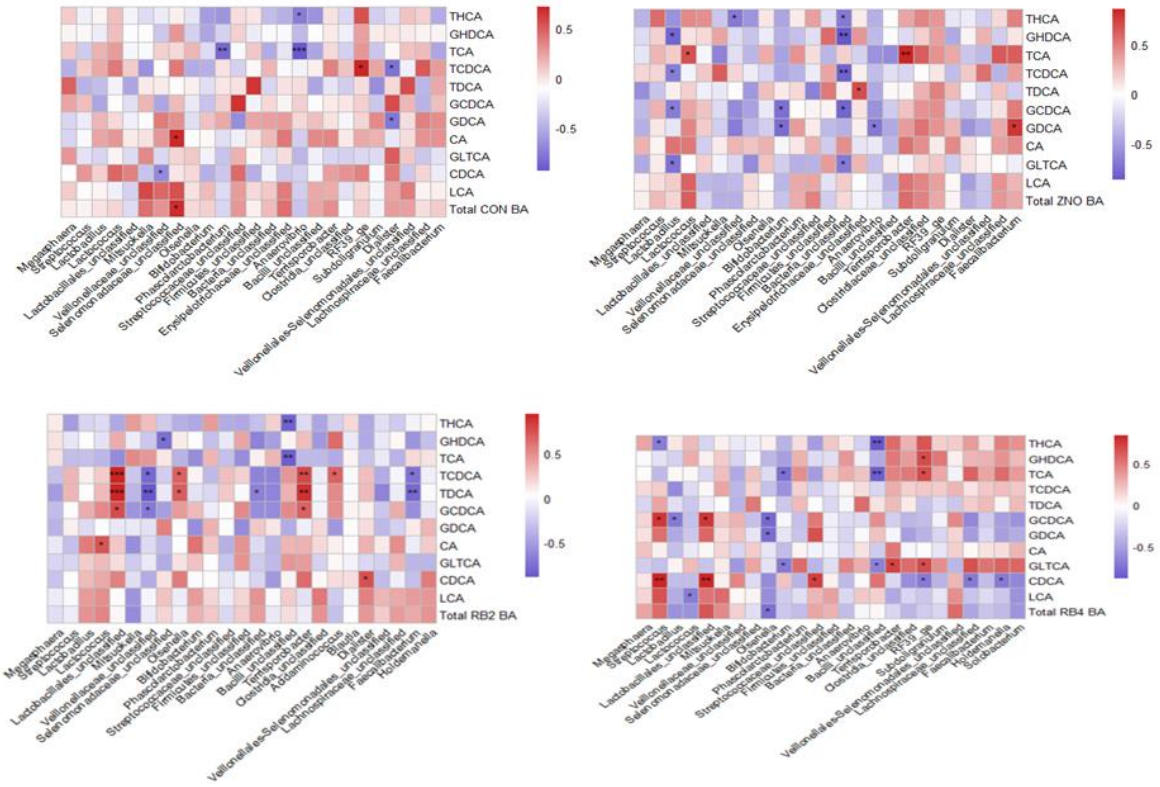


Figure 2.7: Spearman correlation matrices between jejunal genera abundance and bile acid levels. Deoxycholic acid was not detected in the jejunum for the pigs hence not shown. Correlation depicted by colour depth, where red colour denotes a positive and blue colour a negative correlation. The strength of association between the subjects is indicated by the colour intensity and *** P ≤ 0.001, **P ≤ 0.01, *P ≤ 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.

2.5.7 Predicted functions of pig gut microbiota

Pathways regulating oxidative stress and inflammation (e.g., flavone and flavanol biosynthesis, betalain biosynthesis, IL17 signaling, Th17 cell differentiation, lipopolysaccharides biosynthesis and glutathione metabolism) were enhanced in pigs fed CON diet but reduced or absent in pigs on other diets (Figure 6.8a, b and c). Also, RIG-1 signaling pathway (which signifies the occurrence of viral infection) was upregulated in pigs fed the CON diet compared to pigs fed red beetroot supplemented diets (RB2 and RB4). There were no differences (P > 0.05) in the predicted gut microbiota functions for diet RB2 and ZNO. Shigellosis and pathogenic *Escherichia*

coli infection pathways were significantly enriched ($P < 0.05$) in the ileum of pigs fed RB4 diet (Figure 6.8e) relative to other gut segment of pigs in this group.

Importantly, analyses of each gut location with respect to the experimental diets showed that, compared to ZNO pigs, pathways enabling bacteria response and adaptation to environmental changes (e.g., biofilm formation, flagella assembly and two-component system) were upregulated in the caecum of pigs on CON diet. Also, pigs fed RB4 diet had pathways mediating lipid metabolism (i.e., 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.

2.6 Discussion

This study explored the effect of red beetroot supplementation of weaned pig diet as a probable alternative to in-feed ZnO with focus on gut microbial composition and metabolite profile. Red beetroot is a rich source of bioactive compounds with recognised anti-inflammatory, antioxidant and antimicrobial properties. Given these benefits, adding red beetroot to weaned pig diet could promote beneficial microbiota modulation of the gut, aimed at preventing gut dysbiosis and diarrhoea post weaning.

Diet, remains a strong factor shaping and modulating the gut microbiota, towards the achievement of gut health and overall wellbeing (Alou et al., 2016). In this study, the experimental diets had significant influence on the gut microbial composition and diversity. Previous research suggested an average of 7 to 10 days for adaptation of the pig gut to a new diet and achievement of a relatively stable gut microbiota (Chen et al., 2017; Guevarra et al., 2019). Similarly, supplementation of weaned pig diets and gut microbiota modulation with diet containing bioactive plants, feed additives, phytochemicals have been found to prevent weaning stress and PWD with immediate and long-term effects on the gut health (Everaert et al., 2017; Schokker et al., 2018). Recent studies confirmed early establishment of the gut microbiome of young pigs is vital to the prevention of post weaning diarrhoea and improvement of growth performance (Guevarra et al., 2019; Argüello et al., 2019). Therefore, a healthy pig gut microbiota is characterised by an early attainment of a rich and diverse gut microbial

composition. Evaluating, the specific changes in the gut during dietary interventions, will enable understanding the factors responsible for the dynamic shifts in the gut microbiota composition and functions (Isaacson and Kim, 2012).

Present findings aligns with previous studies that showed reduced species richness in the jejunum with in-feed ZnO (Shen et al., 2014; Yu et al., 2017) or ilea digesta (Namkung et al., 2006). Conversely, pigs fed RB2 diet had a species rich gut (Chao1 measure) overall, with increased richness in the jejunum relative to the CON. A comparable number of species were observed in the caecum of all the pigs, however, the caecum was more species rich and diverse than the jejunum and ileum for all the pigs. Which aligns with past findings on abundance of more unique taxa in the caecum compared to the jejunum and the ileum (Gao et al., 2019)

In addition, RB2 and ZNO diets influenced the beta diversity of the pig gut microbiota compared to the CON, meaning these diets might have influenced the replacement and or changes in abundance of certain species in the jejunum, ileum and caecum. Clearly, ZNO diet modulated a species diverse caecal microbiota with more distinct bacteria than CON and RB4. Most likely driven by a decreased caecal Firmicutes abundance resulting in increased relative mean abundance of other phyla (e.g., Bacteroidota). This trend reflects alterations in gut microbiota similar to observations in animals on antibiotics treatment, and an “antibiotic like” mode of action for in-feed ZnO (Soler et al., 2018). Though, RB2 diet compares with ZNO, it was slightly different from the CON but comparable to RB4. Therefore, red beetroot (RB) supplemented diets (RB2 and RB4) did not cause a significant taxonomic shift in the gut microbiota relative to the CON but RB2 did improve the species richness of the gut.

This is the first report on supplementation of red beetroot in weaned pig diet to the best of our knowledge. However observations from ZNO diet resonate with previous findings on pharmacological dose of ZnO in weaned pig diet (Yu et al., 2017). Meanwhile, increased RB levels did not translate to a diverse caecal microbiota despite an increase in dietary fibre, meaning the gut microbiota interact with fibre differently. Importantly, the source and physicochemical characteristics (e.g., solubility, viscosity fermentability) of dietary fibre determines their functions (e.g., microbial specificity and micronutrient availability) in the gastrointestinal tract, as well as impact on gut microbial composition and metabolite production (Awati et al., 2006). Diets containing

high fibre, decrease nutrient (e.g., protein) digestibility, stemming an increased availability of fermentable substrate for gut microorganisms (Gutierrez et al., 2014). In line with higher Proteobacteria abundance observed in RB4 pigs, genera in this phylum tend to increase during weaning stress especially in pigs fed diet rich in protein, fat, and fibre, consequently depleting beneficial bacteria like; *Lactobacillus, Lactococcus* and *Bifidobacterium* (Zijlmans et al., 2015).

Consistent with previous studies, Firmicutes was the dominant phylum, accounting for close to 95% of all phyla observed in the gut locations gut (Kim et al., 2011; Looft et al., 2012; Hu et al., 2016). However, Firmicutes was significantly reduced in the caecum, which contradicts earlier reports of higher proportion of Firmicutes in the caecum (Gao et al., 2019). Rather, Bacteria unclassified, Bacteroidota, Campilobacterota, Desulfobacterota and Spirochaetota were abundant in the caecum. Unlike other studies, Actinobacteriota was second predominant phylum as against Bacteroidota usually reported. Differences in management, experimental diets, sampling age and sites used in these studies may explain this disparity (Thompson et al., 2008; Heo et al., 2013; Guevarra et al., 2018). Remarkably, such gut microbiota alterations have been attributed to lactate accumulation in the gut.

The phylum Actinobacteriota was significantly high in pigs fed CON diet, represented by *Olsenella* and *Bifidobacterium,* important bacteria with health promoting characteristics (Ventura et al., 2004; Picard et al., 2005; Ventura et al., 2009). Actinobacteriota is also an important member of a normal microbiota along with Firmicutes, Bacteroidota and Proteobacteria (Turnbaugh et al., 2007). However, Wang et al. (2020) in their study confirmed gut microbiota changes from lactate accumulation, where phylum Bacteroidetes and Firmicutes are being replace by Actinobacteria and Proteobacteria, with concomitant reduction in butyrate and propionate production. Proteobacteria (e.g., *Campylobacter* and *Salmonella* species) utilize lactate under microaerophilic conditions, to produce carbon dioxide and water (Louis et al., 2022). Hence, lactate accumulations have been linked to gut perturbations with predominant Proteobacteria abundance mostly associated with diarrhoea in animals.

The gut microbiota of 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 reactions (Louis and Flint, 2017). Though lactate prevents the growth of pathogenic organisms by lowering the gut pH, increased levels can be deleterious, causing alterations in gut microbiota, toxicity, and pathogenic colonization of the gut. Emerging studies have demonstrated the influence of lactate on metabolic output and in the aetiology of many diseases (e.g., metabolic acidosis) (Wang et al., 2020; Ma et al., 2022). Essentially, assessment of lactate levels in the gut locations examined in this study will be needed to substantiate this claim.

Notable bacteria genera (e.g., *Megasphaera, Phascolarctobacterium, Negativibacillus and Veillonella*) observed in the gut are prominent lactate utilising bacteria and LAB (e.g., *Streptococcus, Lactococcus, Lactobacillales unclassified* and *Streptococcaceae unclassified*). These genera were strongly associated with SCFA (acetate, propionate and butyrate) levels across the gut locations (jejunum, ileum, and caecum). Although, 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 discovered in RB diets (RB2 and RB4). The nutritional functions of the jejunum and capacity for energy metabolism and fibre fermentation have been alluded to in many studies. In return, the gut microbiota metabolite influences the jejunal immune system, barrier function and cell proliferation (Duarte and Kim, 2022; Chen et al., 2020). Hence, correlations between the gut metabolites and other biomarkers of gut immunity and integrity will aid further elucidation of their contributions of the jejunum to gut health.

Additionally, the host immune system is regulated by continuous interaction between the gut microbiota and dietary metabolites. Reduced gut microbiota association with butyrate levels observed in this study may be due partly to host immune responses as well as the impact of lactate accumulation on the gut (Ivanov and Honda, 2012). Alternatively, 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. However, inter-individual variability in response to the diet as well as variations in gut microbial composition and function cannot be exempted.

On the other hand, RB2 diet increased individual primary and secondary (CA, CDCA and DCA) bile acid levels compared to CON, while DCA was mainly observed in the

colon and faeces, hence not correlated with the gut microbiota abundance. Song et al. (2021) observed dietary supplementation with CDCA, a natural primary bile acid in animal bile, improved growth performance and reduce diarrheal incidence in weaned pigs. Another study by Tian et al. (2020) 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.

Here, the jejunal bile acid profile was in strong association with the jejunal microbiota of RB2 pigs unlike ZNO and RB4 pigs, but similar to observations in the ileum for ZNO pigs. Most bacteria in this region (small intestine) are usually resistant to bile acids, offering protection against pathogenic invasion (Wahlström et al., 2016). Reduced bile acid levels in the gut have been implicated in bacterial overgrowth and inflammation (Ridlon et al., 2014). Conversely, in the caecum across the diet groups, only few genera (e.g., *Streptococcus*, *Lactobacillales unclassified*, *Lactococcus*, *Selenomonadaceae unclassified*, and *Erysipelotrichaceae unclassified*) were (associated) involved in bacterial metabolism of unconjugated bile acids. 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. Also, dietary fibre alters the composition of secondary bile acids, and an increased Proteobacteria with RB4 are some probable causes of this trend.

Dietary components, antibiotics and infections possess the ability to influence bile acid levels due to their effects on bile salt hydrolysing (BSH) species (e.g., *Clostridium spp., Lactobacillus, Bifidobacterium, and Enterococcus*) in the gut microbiota (Li et al., 2013). Bile salt hydrolysing bacteria species have bile acid inducible genes that regulate the conversion of primary bile acids to secondary bile acids. Hence an increased abundance of BSH species in the jejunal or ileal microbiota will enhance conjugation of bile acid and thus increase antimicrobial activity in these segments of the gut. Chiefly, the primary (CA and CDCA) and secondary (DCA and LCA) bile acids, have been recognised for increased bacterial metabolism and potent antibacterial activity. Both conjugated and unconjugated bile acid groups have also been reported to exhibit direct and indirect antimicrobial effects on the gut microbes and enhance production of antimicrobial peptides (Begley et al., 2005; Inagaki et al.,

2006). Total bile acid levels were higher in the jejunum but reduced in the lower intestinal tract, levels in RB2 fed pigs was similar to control pigs but reduced in ZNO and RB4 fed pigs. Which could have provided a level of protection against pathogenic invasion of upper intestinal tract.

Exploring genera differentially abundant in the pigs fed the different diet, *Veillonellaceae unclassified and Selenomonadaceae unclassified* increased in the jejunum of CON pigs relative to RB pigs. T*errisporobacter*, an anaerobic Gram-positive bacterium in the family *Peptostreptococcaceae*, differentially increased in the ileum of RB2 pigs compared to CON and was strongly associated with butyrate, GDCA and GLTCA. Other compositional differences were observed in the cecum, with increased fibre fermenters (e.g., *Romboutsia, Muribauculaceae_ge, Terrisporobacter,* and *Clostridiaceae_unclassified*) linked to SCFA and decreased *Selenomonadaceae unclassified* in ZNO pigs compared to the control. RB2 was not different from ZNO except for increased *Selenomonadaceae unclassified*, while RB4 had increased *Anaerovibrio* inclusive.

Clostridiaceae unclassified, Rumminococcaceae unclassified and *Erysipelotrichaceae unclassified* were significantly higher in the pigs fed ZNO diet. Starke et al. (2014) reported significant increased abundance of *Clostridales* in pigs fed high (2425 mg/kg) dietary zinc. Argüello et al. (2019) also, discovered a higher abundance of the class *Clostridia and Ruminococcaceae* in seronegative and non-shedder pigs to *Salmonella* infection. These strict anaerobes demonstrate a rapid transition of the pig gut microbiota from a suckling microbiota to a post weaning microbiota, helping to protect the pigs from enteric pathogens.

A high abundance of *Erysipelotrichaceae* have been implicated in dysbiosis-related disorders of the gut (Kaakoush, 2015). The high immunogenic characteristic of bacteria in this family have been reported in cases of post treatment with broad spectrum antibiotics like gentamicin in mice (Palm et al., 2014). Which further depicts an antibiotic mode of action for ZnO in weaned pigs. Similarly, many studies have confirmed associations between bacterial belonging to this genus and host lipidemic profiles (Finlayson et al., 2017; Cuevas et al., 2018; Pereira et al., 2020).

There are proofs of strong links between *Erysipelotrichaceae* and host cholesterol metabolites (Martínez et al., 2009; 2013), as well as inhibition of *Erysipelotrichaceae* by dietary supplementation with quercetin (Etxeberria et al., 2015). Quercetin is a flavonoid (polyphenol) found in fruits and vegetables recognized for its health benefit and potential therapeutic effects. Conversely, polyphenol and bioactive pigments (betalains) in red beetroot may have caused a decreased abundance of *Erysipelotrichaceae unclassified* in RB pigs.

Red beetroot has remarkably been described effective in preventing lipid peroxidation, thus decrease oxidative damage (Wang and Yang, 2010). These highlights 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 to hydrolyse triglycerides to fatty acids, in pigs fed red beetroot supplemented diet further attests these inferences. Overall, the predicted functions from the microbiota of each gut location (jejunum and ileum) did not differ from each diet, pathways in response to bacteria adaptation to environmental changes was enhanced by the caecal microbiota of CON pigs.

Summarily, CON pigs had upregulated pathways related to stress response and scavengers for free radicals/ reactive oxygen species - ROS (Williams et al., 2004), compared to pigs fed ZNO and RB2 diets. Endotoxin production and inflammation driven pathways (e.g., lysosome, lipopolysaccharide biosynthesis, IL17 signalling, Th17 cell differentiation) were also enhanced by the gut microbiota of pigs in the CON group, suggesting the probable occurrence of oxidative stress and inflammation in the pigs. There were no marked differences in the predicted gut microbiota functions of pigs fed RB2 and ZNO diet.

This study provides baseline outcomes of red beetroot supplementation in weaned pig diet, however not without some limitations. The absence of colon and faecal microbiota information hindered correlations with metabolite output. Also, whole red beetroot was used in this study, hence, the effect observed on the gut microbiota and metabolite production can only be inferred but not specific to any of its constituents. Future studies investigating individual components will enhance elucidation of their roles and contributions to gut microbiota modulation and pig health.

2.7 Conclusion

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, there were no significant microbiota alterations with red beetroot diets, although RB2 increased the species richness of the gut microbiota. Increased RB to 4% (RB4) was characterized by higher Proteobacteria abundance and a potential decline in butyrate and propionate, which has been linked to increased/accumulated gut lactate. 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 improved gut microbiota metabolites (SCFA and unconjugated bile acids) production in the jejunum and ileum, depicting foregut fibre fermentation. However, SCFA levels (especially butyrate) varied in their association with the gut microbiota in the various gut regions examined. The functional pathway predictions from caecal microbiota were closely associated with differentially abundant caecal bacteria 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.

Chapter 3 **: Dietary red beetroot supplementation prevents intestinal damage and maintains gut barrier function of weaned pigs**

3.1 Abstract

This study explored the effect of red beetroot supplement in weaned pig diet on the gut health assessing the gastrointestinal functionality and changes in the gut structure, integrity, permeability and barrier function. In a 14-day experiment period, pigs weaned at 28 days old were fed with either a control diet or diet supplemented with zinc oxide (3,000 mg/kg), 2% and 4% red beetroot (CON, ZNO, RB2 and RB4; respectively). Digesta and intestinal tissues were collected for determination of microbiota changes, gut morphology and biomarker genes of gut functionality. Blood and faecal samples were also collected for measurement of systemic L-lactate and faecal calprotectin levels, respectively. The result showed that the diets preserved the gut morphology, although a tendency for decreased VH, CD and VH: CD ratio was observed in RB4 pigs. Supplementation of weaned pig diet with either zinc oxide (ZnO) or 2% red beetroot (RB2) prevented intestinal inflammation, decreased caecal *Escherichia coli* population, and promoted intestinal barrier integrity by increased expression of genes encoding fatty acid binding and tight junction proteins (*FABP2, FABP6, OCLN* and *ZO1*; $P < 0.05$). On the contrary, increased diet red beetroot levels (4%) predisposed to GIT dysfunction with increased L-lactate levels, caecal *Escherichia coli* presence and inflammatory cytokines (*IL1B* and *TNF*). All experimental pigs showed commensurate immune response to the ongoing intestinal dysbiosis and post-weaning changes by comparable expression of antimicrobial peptides and caecal abundance of beneficial bacteria (*Akkermansia municiphila*, *Lachnospiraceae spp*. and *Prevotella copri).* Summarily, 2% red beetroot supplementation in weaned pig diet could be a viable alternative to supplemental zinc oxide, in preventing weaning induced gastrointestinal dysfunction. However, further studies will be required to decipher possible causes of dwindling effect, from increased red beetroot levels in weaned pig diet.

3.2 Introduction

The gastrointestinal tract plays a crucial role in food digestion, nutrient absorption, and serves as a barrier between the host and invading enteric pathogens (Vighi et al., 2008). The GIT barrier is therefore essential to its optimum functionality and involves a structural architecture made of vascular endothelia, cellular epithelial lining, mucus secretions and an immunological barrier comprising a network of immune cell secretions e.g., cytokines, inflammatory mediators and antimicrobial peptides. The GIT functionality is described as a state in which the intestinal tract and the microbiome act in synergistic stability, such that the welfare and performance of the animal is not impaired by any form of intestinal dysfunction (Bischoff et al., 2014; Celi et al., 2019). This have been evaluated via established biomarkers from complex host interactions, pathways and mechanisms involving diet, efficient feed digestion and absorption, gut microbiota, effective immune system, intestinal permeability, and barrier function (Carrillo et al., 2017).

At the onset of weaning, the GIT undergoes structural adjustment, following increased stress, reduced feed intake and change in diet from milk to solids. The physiology of the GIT is temporarily disturbed, intestinal integrity compromised, and the absorptive capacity of the gut epithelium reduced. This leads to villus atrophy, crypt hyperplasia, enterocytes loss and shrunken surface area for nutrient absorption. Which may well intensify into gut dysbiosis, intestinal barrier breakdown, increased intestinal permeability, oxidative stress, diarrhoea and malabsorption (Moeser et al., 2017; Li et al., 2018; Rizzetto et al., 2018). Consequently, gut health and animal performance decline drastically as weaning-induced intestinal dysfunction ensues (Zhu et al., 2012; Gresse et al., 2017).

Dietary interventions during this phase of the pig production are necessary to alleviate post-weaning gastrointestinal dysfunctionality and to restore gut health of weaned pigs. The last few years has witnessed an upsurge in research investigating the use of plants, vegetables, herbs and bioactive compounds as dietary supplements in weaned pig diet and as nutraceuticals (Ganesan et al., 2013; Heo et al., 2013). Importantly, plants with high antioxidant, anti-inflammatory and antimicrobial properties are reported to prevent post weaning growth retardation, alleviate intestinal dysfunction and improve gut health (Denev et al., 2014; Rossi et al., 2020; Wan et al.,

2021; Peng et al., 2022). There are numerous studies on the improvement of pig performance and intestinal barrier function with supplementation of feed with plant extract alone or in combinations (Yan et al., 2012; Bontempo et al., 2014). Recent studies in rodents and weaned pigs for example, found that dietary quercetin enhanced gut health by increased expression of tight junction proteins, and as an anti-inflammatory and antioxidant agent (Xu et al., 2014; Zhang et al., 2018; Xu et al., 2021).

Similarly, sustained research effort in the development of novel dietary approaches to alleviate post weaning diarrhoea and stress of pigs have discovered plants with bioactive compounds could replace pharmaceutical doses of ZnO in weaned pig diet. Emerging studies have found that these plant bioactives do not only reduce post weaning diarrhoea but also impact on gut health, microbiota composition and functionality (Bontempo et al., 2014; Sweeney and O'doherty, 2016). These inferences were derived through assessment of biomarkers linked to health and gut functionality, whereby several dietary supplement in weaned pig diets have been tracked for effectiveness in alleviating post weaning stress and GIT dysfunction. Weaning and dietary changes contributes significantly to alterations of the GIT structure, integrity, microbiota and functions. A few biomarkers of gut health and functionality related to this study, are listed in Table 1.5.

Aside changes in the gut microbiota composition and functional capacity, the intestinal barrier function, immunity and oxidative status of weaned pigs can be impaired, thereby upregulating the mRNA expression of inflammatory cytokines (*TNF* and interleukins). Increase in pro-inflammatory cytokines negatively affect expression of tight junction proteins causing increased gut permeability and breakdown of gut barrier with gut metabolites leak into the systemic circulation (Ulluwishewa et al., 2011).

Equally, transcription of genes coding for fatty acid binding and tight junction proteins is a sensitive biomarker for determination of the intestinal permeability. In avian species, decrease *FABP2* expression is indicative of increased intestinal permeability from loss of enterocytes (Chen et al., 2015). However, plasma *FABP2* concentrations is unlikely to provide sufficient information on weaning and dietary impact on GIT functionality (Funaoka et al., 2010; Niewold, 2015).

Current research interest in identification of probable biomarkers to determine GIT changes at weaning and effects of dietary interventions have provided better insight into the diagnosis and alleviation of several GIT conditions and weaning induced gastrointestinal dysfunctionality. Calprotectin a cytosolic protein has been identified as a useful biomarker for detection of several gastrointestinal conditions, especially for quantifying the degree of inflammation (Alibrahim et al., 2015; D'Angelo et al., 2017). Previous studies evaluating faecal calprotectin in pigs observed comparable levels in adult pigs (sow; 13 ± 38 mg/kg of faeces) and healthy adult humans (2 - 47 mg/kg of faeces), but much lower concentrations in piglets at birth (24 ± 60 mg/kg) than human babies (145 ± 78.5 mg/kg) (Lallès and Fagerhol, 2005).

Faecal calprotectin increases during gut dysbiosis, due to increased migrations and infiltration of the intestinal tract by neutrophils and macrophages. Elevated levels is commonly observed in humans with inflammatory bowel disease (IBD), hence, it is validated a non-invasive biomarker of gut inflammation (Ikhtaire et al., 2016; Shi et al., 2023). Considering the transient nature of circulating cytokines during inflammation, faecal calprotectin avails the advantage of constant and timed monitoring of gastrointestinal inflammation and gut health of weaned in pigs during dietary intervention and at weaning (Barbosa et al., 2021; Dang et al., 2023).

In addition, L-lactate levels measured in the gut or plasma is a potential biomarker of gut permeability and health. Lactate is a product of microbiota fermentation in the gut and immediately used up by other gut bacteria (lactate utilizing bacteria), hence, it is rarely detected. An Increased lactate levels and/or accumulation suggests a disrupted intestinal barrier integrity cascading to an increased systemic lactate level. The occurrence of high faecal lactate in animals, is therefore associated with gut microbiota imbalance, sequel to increased lactate-producing bacteria and reduced lactate utilizing bacteria population (Mayeur et al., 2013; Blake, 2017;)

Chapter 2 demonstrated improved gut microbial diversity and metabolite modulation with supplemental red beetroot at 2%. Conversely, the microbiota shifts with increased Proteobacteria abundance linked to lactate accumulations requires further elucidation. Not only that, the impact of reduced SCFA (e.g., butyrate) and bile acids (unconjugated) production with increased RB levels (RB4), on the GIT functionality necessitate further investigation. Red beetroot, as a plant rich in bioactive compounds

(betalains, vitamins, fibres and nitrates), with high antioxidant and anti-inflammatory potential (Georgiev et al., 2010; Singh and Hathan, 2014; Clifford et al., 2015; da Silva et al., 2019) possess the potential of improving GIT integrity, permeability and barrier function post weaning. This chapter is focused on the effect of red beetroot supplementation on the gastrointestinal functionality, exploring some biomarkers of gut health.

3.3 Materials and methods

3.3.1 Materials

Reagents, chemicals and materials for sample processing, storage, and histology were purchased from Merck Sigma Aldrich (Dorset, UK), Starlab UK Ltd (Blake lands, UK) and Sarstedt Limited (Leicester, UK). Invitrogen RNAlater and RNase-free water were purchased from Thermofisher scientific (Loughborough, UK). The reagent used for RNA isolation, TRIsure was obtained from (Bioline Ltd, UK), Lactate handheld and sensor-strips were purchased from (EKF Diagnostics UK). Pig calprotectin competitive ELISA kit was ordered from CUSABIO (China) and delivered through Generon (Slough, UK). All reagents for gene expression analysis / polymerase chain reaction (PCR); iScript cDNA synthesis kit, iTaq Universal SYBR Green Super mix were purchased from Bio-Rad (Watford, UK). Milli-Q water and nuclease free water was from Merck Millipore (Gillingham, UK).

3.3.2 Methods

3.3.2.1 Experimental animals, sample collection and processing

The experimental animals and diet composition has been described in chapter 2; section 2.3.2.2. In addition to details previously provided, plasma separated from blood samples collected was aliquoted into 2 mL tubes and faecal samples (approx. 1.0 g) were collected into 2 mL Eppendorf tubes. These samples were quickly snap frozen and stored at -80°C until analyses. The intestinal tract was separated as described by Liu et al. (2018). Precisely, the large intestine was first separated from the small intestine, then the small intestine was divided into three segments, the proximal-10 cm to the pylorus as the duodenum, the middle portion as jejunum and the distal 5 cm-

section prior to the ileocecal junction as the ileum. Three pieces (sized; 3.0 - 5.0 cm) were taken from each gut segment and washed with ice cold phosphate buffered saline solution (PBS; 10 mM, pH 7.4). One section was fixed in 10% neutral buffered formalin for histology, the second cut was wrapped in foil, snap frozen and stored (-80°C), and the third was immersed in 5 mL RNAlater (Sigma Aldrich) then stored (-20°C) until RNA isolation.

3.3.2.2 Histological and morphological assessment of intestinal tissue

The intestinal segments (duodenum, jejunum, and ileum) were fixed in 10% neutral buffered formalin for 48 hours, rinsed with distilled water, then transferred into a 30 mL universal bottles containing 70% ethanol. Fixed tissues were submitted to the histopathology facility at St James' hospital, Leeds UK, for processing, waxing, embedding, sectioning and staining (haematoxylin and eosin). Histology slides were scanned at x 20, on Zeiss Axio Scan Z1 slide scanner equipped with Colibri2 LED light source, HXP 120v (Germany). Four sections per each gut segment per animal were scanned, following the method described in Håkenåsen et al. (2020), the height of well-defined villi (VH) and depth of the corresponding crypt (CD) (approx. 40 villi and crypts per sample) were measured with QuPath (v 0.3.2). The ratio between the villus height and crypt depth (VH: CD) was also calculated.

3.3.2.3 Assessment of systemic and gut lactate levels

To determine the role of the dietary supplements on intestinal integrity and barrier function, L-lactate levels in the ileum and colon digesta as well as the systemic circulation were assessed. This was conducted with an L-lactate Scout (SCT) handheld and lactate sensors (EKF Diagnostics) with detection range; 0.5 - 25.0 mmol/L (Meléndez et al., 2021), using protocols described in Sutton (2020). Plasma samples (0.5 mL) were centrifuged, and the supernatant separated into clean sterile tubes. Sample lactate levels were displayed on the handheld device after adding 0.2 µL of plasma/digesta to the lactate sensor inserted into the device. For pig digesta, ileal digesta (0.3 g diluted 1:5 in 10 mM PBS, pH 7.4) and colon digesta (1.0 g, 1:1 dilution) were homogenised, centrifuged at 14,000 x g for 2 min, then the supernatant was separated into 2 mL Eppendorf tubes. L-lactate was measured in duplicate as described above, and readings were automatically haematocrit adjusted by the device as indicated in the manufacturer's instructions.

3.3.2.4 Measurement of faecal calprotectin levels

Faecal calprotectin is a validated non-invasive biomarker of intestinal inflammation influenced by dietary interventions. Pig faecal calprotectin levels were determined using a competitive calprotectin ELISA kit (Generon, UK) with detection range 0 to 20 ng/mL (Rebucci et al., 2022; Barbosa et al., 2021). Following the manufacturer's instructions, 100 mg faecal sample was suspended in 0.5 mL 10 mM PBS, pH 7.4, vortexed for 1 min, then mixed on a roller shaker for 20 min (5000 rpm). The mixture was centrifuged at 1000 x *g*, 4°C for 20 min, after which the supernatant was further diluted (1:5) with sample diluent before conducting the assay. All reagents were brought to room temperature (Rt), horseradish peroxidase (HRP) conjugate was diluted 1:100 fold with HRP conjugate diluent and the wash solution was diluted 1:25 fold with distilled water. Calprotectin standard was carefully reconstituted with 1.0 mL of sample diluent to give 20 ng/mL, then a series of dilution (S0 - S6) was carried out with 150 µL standard stock and equal volume of sample diluent to give the desired concentration range (0 - 20 ng/mL). All samples, standard and blank were assessed in duplicate with 50 µL of freshly prepared standard and sample added to designated wells. Following this, 50 µL of diluted HRP conjugate was added to each well except the blank, the plate was covered with a plate sealer and incubated at 37°C for 30 min. The wells were aspirated, washed four times with wash solution, and dabbed against an absorbent paper to get rid of excess liquid. Afterwards, 90 µL of 3, 3′, 5, 5′-tetramethylbenzidine (TMB) substrate was added to each well, light protected to prevent TBM degradation and incubated at 37°C for 20 min. A colour change was observed (light blue to varying graduations of blue), 50 µL of stop solution was added to each well, tapping the plate gently to ensure a thorough mix. The optical density (OD) was read within 5 min after a change in colour from a gradient of blue to a yellow solution, on a microplate photometer (Multiskan FC, Thermofisher scientific, UK) set at 450 nm. Calprotectin concentration was calculated in nanograms per microgram (ng/mg) of faeces using average optical density of samples after a four-parameter logistic (4PL) curve fitting for the standard in R.

3.3.2.5 Determination of transcriptional changes in gut tissue and caecal microbial presence

The effect of diets on inflammatory genes and tight junction protein expression was assessed as a biomarker of gut immunity, integrity and function. First, ribonucleic acid (RNA) was isolated from gut tissue samples stored in RNAlater, transcribed to complementary deoxy-ribonucleic acid (cDNA), then primers for the target genes were designed before a quantitative polymerase chain reaction (qPCR) was conducted.

3.3.2.5.1 RNA isolation and real time qPCR

Total RNA was extracted from pig jejunum and ileum tissue samples in RNAlater using TRIsure reagent (Bioline Ltd, UK.), according to the manufacture's recommendations. Briefly, tissue samples were allowed to thaw on ice, approximately 50 -100 mg of gut tissue was taken into a cold 2 mL RNase-free tube containing 1mL TRIsure. The sample was homogenised with a tissue homogeniser (VWR, International Ltd, Poole, UK) for 45 sec, homogenate was incubated for 5 min at room temperature (Rt) then 0.2 mL chloroform was added per 1 mL TRIsure used. Tubes were capped securely, whirled on a vortex machine (VWR, UK) for 15 sec, further incubated for 3 min then centrifuged at 12,000 x g, 4°C for 15 min to separate the sample into phases (a colourless upper aqueous layer, an interphase and pale green organic layer).

The upper aqueous layer (0.5 mL) was carefully separated into a new sterile RNase-free tube and an equal volume of cold isopropyl alcohol was added to precipitate the RNA. The mixture was incubated for 10 min at Rt, centrifuged at 12,000 x *g*, 4°C for 10 min. After, the supernatant was removed leaving the RNA pellet, which was washed twice with 75% ethanol (1 mL per 1 mL TRIsure used). The sample was vortexed, centrifuged at 7500 x g for 5 min at 4°C, each time removing the ethanol and leaving the RNA pellet which was subsequently air-dried and dissolved in RNase-free water. RNA quality and concentration was determined using the Nano-Quant plate (Tecan Spark 10M microplate reader) at 260 and 280 nm. Isolated RNA samples were stored at -20°C until further analysis.

3.3.2.5.2 Complementary DNA (cDNA) synthesis, primer design and qPCR

Each RNA isolate was reverse transcribed to cDNA using the iScript™ cDNA synthesis kit (Bio-Rad). The reaction contained 2 µL 5x iScript buffer, 0.5 µL of iScript reverse transcriptase enzyme, RNA template in volume containing a 0.5 µg of total RNA all made up to a final volume of 10 µL with RNase- free water. The reaction mixture was incubated on a pre-programmed thermal cycler (Prime, Biby scientific Ltd, UK) set at 5 min incubation at 25°C, followed by 30 min at 42°C terminated at 5 min at 85°C then held at 4°C. The resulting cDNA was diluted 20 times and stored at -80°C prior to use.

Quantitative real time PCR was performed to determine the transcription levels of some selected representative genes for; inflammation (interleukin-1B *(IL1B*), *IL10*, *IL2*, interferon gamma (*IFNG*), tissue necrosis factor alpha (*TNF*)), immunity/antimicrobial peptides (mucin 2 (*MUC2*), *MUC4*, regenerating islet-derived 3 gamma (*REG 3G*), C-C motif chemokine ligand 20 (*CCL20*)) and tight junction/integrity functions (Zona occludens; *ZO1*, Claudin 3; *CLDN3*, Occludin; *OCLN* and fatty acid binding proteins (*FABP2, FABP6*). Primers for selected genes were designed and blasted using the national centre for biotechnology information (NCBI) database (Table 3.1) centred on 18 - 24 base length, melting temperature of 60°C +/-5°C and a GC content between 40-60%. Primer efficiency and amplification linearity was verified (for target and housekeeper genes) before quantification and PCR product sizes were confirmed by 2% agarose gel electrophoresis (Figure 6.9).

Transcription levels in samples (cDNA) were evaluated in duplicate in 10 µL PCR reactions using iTaq™ Universal SYBR Green Super mix (Bio-Rad, UK) on a StepOne Plus 96-well, Real-Time PCR System (Applied Biosystems, Nottingham, UK). Each reaction contained cDNA template (2 µL), 5 µL of 2x SYBR reagent, and 0.4 µL each of a 400 nM forward and reverse primer, made to a final volume of 10 µL with RNase-free water. The programme for amplification was set at 95°C for 30 sec (activation step), followed by 40 cycles of denaturation at 95°C for 15 sec and annealing/extension at 60°C for 60 sec. A melt curve analysis (65°C to 95°C, 0.5°C incremental increases every 5 sec) was performed at the end of each PCR run. Gene expression levels was calculated using ($2^{-\Delta\Delta CT}$) method by Livak and Schmittgen (2001), with normalization to the housekeeper gene (β-actin).

3.3.2.5.3 Microbial genomic DNA extraction

DNA samples used were from the genomic DNA extraction conducted and described in chapter 2; section, 2.3.2.4. To complement 16S rRNA analyses and due to high number of genera below 1% relative abundance according to Jian et al. (2020), the population of some representative bacteria species recognised for their effect on the gut function and health were assessed in the caecum using qPCR Primer sequences were obtained from the literature and control blasted using NCBI primer-blast tool (Table 3.2). DNA samples were diluted 1:100 fold and the qPCR for the bacterial targets was conducted with 30 ng genomic DNA, amplification set for; 2 min activation step at 95°C followed by 40 cycles of denaturation at 95°C for 15 sec and 60°C for 60 sec. The Ct-values of targeted bacterial species were normalized to the universal microbial primer and the relative bacterial abundance was determined as described above.

3.4 Statistical analyses

Data collected was normalized using Shapiro-Wilk test and square root transformed where necessary to ensure normal distribution before further statistical analyses. Results were expressed as mean and standard error. Statistical analysis was performed by ANOVA, using the general linear model in SPSS (v. 26.0). Differences between the diet groups at the selected gut location was determined by Tukey's honest significant difference (HSD) *post hoc* test. The differences were considered significant at $P \leq 0.05$ level. Then, Pearson's correlation analysis was used to establish relationship between the different biomarkers assessed.

Table 3.1: Primer sequences for target genes

Gene	Forward primer (5´ to 3´) Reverse primer (5´ to 3´)	Size (bp)	Accession number
Reference genes			
ACTB	GTCACCAACTGGGACGACAT GTACATGGCTGGGGTGTTGA	174	XM_021086047.1
Pro and anti-Inflammation target genes			
IL1B	ACTTTGTCTGTGATGCCAACG TCATGCAGAACACCACTTCTC	147	NM_214055.1
IL2	CAGTTTTGGAACTAAAGGGATCTG AGTCAGTGTTGAGTAGATGCTTTG	125	NM_213861.1
IL10	ACCTGGAAGACGTAATGCCG GGGCAGAAATTGATGACAGCG	125	NM_214041.1
TNF	GCCCTTGGAAATGCTCTCTA ACCCCTTGTCTGTCTACTCA	93	X54001.1
IFNG	GCCATTCAAAGGAGCATGGA TTCACTGATGGCTTTGCGCT	144	NM_213948.1
Immunity target genes			
REG 3G	ATCCTTCGTGTCCTCCCTGA GCATTTGGTTCCAAGCCCTC	96	XM_021085605.1
CCL20	AGCTCGCCAATGAAGCTTGT CTGCACACACGGCTAACTTT	72	NM_001024589.1
MUC2	AGCTCCAGAGAGAAGGCAGAAC AGGATGTACTCGACCTGGGAG	220	XM_021082584.1
MUC4	CTCACACCACCACTTCAGTCCTAA GTAGTTGTCATAGTGTTTCCACCC	169	XM_021068272.1
Tight junction and fatty acid binding proteins			
FABP2	TTGAGCATTGTTCTTGGAGCAC CAGCAACCTTATGCTTGATGAG	102	NM_001031780.1
FABP6	GTGGTGAACAGCCCCAACTA TGCTTACACGCTCGTAGCTC	100	NM_214215.2
CLDN3	AGGACTACGTATGAGGGGGC GACTGGTCTCGGATGCAAGG	99	NM_001160075.1
OCLN	TCAGGTGCACCCTCCAGATT TATGTCGTTGCTGGGTGCAT	169	NC_010458.4
ZO1	TCAAGGTCTGCCGAGACAAC TCACAGTGTGGTAAGCGCAG	139	XM_003480423.4

Beta Actin – (ACTB); Interleukin 1B - (IL1B), Interleukin 2 - (IL2), Interleukin 10 - (IL10); Tissue necrosis factor - (TNF); Interferon gamma - (IFNG). Regenerating islet-derived 3 gamma - (REG 3G) -; C-C motif chemokine ligand 20 - (CCL20); mucin 2 – gel forming (MUC2); mucin 4- cell surface associated (MUC4). Fatty acid binding protein - (FABP2; FABP6); Claudin 3 - (CLDN3); Occludin (OCLN); Zona occludens/ tight junction protein- (ZO1).

Table 3.2: Primer sequences for bacterial targets

Bacterial gene	Forward primer (5´ to 3´) Reverse primer (5´ to 3´)	Size (bp)	Accession number
Universal Primer	CGGTGAATACGTTCYCGG AAGGAGGTGATCCRGCCGCA	172	(Suzuki et al., 2000)
Akkermansia muciniphila	CAGCACGTGAAGGTGGGGAC CCTTGCGGTTGGCTTCAGAT	329	NR_074436.1
Faecalibacterium prausnitzii	ATACCGCATAAGCCCACGAC ATGTGGCCGTTCAACCTCTC	149	NR_028961.1
Escherichia coli	TGATTTCCGTGCGTCTGAATG ATGCTGCCGTAGCGTGTTTC	115	(Molina et al., 2015)
Lactobacillus spp.	AGCAGTAGGGAATCTTCCA CACCGCTACACATGGAG	LAC1; Lab0677	(Su et al., 2008; Wang et al., 2020)
Lachnospiraceae spp.	AACAGAGGAGACAGGTGGTG GCTTCCCTTTGTTTACGCCA	228	(Park et al., 2020)
Prevotella copri	ACCACTTGGGGATAACCTTG TACATGCAAAAAGCCTCACGAGGC	347	(Verbrugghe et al., 2021)
Enterotoxigenic Escherichia coli (ETEC).	GGCACTAAAGTTGGTTCA CACCCTTGAGTTCAGAATT	K88 AD	(Alexa et al., 2001; Wang et al., 2020)

3.5 Results

3.5.1 Effect of red beetroot supplement on gut morphology

As shown in Table 3.3, the diets did not potentiate significant changes to the histo-morphological assessment of the gut tissues from the duodenum, jejunum and ileum, measured via the villus height (VH), crypt depth (CD) and villus height: crypt depth ratio (VH:CD). Although slight decrease in the crypt depth and villus height was observed in the jejunum and ileum of RB4 pigs. Representative images from the gut locations for each diet is presented in Figure 3.1.

Table 3.3: Gut histomorphometric measurement (µm) of weaned pig fed different diets

Diets	CON	ZNO	RB2	RB4	*P* value
Duodenum					
Villus height	282.84±4.31	254.50±20.57	301.60±15.67	300.01±31.35	0.36
Crypt depth	279.62±14.25	252.34±19.33	280.07±13.54	283.56±26.70	0.64
VH:CD ratio	1.02±0.06	1.01±0.04	1.08±0.45	1.05±0.03	0.68
Jejunum					
Villus height	252.54±14.45	229.97±29.39	254.52±19.71	228.08±12.53	0.59
Crypt depth	243.92±15.73	222.37±32.71	259.04±28.61	205.64±10.09	0.40
VH:CD ratio	1.04± 0.03	1.05±0.02	0.99±0.04	1.11±0.06	0.27
Ileum					
Villus height	215.47±24.65	221.50±11.42	223.53±26.09	205.11±13.22	0.86
Crypt depth	226.48±26.88	228.51±12.59	233.63±30.35	228.44±6.16	0.83
VH:CD ratio	0.99±0.02[a]	0.97±0.03[a]	0.94±0.01[a]	0.90±0.04[b]	0.03

Data expressed as means ± standard error. Superscripts along the table indicate significant differences (P< 0.05) in that gut location for the corresponding measurement.

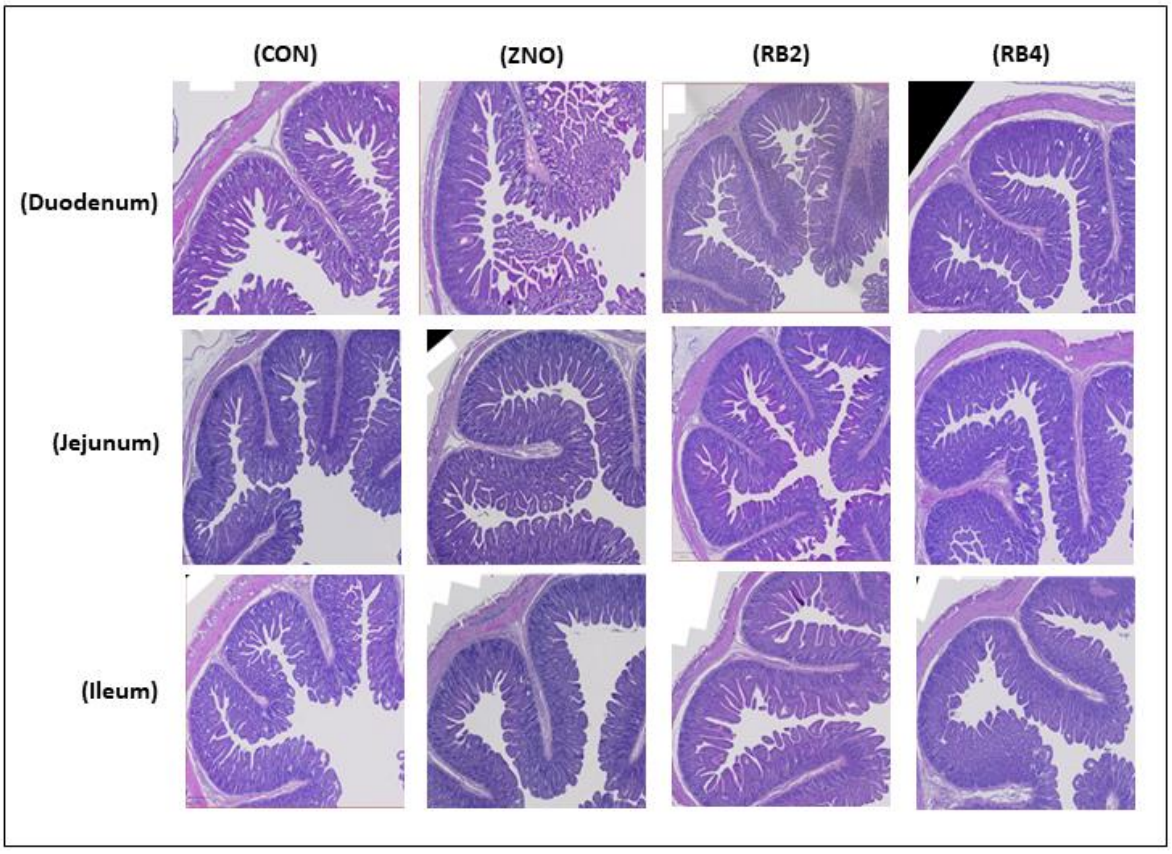


Figure 3.1: Representative haematoxylin and eosin sections of weaned pig gut. CON, ZNO, RB2 and RB4 represents control diet and diets supplemented with zinc oxide, 2% and 4% red beetroot respectively. (Each micrograph is of magnification 20^{x} and scale bar - 400 µm).

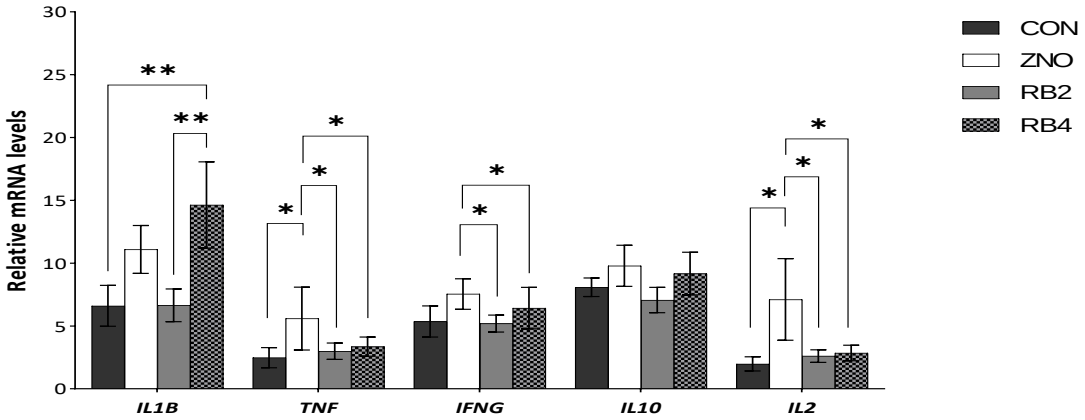
3.5.2 Effect of red beetroot on gut inflammation

To determine the role of red beetroot supplemented diets on gut inflammation, changes in the mRNA levels of inflammatory genes (*IL1B, IL10, IL2, TNF* and *IFNG*) in the jejunum and ileum were assessed. As shown in figure 3.2a, mRNA levels of pro-inflammatory genes (*IL1B, TNF* and *IFNG*) increased in the jejunum with ZNO diet. *IL1B* was higher in pigs fed RB4 compared to CON ($P = 0.003$) and RB2 ($P < 0.001$) pigs. Also, jejunal *IFNG* transcription levels were comparable in CON and RB pigs, but high in ZNO pigs than RB pigs (RB2; $P = 0.03$; RB4; $P = 0.02$). However, jejunal *IL2* expression reduced in CON ($P = 0.03$), and RB fed pigs (RB2; $P = 0.03$, RB4; $P = 0.04$) compared to ZNO pigs.

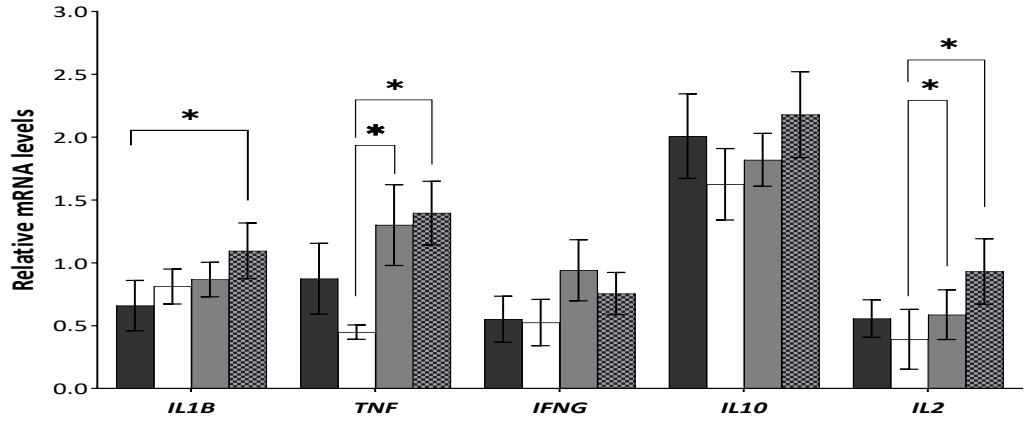
As presented in figure 3.2b, comparable mRNA levels of *IL10* and *IFNG* was observed in the ileum. *IL2* and *TNF* mRNA levels reduced in ZNO pigs, while levels increased in the RB pigs (RB2; $P = 0.01$, RB4; $P = 0.04$), however comparable to CON pigs. Also, in the ileum, *IL1B* levels in RB4 pigs was significantly higher ($P = 0.05$) than CON.

Faecal calprotectin levels observed ranged between (210 and 985 ng/mg of faeces), although levels were comparable between the diet groups, RB4 pigs showed higher levels ($P = 0.01$) relative to pigs fed ZNO diet (Figure 3.2c). In addition, *IL10* mRNA levels were positively associated with other targets evaluated for gut inflammation (TNF; $R = 0.55$, $P < 0.001$, IL1B; $R = 0.71$, $P < 0.001$, IL2; $R = 0.60$, $P < 0.001$, and IFNG; $R = 0.49$, $P = 0.002$).

(a)



(b)



(c)

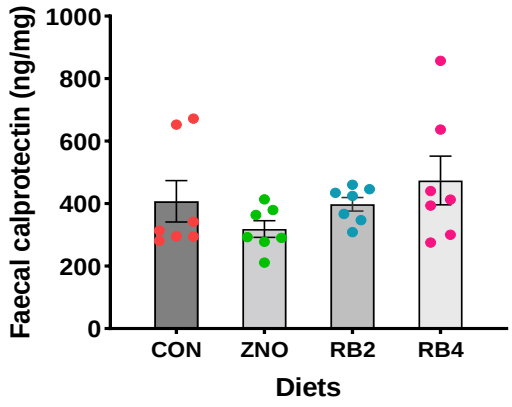


Figure 3.2: Assessment of gut inflammation (a) Jejunum (b) Ileum and (c) evaluation of faecal calprotectin levels. Significant difference between diets depicted by * P ≤ 0.05, ** P ≤ 0.01, *** P ≤ 0.001 between subjects linked by the line. CON, ZNO, RB2 and RB4 represents control diet and diets supplemented with zinc oxide, 2% and 4% red beetroot, respectively.

3.5.3 Effect of red beetroot on gut immunity and caecal microbiota

As shown in Figure 3.3a, the diets had no influence on jejunal REG 3G (P = 0.75), CCL20 (P = 0.85), MUC2 (P = 0.16) and MUC4 (P = 0.65) transcription levels. In the ileum, MUC2 and MUC4 mRNA levels were not quantified due to high Ct values. REG 3G levels were higher for CON than ZNO fed pigs (P = 0.02) but not different from RB pigs (P > 0.05) in the ileum.

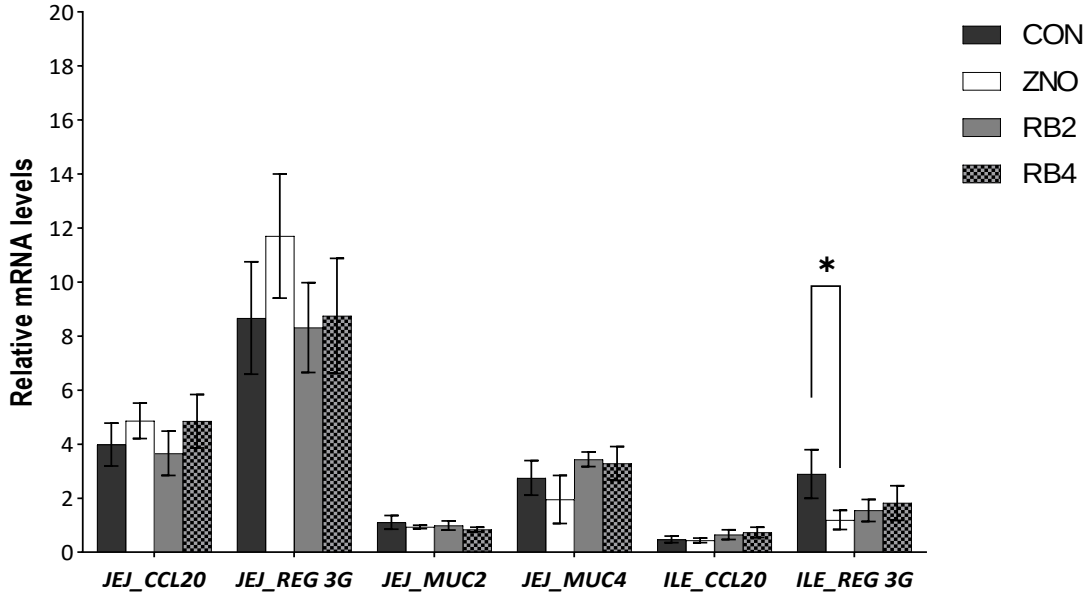
Furthermore, the population of specific bacterial species with recognised gut health functions were evaluated using qPCR method. Results showed, comparable caecal population of *Prevotella copri*, *Lachnospiraceae spp.* and *Akkermansia muciniphila* across the diet groups (Figure 3.3b). However, in CON pigs *Lactobacillus spp.* was significantly increased (P = 0.03) compared to ZNO pigs.

Again, pigs fed RB2 (P ≤ 0.001) and ZNO (P = 0.003) diet had a decreased caecal *Escherichia coli* abundance compared to CON pigs. *E. coli* population in RB4 pigs was similar to CON pigs (P = 0.86) but higher (P ≤ 0.02) compared to ZNO and RB2 pigs. Although, comparable *Faecalibacterium prausnitzii* abundance was observed between CON fed pigs and others, pigs on RB4 diet had a higher abundance (P = 0.04) than ZNO pigs. The relative abundance of enterotoxigenic *Escherichia coli* (ETEC) in the caecum was below quantifiable thresholds (Ct ≥ 34 cycles).

3.5.4 Effect of red beetroot on gut integrity, permeability and barrier function

The mRNA levels of genes encoding for fatty acids binding protein (*FABP2*) reduced (P < 0.01) in the jejunum of RB pigs compared to ZNO fed pigs, but comparable levels were observed in the ileum of all the pigs across the diet groups (Figure 3.4a and 3.4b). The diets significantly influenced the gut (jejunum; P = 0.003, ileum; P = 0.05) expression of *FABP6*, with increased levels (P < 0. 01) in the jejunum of ZNO fed pigs while RB fed pigs was not different from CON pigs. In the ileum, RB2 fed pigs had significantly higher *FABP6* levels (P < 0.05) compared to ZNO and CON pigs, RB4 pigs was not different from CON and RB2 pigs.

(a)



(b)

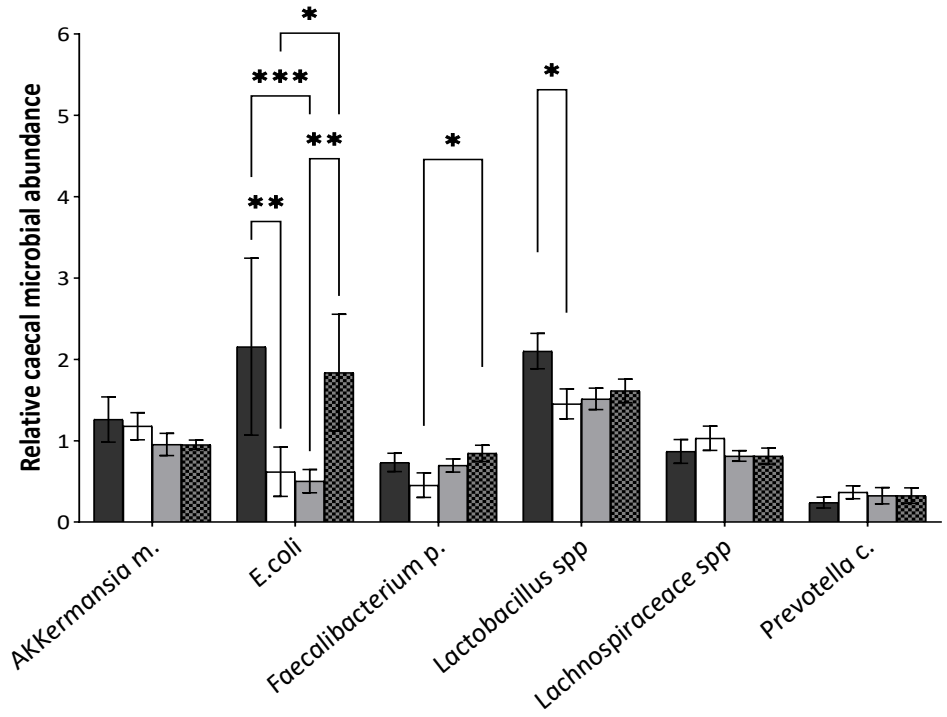


Figure 3.3: Effects of diets on (a) gut immune status assessed via gut expression of mucins and antimicrobial peptides in the jejunum and ileum and (b) caecal bacteria abundance. Significant difference between diets depicted by * P ≤ 0.05, ** P ≤ 0.01, *** P ≤ 0.001 between subjects connected by the line. CON, ZNO, RB2 and RB4 represents control diet and diets supplemented with zinc oxide, 2% and 4% red beetroot, respectively.

Considering the mRNA levels of the tight junction (TJ) proteins (Figure 3.4a), jejunal expression of *ZO1* (P = 0.21) and *CLDN3* (P = 0.65) was similar in all the pigs. However, jejunal OCLN expression was significantly higher (P = 0.02) in RB2 pigs than in RB4, but comparable to CON and ZNO pigs. Comparable ileal *OCLN* was observed in all the pigs, while ilea *CLDN3* reduced in RB2 pigs relative to CON and ZNO pigs (Figure 3.5b). Ileal *ZO1* transcription level also increased in RB pigs but significantly in RB2 pigs than in CON (P = 0.04) and ZNO (P = 0.03) pigs. Ileal expression of FABPs and TJ proteins in RB2 and RB4 fed pigs was similar.

Additionally, mRNA levels of FABPs and TJ proteins were positively associated with the expression of inflammatory target genes. In the jejunum; (*FABP2* vs (*IFNG*; R = 0.57, P < 0.001, *TNF*; R = 0.61, P = 0.001), and *ZO1* vs (*TNF*; 0.55, P < 0.001, *IL2*; R = 0.54, P < 0.001), in the ileum; (*FABP2* vs *ZO1*; R = 0.74, P = 0.001, *FABP6*; R = 0.83, P < 0.001). However, there was a negative correlation between jejunal *OCLN* and *IFNG* (R = - 0.56; P < 0.001; *TNF*; R= - 0.41, P = 0.01).

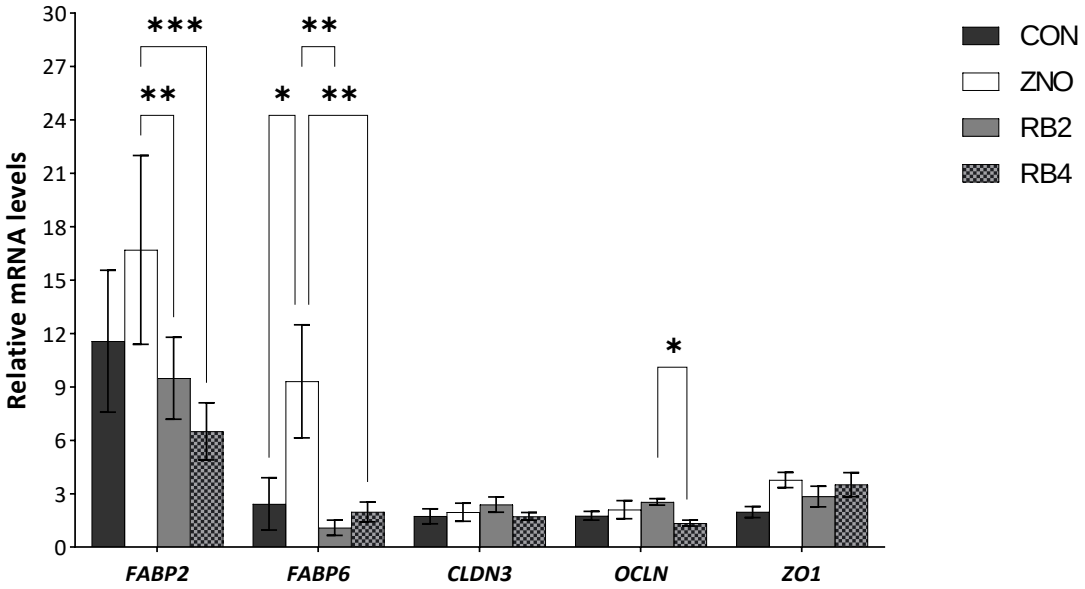
Systemic and gut L-lactate levels assessed is presented in Table 3.4, ileal L-lactate was significantly lower (P < 0.05) in RB2 and ZNO pigs compared to CON pigs but RB4 pigs were similar to CON pigs. Plasma and colon lactate levels did not differ between the pigs across the different diet groups.

Table 3.4: Gut and systemic L-lactate levels of weaned pigs fed different diets

	CON	ZNO	RB2	RB4	P value
Gut location					
Ileum (mM/g)	96.76±26.41[a]	44.72±15.53[b]	48.83±11.13[b]	76.85±20.18[a]	0.03
pH	6.97	6.92	6.91	6.59	
Colon (mM/g)	4.08±1.64	3.39±2.29	4.02±1.78	4.80±1.95	> 0.05
pH	6.05	6.26	6.16	6.00	
Plasma (mmol/L)	9.68 ± 1.18	7.85±1.01	8.01± 0.86	8.98±1.07	> 0.05

Lactate level expressed as means and (standard error). Superscripts across the table indicate significant differences (P< 0.05) between the diets for that gut location evaluated.

(a)



(b)

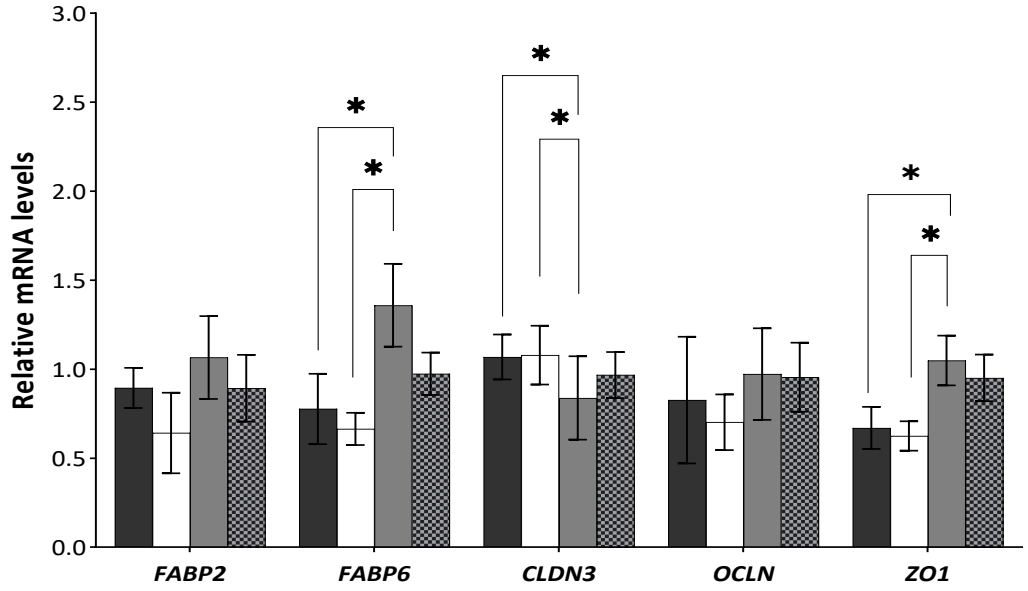


Figure 3.4: Effect of diets on gut integrity, permeability and barrier function assessed by expression levels of fatty acid binding and tight junction proteins in the (a) Jejunum and (b) Ileum. Significant difference between diets depicted by * P ≤ 0.05, ** P ≤ 0.01, *** P ≤ 0.001 between subjects linked by the line. CON, ZNO, RB2 and RB4 represents control diet and diets supplemented with zinc oxide, 2% and 4% red beetroot, respectively.

3.6 Discussion

Weaning remains a critical transition period that exposes the young pig to many endogenous and exogenous stressors resulting in weaning-induced intestinal dysfunctions. The small intestine, atrophies from reduced feed intake and nutrient absorption, resulting in severe impairment of the gut structure and function (Montagne et al., 2003; Lallès et al., 2004; 2007). As a result, modulating weaned pig gut health during post weaning period through strategic dietary intervention and supplementation is fundamental to alleviating weaning induced intestinal dysfunctions.

Past studies have shown that some plants containing bioactive compounds with anti-inflammatory and antioxidant properties improve gut function and health of weaned pigs (Li et al., 2012; Gan et al., 2019; Wang et al., 2020). Red beetroot is an example of plant with similar properties; hence, this chapter evaluated its influence on the gut health of weaned pigs with focus on the gut structure, inflammation, immunity, integrity, permeability and barrier function. Current outcomes showed red beetroot at 2% inclusion has promising effects on gut functionality and in some cases better responses than zinc oxide supplemented weaned pig diet.

Past investigations on effect of dietary supplement on the gut structure of weaned pigs reported significant changes, not only due to weaning, but also due to the dietary composition provided the pigs (van Beers-Schreurs et al., 1998; Ngoc et al., 2012). Failure in morphology observed in the foregut at weaning is characterised by an increased crypt depth (crypt hyperplasia), decreased villus height and/ or a decrease in villus height /crypt depth ratio. This is followed by epithelial sloughing (reduction in mature enterocyte population) thus a reduced absorptive capacity and post weaning diarrhoea (Pluske et al., 1997).

In this study, the experimental diets preserved the gut morphology. However, the VH, CD and VH: CD reduced in the jejunum and ileum of pigs fed RB4 diet. Shorter villi and deeper crypts have consistently been associated with increased enterocyte loss or reduced cell renewal, which leads to impaired intestinal absorptive capacity and post weaning diarrhoea (Nabuurs et al., 1993; Yin et al., 2019). The VH:CD ratio has been designated an important criterion for assessing nutrient digestion and absorption

capacity (Pluske et al., 1997; Montagne et al., 2003), hence, very likely these functions were somewhat impaired in pigs fed RB4 diet.

Equally, weaning transition is associated with microbial disruptions and dysbiosis causing intestinal inflammation. This is evident by an immediate immunological response stimulating increased production/expression of inflammatory cytokines in the gut (Pié et al., 2004). During gut inflammation, pro and anti-inflammatory cytokines are significantly modulated and regulated. More so, in event of a pathogenic invasion of the gut or a dietary modulation to alleviate weaning stress, there could be an increased expression of pro-inflammatory cytokines; *TNF, IL6, IFNG* and *IL1B* (Lippolis, 2008). Consequently, the gut permeability and barrier function become compromised due to reduced expression of tight junction proteins (Zonula occludens, Occludin, Claudins) (Capaldo and Nusrat, 2009; Vancamelbeke and Vermeire, 2017). The presence of intestinal inflammatory cytokines therefore is a proven biomarker to assess the GIT barrier function (Kim et al., 2009; Bischoff et al., 2014; Celi et al., 2017).

Results here showed that pigs fed RB2 diet had comparable jejunal expression levels of inflammatory cytokines (*IL1B, TNF, IFNG*) with CON pigs, but these inflammatory cytokines increased in ZNO pigs. However, in the ileum, pigs fed ZNO diet showed significant ability to downregulate *TNF* expression, while pigs on RB diets had comparable levels with CON pigs. Meaning, pigs on ZNO diet had inflammation in their jejunum, which tends to resolve in the ileum or alternatively suggests the active sites of these dietary supplements in the gut (ZNO; ileum, RB2; jejunum and ileum).

In addition, there was a noticeable increased jejunal and ileal *IL1B* mRNA levels with an increased red beetroot level to 4% (RB4 diet). Whereas *IL1B* expression in pigs fed RB2 diet was similar to CON (in the jejunum and ileum) and ZNO (ileum). Increased red beetroot supplementation to 4% (RB4) potentiated an increased expression of *IL1B* in the small intestine (jejunum and ileum), indicating RB4 diet may initiate gut inflammation. Comparable IL10 and IL2 mRNA levels across the diet groups signals an adequate immune response commensurate to curtail the severity of an ongoing inflammation.

Faecal calprotectin (CP) is a promising biomarker of intestinal inflammation observed with significant correlation with the severity of inflammation (Niewold, 2015; Jukic et

al., 2021). In the gut, calprotectin contributes to mucosal injury and inflammation hence strongly elevated faecal calprotectin occur in gut bacterial infections, gastrointestinal diseases, oxidative stress and drug induced enteropathies (Vavricka et al., 2018; Fukunaga et al., 2018). It has been utilised in monitoring Crohn's disease and patient with responding or relapsed IBD, hence a reliable marker for disease activity. Only few studies in pigs have measured faecal calprotectin as a marker for gut health or inflammation, and most studies in pigs utilised animals challenges with enterotoxigenic *E. coli* (Barbosa et al., 2021). However, Xiao et al. (2014) reported increased expression of calprotectin to dietary interventions in a study involving the dietary supplementation of piglet feed with chitosan. Decrease faecal CP levels by any diet indicates inhibition of inflammation and a reduced diarrhoea propensity. Current findings depict a potential of gut inflammation in RB4 fed pigs, with higher faecal calprotectin than ZNO pigs, while RB2 and CON were intermediary.

Gut inflammation also initiates the secretion of antimicrobial peptides (e.g., *REG 3G*). Muniz et al. (2012) and Sun et al. (2021), described the importance of *REG 3G* secretion in the gut towards attaining immunity. During inflammation, immune cells are activated, and antimicrobial peptides (AMPs) are secreted to eliminate microbes in the GIT, maintain gut immunity and homeostasis (Niyonsaba et al., 2009; Muniz et al., 2012). Similarly, *CCL20* has been reported to possess strong bactericidal activity with greater potency than human β-defensins (Hoover et al., 2002), but *REG 3G* are secreted by Paneth cells and are highly expressed during Salmonella infection (Cash, 2006; Godinez et al., 2009; Vaishnava et al., 2011). Mucins (e.g., *MUC2, MUC4*) are produced by the enterocytes (goblet cells) to prevent bacterial adhesion to the intestinal epithelium and translocation (Liu et al., 2020), while *MUC4 a*re continuously expressed in the transmembrane (Johansson and Hansson, 2016). Animals deficient in *MUC2* are devoid of intestinal mucus and are said to develop inflammation with diarrhoea and increased bacterial translocation (Burger-van Paassen et al., 2009; Kim and Ho, 2010). Hence, mucins play essential roles of gut protection, maintenance of intestinal homeostasis, epithelial barrier stability and adhering antimicrobial peptides to the gut epithelium (Johansson et al., 2011; Vallance et al., 2013; Zarepour et al., 2013).

Here, the diets did not influence expression of the antimicrobial peptides (*CCL20*, *REG 3G*) and mucus genes (*MUC2, MUC4*) examined. Perhaps, the experimental diets preserved the gut homeostasis despite ongoing gastrointestinal immunological changes. Burger-van Paassen et al. (2012) in their study revealed that the expression of *REG 3G* can be influenced by the levels of MUC2. Correspondingly, the pigs had similar abundance of mucin degrading bacteria species; *Akkermansia muciniphila* in the caecum. This may inform the cause of the comparable mucin expression levels observed.

Xu et al. (2021), reported an increased SCFA (short chain fatty acids)- producing bacteria e.g., *Prevotella copri* and anti-inflammatory microorganism - *Akkermansia muciniphila* (Dalile et al., 2019; Kim et al., 2020) in pigs fed quercetin supplemented diet. In contrast, observations from the caecal microbiota in this study confirmed the presence of these species in similar abundance in all the experimental pigs. *Akkermansia muciniphila* has a niche for the intestinal mucus layer and believed to be crucial to maintaining the integrity of the intestinal epithelium (Everard et al., 2013). Although, there was an increased caecal abundance of the anti-inflammatory bacteria species; *Faecalibacterium prausnitzii* in RB4 pigs when compared to ZNO, this could attest to the stride to alleviate gut inflammation, maintain gut immunity, homeostasis and health (Ferreira-Halder et al., 2017). The increased *E. coli* population in RB4 fed pigs supports our findings of a high Proteobacteria abundance and an increased abundance of opportunistic bacteria in the gut microbiome of pigs fed this diet (Chapter 2).

One of the leading causes of weaning induced GIT dysfunctionality is the breakdown in intestinal integrity, permeability and barrier function sequel to gut inflammation. Uncontrolled synthesis of pro-inflammatory cytokines has a negative impact on the gut integrity and barrier function (Al-Sadi and Ma, 2007; Beaurepaire et al., 2009). Importantly, the role of fatty acid binding proteins (*FABP2, FABP6*) in rebuilding the gut integrity and maintenance of intestinal permeability have been demonstrated in past studies (Berkeveld et al., 2008; Schroyen et al., 2012). FABPs expression is a sensitive biomarker for deciding the relative amount of the intestinal epithelium and GIT functionality during the post-weaning phase.

Tight junction proteins are present in the para-cellular space of the gut where they mediate cell-cell communication, control adherence to the intestinal epithelial layer, and regulate the flow of water ions and electrolytes in and out of the gut lumen and mucosal tissues. Therefore, they are responsible for maintaining the intestinal integrity and barrier function (Godinez et al., 2009; Ulluwishewa et al., 2011; Wells et al., 2017). Tight junction proteins are regulated by SCFAs and are reportedly sensitive to changes in diet, pathogen invasion, antibiotics and gut inflammation, which tend to modify, stabilised or disrupt them (; Zhang and Guo, 2009; Yin et al., 2019).

This study assessed dietary effects on intestinal permeability and integrity by quantifying the expression of fatty acid binding proteins (FABPs), tight junction proteins (TJs) in intestinal tissues and gut-systemic L-lactate levels. There was an increased expression of FABPs in the jejunum of ZNO pigs and reduced Occludin (*OCLN*) in RB4 pigs, a response to increased inflammatory cytokines (RB4; *IL1B*, ZNO; *IL1B*, *TNF, IFNG*) in the jejunum of pigs in these groups. Al-Sadi and Ma (2007) also reported a decrease Occludin mRNA level but an increased in *IL1B*. An increased ileal *FABP6* expression in RB pigs suggests the likelihood of inflammation in the jejunum of ZNO pigs and ileum of RB pigs. More so, a strong positive correlation between the jejunal expression of FABPs (*FABP2 and FABP6*), TJs (*ZO1*) and the inflammatory cytokines (*IFNG, TNF*) depicts inflammation was more pronounced in the jejunum than ileum.

The disruptive properties of *TNF* and *IFNG* on the tight junction barrier have been established and reported in past studies (Al-Sadi et al., 2009; Boivin et al., 2009; Kaminsky et al., 2021). Correlations between the ileal FABPs and TJs (*ZO1*) mRNA levels suggest a compensatory response to alleviate the ongoing inflammation and further prevent deterioration of the gut barrier function. Obviously, the dietary supplements evaluated differ in their mechanism of action. ZNO diet increased FABPs, *CLDN3* and maintained *OCLN* expression levels, which is similar to report by Zhang, B. and Guo (2009). RB diets (notably RB2), increased *FABP6* and *ZO1*, while *FABP2* and *OCLN* expression levels were maintained. This is the first report evaluating the effect of red beetroot supplement on gastrointestinal functionality of weaned pigs. Outcomes from this study resonates with previous reports of close associations

between the intestinal permeability and *ZO1* (Zhang and Guo, 2009) or *OCLN* levels (Bruewer et al., 2003; Farhadi et al., 2003; Moeser et al., 2007; Al-Sadi et al., 2011).

L-lactate is not a direct biomarker of intestinal function, but the gut-systemic L-lactate levels can determine the gut barrier function. L-lactate is most abundant in the blood compared to D-lactate, hence, physiologically more significant. It is a product of bacterial (e.g., *Lactobacillus spp.*) fermentation of carbohydrate substrate in the gut (Duncan et al., 2004). However, a high plasma L-lactate concentration indicates an increased intestinal permeability, (upshot of intestinal barrier failure) that allows seepage of lactate from the gut into the blood stream (Kurundkar et al., 2010; Gilani et al., 2016). In a study carried out by Pinedo-Gil et al. (2018), measuring fish plasma lactate after diet containing red beet and betaine, no improvement in systemic response to stress was observed. Similarly, plasma L-lactate levels observed in this present study rules out a systemic stress from weaning. However, increased ilea L-lactate in CON and RB4 pigs confirm the earlier inference of increased lactic acid producing bacteria abundance (lactate production) with strong links to microbiota shift of increased phylum Actinobacteriota and Proteobacteria respectively.

3.7 Conclusion

At weaning, the normal gut morphology, integrity and barrier function is disrupted, leading to a decline in gastrointestinal functionality and performance of weaned pigs. Supplementation of weaned pig diet do not only modulate the gut microbiota, but enhances its ability to function effectively. In this study, the experimental diets maintained the gut morphology; however, pigs fed diet RB4 exhibited tendency of a decreased GIT functionality. Pigs in this group had shorter villi, crypt depth, increased L-lactate levels, genes encoding for inflammation and caecal *E. coli* population. Whereas pigs fed ZNO and RB2 diet, reduced caecal *E. coli* proliferation, alleviated gut inflammation, preventing damage to gut permeability and functionality. These diets presented superior benefits over CON in enhancing beneficial caecal microbiota, gut permeability and functionality. Put together, red beetroot supplementation at 2% (RB2) presents promising gut health benefits with potentials to replace ZnO in weaned pig diet.

Chapter 4 **: Effect of red beet supplementation on antioxidant and inflammatory markers in weaned pigs**

4.1 Abstract

This study evaluated the anti-inflammatory and antioxidative response of weaned pigs to red beetroot supplemented diets. After a 14-day feeding trial in which pigs were fed one of four diets comprising a control diet (CON), diet supplemented with zinc oxide (3000 mg/kg - ZNO) and diet supplemented with red beetroot (RB) at 2% (RB2) and 4% (RB4). Blood samples and liver tissue were collected for evaluations of systemic and hepatic biomarkers of oxidative stress and antioxidant defence enzyme activity. Results highlight probable hepatotoxicity risk in pigs fed pharmacological dose of zinc oxide (ZNO diet) due to hepatic oxidative stress from malondialdehyde (MDA) levels, reduced catalase activity and depleted glutathione, compared to the CON. All the pigs had comparable plasma reducing and radical scavenging ability, but hepatic reducing ability of ZNO pigs was 2-fold higher ($P < 0.05$) than other pigs. Hepatic expression of antioxidant genes (*GPX1a, SOD, CAT* and *GCLC)* was not impaired by the dietary supplements; zinc oxide, 2% and 4% red beetroot. However, red beetroot supplementation of weaned pig diet at 4% presented inflammation with increased serum IL1B, downregulated *HMOX1* expression, and a waning antioxidant defence. RB2 diet showed hepatoprotective ability by maintaining hepatic antioxidant enzyme (CAT, SOD) activity and glutathione levels, while systemic antioxidant enzyme activity (CAT and SOD) increased. The anti-inflammatory and antioxidative status of pigs fed red beetroot supplemented diet was linked to the bioavailable betalains compounds, especially betanidin observed in the serum and liver. Nonetheless, the synergistic effect of other bioactive compounds and their metabolites cannot be excluded from the responses observed. Therefore, weaned pig diet supplemented with 2% red beetroot (RB2) improved anti-inflammatory and antioxidative response, thus a potential for application in the pursuit of zero zinc oxide in pig production. Future research investigating the impact of pure bioactives compounds and betalains from red beetroot on weaned pigs are warranted.

4.2 Introduction

As pig production moves to the era of zero zinc oxide in weaned pig diet, focus on dietary supplementation with plants rich in bioactive compounds appear promising. At weaning, young pigs combat different kinds of stress ranging from dietary to environmental, physiological, and biochemical that affect the normal function of the body (Pluske et al., 1997; Van der Meulen et al., 2010). During this period, pigs face multiple changes in the gastrointestinal tract and immune system that can result in oxidative stress. Several factors cause oxidative stress at weaning, one of which is dietary stress from poor quality diet and an immature GIT (Wijtten et al., 2011; Kick et al., 2012). Weaning induced GIT changes results in gut dysbiosis and inflammation, triggering the activation of cytokines and production of reactive oxygen species (ROS) (Amarakoon, 2017).

Gut inflammatory response produces reactive species such as nitric oxide (NO) with antimicrobial properties (Bäumler and Sperandio, 2016), thus ROS production is an immune response to antigen/pathogenic invasion. However, continuous exposure of weaned pigs to exogenous and endogenous stressors increases the production of oxidants (e.g., superoxide - $O_2^{\bullet-}$, hydrogen peroxide – H_2O_2, hydroxyl radical – $^{\bullet}OH$) that sets the system off balance resulting in oxidative stress (Rahal et al., 2014; Reuter et al., 2010; Pi et al., 2010). Oxidative stress occurs from disruption of the critical balance between oxidants and antioxidants (e.g., superoxide dismutase - SOD, glutathione peroxidase - GPx, glutathione - GSH, catalase - CAT), usually due to depletion of antioxidants from excess ROS or increase toxins or injury to the system. Oxidative stress is therefore characterised by excess free radicals (ROS) and insufficient protection by antioxidants (Sies et al., 2017; Flohé, 2020;).

Although the relationship between antioxidant status and post weaning stress/ diarrhoea of pigs is still poorly understood, Zhu et al. (2012) demonstrated weaning induced oxidative stress by increased plasma malondialdehyde levels in weaned pigs. Spees et al. (2013) also reported increased concentration of ROS in the intestine with increased *E. coli* population 7 days post weaning. Yin et al. (2014) observed increased lipid peroxidation a mark of oxidative stress in pigs three days post weaning and recovery by upregulation of antioxidant genes and proteins (e.g., *GPx4*, Uncoupling protein 2 – *Ucp2*, Nuclear factor erythroid 2-related – *Nfr2r*).

The role of weaning and post weaning diarrhoea in causing oxidative stress may be variable, due to host response in ensuring homeostasis, and a balance between oxidising reactive oxygen species (ROS) and the antioxidant defence system. In extreme cases however, the host may be overwhelmed by continued assault to the system leading to irreparable oxidative injury, liver damage and cell death (Yin et al., 2013). In which case, weaned pigs show reduction in growth, anorexia from dietary stress, inflammation, immune break down and leaky gut syndrome (diarrhoea) from an impaired intestinal barrier function (Wei et al., 2017; Amarakoon, 2017).

The most viable way to alleviate post weaning oxidative stress is to prevent nutritional stress by providing weaned pigs with high quality feed containing antioxidants. Antioxidant rich fruits, vegetable or their wastes are essential for the reduction of oxidative stress in immune cells (Víctor et al., 2002). Plants polyphenols (e.g., resveratrol, catechin, quercetin, and curcumin) are exogenous sources of dietary antioxidants with the ability prevent oxidative stress (Menon and Sudheer, 2007; Landete, 2013). Additionally, polyphenols may enhance the antioxidant defence system by scavenging for free radicals and upregulate anti-inflammatory action (Kumar and Pandey, 2013; Alkadi, 2020), consequently, systemic/gut inflammation can be prevented, and animal health improved.

Red beetroot (RB) contains important bioactive compounds (e.g., betalains and polyphenols, ascorbic acid, carotenoids, phenolic acids) with proven health benefits (e.g., antioxidative, anti-inflammatory, antimicrobial and hepatoprotective) (Lee et al., 2005; Georgiev et al., 2010; Kumar and Brooks, 2018; Masih et al., 2019). RB has been highlighted as a resource for development of dietary supplements and nutraceuticals for health conditions emanating from inflammation and oxidative stress (Ninfali and Angelino, 2013; Chhikara et al., 2019). It has high mineral content (calcium, potassium, sodium, phosphorus, chloride) and trace elements (Iron, zinc, iodine, selenium, copper, manganese) that mediate the hepatic expression of antioxidant enzyme genes (SOD, GPx), responsible for the reduction of oxidants (Kujala et al., 2001).

Notably, red beetroot is the main source of betalains, which comprise cationized antioxidants e.g., betanin (betanidin 5- 0- betaglucoside), reported to inhibit lipid peroxidation and heme decomposition at low concentrations (Kanner et al., 2001;

Lichtenthäler and Marx, 2005). Fernando et al. (2022) observed increased radical scavenging activity of betanin, compared to other betalains (Neobetanin, indicaxanthin, vulgaxanthin1) at 25 µg/mL. Also, many scientific literature have demonstrated *in vitro* and *in vivo* benefits of different forms of red beetroot product (crisped, pulverized, beet juice, beet waste, cooked etc.) and betalain compounds in animal species and humans (Gandía-Herrero et al., 2016; da Silva et al., 2019; Wang et al., 2022), but data on effect of red beetroot in weaned pigs is grossly deficient.

Meanwhile, betalains have demonstrated stronger radical scavenging properties compared to other known antioxidants (e.g. ascorbic acid, tocopherols and rutin) (Gliszczyńska-Świgło et al., 2006; Swarna et al., 2013). There is evidence suggesting higher antioxidant activity in degraded betalains such as neobetanin and betanidin (Wootton-Beard et al., 2011; Mikołajczyk-Bator and Czapski, 2018). Also, a possible synergistic effect between polyphenols, betalains, their metabolites and other compounds in red beetroot contributing to the overall antioxidant effect or health benefits observed (Georgiev et al., 2010; Fernando et al., 2022). This study therefore explored the impact of red beetroot in alleviating weaning induced oxidative stress and inflammation. The presence of systemic inflammation and lipid peroxidation as measures of oxidative stress was established, then systemic and hepatic antioxidant defence responses of pigs fed the different diets was evaluated.

4.3 Materials and methods

4.3.1 Materials

Solvents used for this study; (methanol and formic acid (UHPLC-MS grade), butanol and dimethyl sulfoxide (DMSO) were purchased from Fischer scientific (Loughborough, UK). The chemicals; ((±)-6-hydroxy-2,5,7,8-tetramethylchromane-2-carboxylic acid (Trolox), 2,2´-azino-bis(3-ethylbenzothiazoline-6-sulfonic acid) di-ammonium salt (ABTS), potassium peroxodisulfate, sodium acetate-trihydrate, Trichloroacetic acid, (TCA), 1,1,3, 3-tetramethoxypropane (TEP), Thiobarbituric acid (TBA), butylated hydroxytoluene (BHT), sodium dodecylsulfate salt (SDS), Iron-(Miller III et al.)-chloride-hexahydrate, 2, 4, 6-tri (2-pyridyl)-s-triazine, 5, 5´-Dithiobis (2-nitrobenzoic acid) (DTNB), 5-sulfosalicylic acid (SSA), nicotinamide adenine dinucleotide phosphate (NADPH), sodium dihydrogen phosphate monohydrate and

disodium hydrogen phosphate dehydrate) were all purchased from Sigma - Aldrich (Dorset, UK). The cytokine and chemokine 9-plex porcine ELISA kit (ProcartaPlex™ Panel 1) was obtained from Thermofisher scientific, (Loughborough, UK). Superoxide dismutase activity kit and SOD from bovine erythrocytes (≥ 3000 units/mg protein) were purchased from Sigma-Aldrich (Dorset, UK). Catalase enzyme kit was purchased from Cambridge Bioscience Ltd (Cambridge UK). All aqueous solutions were prepared using deionized water purified by Milli-Q water (Millipore, Gillingham, UK).

4.3.2 Methods

4.3.2.1 Experimental animals, sample collection and processing

The animal feeding trial is as described in chapter two (see section 2.3.2.2), thirty-eight pigs were sampled at the end of the feeding trial. Blood (10 mL) was collected from the jugular vein of each pig into labelled heparinized and plain tubes for plasma and serum respectively. The pig liver was removed, cut into small piece (3.0 - 5.0 cm) and stored in 5.0 mL RNAlater, another piece (10 - 20 g) was wrapped in a foil and immediately snapped frozen. All samples were stored at -80°C until further analyses.

For the serum, blood sample collected into plain tube was left to clot at room temperature (25°C) for 20 to 30 min, centrifuged at 4°C, 1,500 x *g* for 15 min. The supernatant was collected into sterile tubes and immediately snap frozen. Plasma was derived, after blood in heparinized tubes was centrifuged at 2,000 x *g*, 4°C for 10 min, the supernatant was collected into sterile tubes and snap frozen. Frozen pig liver sample (1.0 g) was homogenised in 9.0 mL ice-cold buffer (100 mM Tris-HCl, pH 7.2) using a tissue grinder (VWR, UK), then centrifuged at 12,000 x *g* for 10 min at 4°C. The supernatant was collected and aliquoted into 2 mL Eppendorf tubes then stored at -80°C until analysed.

Protein in the serum, plasma and liver samples was quantified using the BCA protein assay (Pierce™ kit, Thermofisher scientific) based on Bicinchoninic acid colorimetric method, with bovine serum albumin (BSA) (20 - 2000 µg/mL) as standard. Samples were diluted in phosphate buffered saline (PBS; 1:20 v/v), to ensure readings were within the standard curve and transferred to a 96 well plate in duplicate (2 x 10 µL). The assay was performed by adding 200 µL working reagent to each well, incubated at 37°C for 30 min and absorbance determined at 570 nm on a microplate reader

(Tecan Spark 10 M). Serum and liver protein were expressed in mg/mL and mg/g, respectively.

4.3.2.2 Evaluation of circulating systemic inflammatory markers

Circulating inflammatory cytokines (Interleukins (IL) 1B, IL4, IL6, IL8, IL10, IL12p40, interferon alpha (IFNA), IFNG, and TNF) was determined in serum samples with a porcine cytokine and chemokine multiplex panel (Procartal 9-plex panel, Thermofisher scientific) applying the Luminex xMAP technology. Serum samples were analysed at the Clinical histopathology unit of the Leeds Institute of Cancer and Pathology, St James' University Hospital, Leeds, UK. Data was analysed with Bio-Plex ™ manager using a four-parameter logistic (4PL) algorithm to determine best curve fit.

4.3.2.3 Betalain extraction and quantification in feed and pig samples

Betalains in feed samples, serum and liver homogenate were extracted using method described in (Sawicki et al., 2016) with slight modifications. Briefly, 0.2 g of feed sample (in triplicate) was mixed with 3 mL of extraction solvent (water/methanol/formic acid; 84.95/15/0.05, v/v/v) for 90 sec by continuous vortexing. The mixture was sonicated in a water bath (Grant Instruments, UK), for 10 min at 5.0 ± 3°C and centrifuged at 3200 × *g*, at 5°C for 10 min. The supernatant was separated into a tube and extraction repeated on the residue to ensure maximum betalain extraction. Combined supernatant from the extractions were vortexed and stored at -20°C until further analysis.

For serum and liver homogenates, betalain was extracted using solid phase extraction (SPE) method, with 33 µm, 200 mg/6 mL Strata-X polymeric reversed phase column (Phenomenex, Torrance, USA). The cartridges were conditioned with 4 mL methanol and equilibrated with 4 mL distilled water, then 800 µL of sample (serum and liver homogenate) was passed through the cartridge. Next, the cartridge was washed with 1 mL of water and betalain was eluted with 1.4 mL of methanol. Eluents were evaporated on a rotary evaporator (Genevac EZ-2 Series, Ipswich, UK), freeze dried for 12 hr (Freeze dryer, Labconco, USA) and re-suspended in 60 µL of water. The solution was, centrifuged at 21,000 x *g*, 4°C for 15 min, 30 µL of the supernatant was collected for injection into Ultra-high-performance LC (UHPLC-MS).

Betalain compounds in samples were identified and quantified on UHPLC-MS, a Thermo-Vanquish UHPLC coupled with a triple-stage quadrupole (TSQ Quantiva) mass spectrophotometer and electrospray ionization (ESI-MS) source (Thermo Scientific, Cambridge, UK). HPLC separation was by Kinetex XB-C18 column (100 mm × 2.1 mm, 2.6 µm) at 40°C. The mobile phase A and B consist of 2% (v/v) formic acid in water and UHPLC-MS grade methanol respectively, delivered at a constant flow rate of 0.2 mL/min. The gradient programme involved a proportional increase in solvent B from 5% to 25% for (0 - 5 min), 25% - 95% (5 -10 min), sustained at this gradient for 10 - 20 min before declining to 5% (20 - 32 min). Betalain detection was at 536 nm and 486 nm wavelength for betacyanins and betaxanthins respectively with molecular weight monitoring by ESI-MS (in the positive ion mode). Betalain identification was by comparing the retention time and m/z $[M + H]^+$ values (see Table 4.3) reported in previous research (Sawicki et al., 2020; Nemzer et al., 2011). Compounds were quantified using standard curve with concentration ranged 0.5 - 100 µM, prepared from purified betalains.

4.3.2.4 Evaluation of oxidative stress; Thiobarbituric acid reactive substances (TBARS) assay

TBARS assay determines the presence of lipid peroxidation a biomarker of oxidative stress. The assay measures the generation of an advanced product of lipid degradation under the influence of ROS and Thiobarbituric acid (TBA). A coloured product formed (malondialdehyde) is measured spectrophotometrically to determine the presence of oxidative stress (Ghani et al., 2017). Oxidative stress in the weaned pigs was determined by measuring malondialdehyde (MDA) levels in plasma and liver samples.

Plasma and liver sample aliquots (200 µL; 1:5 dilution for liver samples) were deproteinated by mixing with an equal volume of trichloroacetic acid (TCA, 5%), vortexed and centrifuged at 12,000 x *g* for 5 min. The supernatant (200 µL) was collected into 2 mL safe seal tubes, to which 200 µL of 2-thiobarbituric acid (1% TBA) and 20 µL of sodium dodecylsulfate and butylated hydroxytoluene (0.5% SDS-BHT) solution were added, mixed and incubated for 15 min in a water bath (100°C). The mixture was cooled on ice and butanol (99%; 1 mL) added to extract the trimethine dye. The mixture was vortexed for 30 sec, centrifuged at 12,000 x *g*, 4°C for 5 min,

then (200 µL) supernatant was transferred into a 96-well plate in duplicates. Fluorescence was measured on a microplate reader at an excitation/emission wavelength of 540/590nm and optimal gain (Yoshino et al., 2012; Weiss and Deutschman, 2014). MDA concentration in samples was calculated using 1, 1, 3, 3-tetramethoxypropane (0 - 2640 nM) as standard and expressed in nano-molar MDA/mg protein.

4.3.2.5 Determination of antioxidant enzyme activity and glutathione concentration

Antioxidant enzyme assays were conducted in duplicates using aliquots of plasma sample and liver homogenate. Superoxide dismutase (SOD; EC 1.15.1.1) and catalase (CAT; EC 1.11.1.6) activity was determined using commercial kit from Sigma-Aldrich and (Cambridge Bioscience Ltd) respectively. Following the manufacture's direction, SOD activity in plasma and liver was determined by adding 200 µL of working solution (WST- [2-(4-Iodophenyl) - 3-(4-nitrophenyl)-5-(2, 4-disulfophenyl) - 2H-tetrazolium, monosodium salt]) and 20 µL of enzyme working solution (xanthine oxidase) to (20 µL) sample. The mixture was incubated at 37°C for 30 min. SOD activity was measured at 450 nm from the rate of decreased in colour intensity of the product formed after reaction of tetrazolium salt with superoxide anion present in the sample (Yusuf et al., 2012; More and Makola, 2020). SOD from bovine erythrocytes with concentration ranged 0.001 - 200 U/mL was used as standard, a curve of concentration and inhibition rate was fitted with four parameter logistic (4PL) method using an online data analytical tool (MyAssays).

Catalase (CAT) is an antioxidant enzyme responsible for the detoxification of hydrogen peroxide (H_2O_2; a toxic product of aerobic metabolism and pathogenic ROS production) to water and oxygen (catalytic activity). The assay employs the peroxidatic function (ability to serve as electron donors; equation 4.1) of CAT to determine the enzyme activity, based on the reaction of the enzyme with methanol in the presence of an optimal concentration of hydrogen peroxide to produce formaldehyde. The formaldehyde formed is measured colorimetrically after reaction with a chromogen (amino-3-hydrazino-5-mercapto-1, 2, 4-triazole) to form a purple-coloured oxidised heterocyclic aldehyde (Wheeler *et al.* 1990; Vives-Bauza *et al.* 2007)

$$(Catalytic\ activity) \quad H_2O_2 \xrightarrow{Catalase} O_2 + 2H_2O_2$$

$$(Peroxidatic\ activity) \quad H_2O_2 + AH_2 \xrightarrow{Catalase} A + 2H_2 \quad \textbf{Equation: 4.1}$$

A Cayman catalase assay kit (Cambridge, UK.) was used according the manufacture's direction (Wiecek et al., 2018; Cong et al., 2021). The reaction involved the addition of 100 µL of diluted (1:20) assay buffer, 20 µL sample, standard or positive control and 30 µL methanol to 96-well plate in duplicates. The reaction was initiated by adding an optimal concentration of H_2O_2, (20 µL, 8.82 M) at a precise time. The plate was covered and incubated on a shaker for 20 min at Rt. Then 30 µL potassium hydroxide was added to each well to terminate the reaction followed by 30 µL of the chromogen. The plate was again incubated for 10 min, then 10 µL potassium periodate was added to each well and incubated for 5 min at Rt. Finally, the absorbance was measured at 540 nm using a plate reader (Tecan Spark 10M), then blank corrected and the average absorbance of each standard and sample was calculated. CAT activity was calculated (nmol/min/mL) from a standard plot of formaldehyde concentration and absorbance. Standard formaldehyde concentration ranged 0 - 100 µM was prepared and bovine liver catalase was used as control.

Glutathione is an endogenously produced antioxidant capable of preventing damage caused by reactive oxidative species (ROS). It is involved in the detoxification of xenobiotic, removal of hydroperoxide and maintenance of oxidation state. Glutathione occurs mainly in a reduced (GSH; about 90 - 95% of the total glutathione) or oxidised state (GSSG; > 10%). It is a sensitive indicator of the ability of an organism to resist toxic challenge and overall health of the cell. The glutathione assay measures total glutathione (GSSG+GSH) in a biological sample, based on a catalytic action by GSH or GSSG in the reduction of Ellman's reagent (DTNB) in the presence of nicotinamide adenine dinucleotide phosphate (NADPH; 0.16 mg/mL) and glutathione reductase. The catalytic action of glutathione in the system is dependent on the continual enzymatic generation of GSH present or formed from GSSG (Tietze, 1969; Pastore et al., 2001).

The reagents were prepared as specified following procedures described for glutathione assay in (Akerboom and Sies, 1981; Nair et al., 1991). To prepare the sample for the assay, (0.3 mL) of sample was mixed with equal volume of 5-sulfosalicylic acid solution (SSA; 5%, 0.3 mL), incubated at 4°C for 10 min then centrifuged (10,000 x *g* for 10 min) to remove the precipitated protein. Potassium phosphate buffer (100mM, pH 7.0 with 1mM EDTA) was used as assay buffer while the working reagent was made from equal volume of 5,5-dithio-bis-(2-nitrobenzoic acid) (DTNB; 228 µL; 1.5mg/mL) and glutathione reductase (228 µL; 6U/mL) diluted in 8 mL of assay buffer.

The reaction involved pipetting 10 µL of SSA (reagent blank), standard and sample into a 96 well plate in duplicates. Then, 150 µL of the working reagent was added to the mixture and incubated on plate rocker (Stuart scientific, UK) for 5 min at Rt. Following this, 50 µL of NADPH solution was added, mixed and absorbance was measured at 412 nm with kinetic read every 15 sec up to 60 sec, values from the glutathione standard solutions were used to determine the standard curve and the change in absorbance per min (ΔA_{412}/min) was determined equivalent to 1nmole of reduced glutathione per well.

4.3.2.6 Systemic and hepatic total antioxidant capacity (FRAP and TEAC assays)

The antioxidant capacity of serum, plasma and liver homogenates was analysed using the FRAP and TEAC method as described in (Han et al., 2021). The ferric reducing antioxidant power (FRAP) assay is based on the antioxidants in samples acting as a reductant (reducing agent), thus converting ferric (Fe^{3+}) to ferrous (Fe^{2+}) by reaction with a chelating agent (2,4,6-tris (2-pryridyl)-s-triazine; TPTZ) to form an iron complex Fe^{2+}-TPTZ (blue coloured compound) at pH 3.6. The increase in absorption of the compound formed at 593 nm is proportional to the antioxidant capacity of the sample, expressed as equivalent to a standard antioxidant (Apak, 2019). The ferric reducing antioxidant power (FRAP) reagent was prepared from 30 mM sodium acetate buffer (pH3.6), 20 mM Iron III chloride-hexahydrate and 10 mM 2,4,6-tris (2-pryridyl)-s-triazine (TPTZ), mixed in the ratio (10:1:1; v/v/v). The reducing capacity of the sample (10 µL) was determined by mixing with the FRAP reagent (300 µL). Samples were

incubated in the dark at 37°C for 15 min and absorbance read at 593 nm on a plate reader.

The Trolox equivalent antioxidant capacity (TEAC) or ABTS assay assess the capacity of a compound to scavenge 2, 2'-azino-bis (3-ethylbenzothiazoline-6-sulfonic (ABTS) radicals. The antioxidant capacity of a sample is thus determined by its ability to diminish the colour intensity after reaction with $ABTS^{\bullet+}$ radical. The reaction is time-dependent, and the blue-green radical is measured with a spectrophotometer at a range of 690 - 734 nm. The rate at which the colour intensity of the radical decreases (quenched) is proportional to the concentration of the antioxidant in the sample (Arts et al., 2004; van den Berg et al., 1999).

The assay was conducted as described in (Re et al., 1999), the radical cation ($ABTS^{+}$) was prepared by mixing 14 mM of 2,2'-azino-bis (3-ethylbenzothiazoline-6-sulfonic) ABTS® with 4.9 mM potassium peroxodisulfate in ratio (1:1; v/v). The mixture was allowed to stand in the dark at room temperature for 12 to 24 hr for complete interaction. Then, the $ABTS^{+}$ working solution was prepared by dilution of the stock in distilled water until an initial absorption of 0.700 ± 0.020 was reached at 732 nm. The antioxidant capacity of sample (10 µL) was measured by adding (300 µL) $ABTS^{+}$ working solution in a 96 well plate. The plate was incubated in the dark for 6 min at Rt and absorbance read at 734 nm on a microplate reader. Trolox was used as standard for both assays in concentrations ranged 0 to 1.0 mM, prepared in ethanol-water mixture (75:25; v/v). Results were expressed in µg Trolox equivalent (TE)/mg protein in the sample.

4.3.2.7 Hepatic antioxidant gene expression analyses

The influence of diets on the expression of antioxidant genes in the liver was analysed using a quantitative polymerase chain reaction (qPCR). Selected antioxidant genes used are presented in table 4.1, primer sequences for the genes were designed and blasted using NCBI primer-BLAST tool (Ye et al., 2012), setting the template for effective primer design of an average melting temperature of 60°C, GC content between 40-60% and maximum, 18 - 24 bases. Primer efficiency and amplification linearity was verified for the selected and housekeeper genes and PCR product sizes were confirmed by 2% agarose gel electrophoresis (Figure 6.9) before quantification

in samples. RNA isolation, cDNA synthesis and qPCR were conducted as described in Chapter 3; sections 3.3.2.5.1 and 3.3.2.5.2). Total RNA was isolated from pig samples stored in RNAlater, after thawing the samples on ice, 50 mg liver tissue was placed in 1 mL TRIsure reagent (Bioline Ltd, UK). The complementary DNA (cDNA) was diluted 1:20 before qPCR run and the programme for amplification was as described previously. Hepatic antioxidant gene transcription levels were calculated using ($2^{-\Delta\Delta CT}$) method by Livak and Schmittgen (2001), normalized with β-actin (housekeeper gene).

4.4 Statistical analyses

Data was presented as mean ± SE (Standard error), checked for normality using Shapiro-Wilk test and square root transformed where necessary to ensure normal distribution before further analyses. Statistical analysis was performed by one-way ANOVA, using the general linear model in SPSS (v. 26.0). Differences in antioxidant capacities and enzyme activities between the diet groups was determined by Tukey's honest significant difference (HSD) post hoc test, then unpaired t-test was used for pairwise comparison between two diet groups (e.g., CON vs RB2, ZNO vs RB2). Differences in the concentration of each betalain compound was evaluated using Tukey's multiple comparison test in Graph Pad prism (v.9.4.1). An unpaired t-test with Welch's correction was used to compare the betalains concentration between RB2 and RB4 diet. Pearson's correlation analysis was conducted to determine the relationship between betalain composition in liver samples, antioxidant capacity, enzymes activity and target genes. Differences between groups were considered significant at $P \leq 0.05$ level, results were presented in tables and graphs (Excel 2016; Graph Pad prism v.9.4.1).

Table 4.1: Antioxidant biomarker gene primer sequences

Genes	Forward primer (5´ to 3´) Reverse primer (5´ to 3´)	Size (bp)	Accession number
Reference gene			
ACTB	GTCACCAACTGGGACGACAT GTACATGGCTGGGGTGTTGA	174	XM_021086047.1
Antioxidant biomarker genes			
CAT	TGTGAACTGTCCCTTCCGTG TCCCCAGAATAGCGGGTACA	162	XM_021081498.1
GPX1a	GGTCTCCAGTGTGTCGCAAT TCGATGGTCAGAAAGCGACG	106	NM_214201.1
GCLC	GGCGACGAGGTGGAATACAT CTGGTCTCCAAAGGGTAGGA	145	XM_021098555.1
HMOX1	GCAGAGGGTCCTCGAAGAAG GTCACGGGAGTGGAGTCTTG	165	NM_001004027.1
SOD	AGGGCACCATCTACTTCGAG GATCCTTTGGCCCACCATGT	183	NM_001190422.1

Catalase - (CAT); Glutathione Peroxidase – (GPX1a); Glutamate Cysteine Ligase Catalytic - (GCLC); Heme Oxygenase - (HMOX1); Superoxide Dismutase – (SOD).

4.5 Results

4.5.1 Betalains and related compounds in feed and biological samples

The betalain content in the experimental diets was assessed alongside serum and liver samples. Betalains were not detected in CON and ZNO diet hence they were not included in the data analyses. As in Table 4.2, all the betalain compounds identified increased (by a 2 to 4-fold) in RB4 diet compared to RB2 diet as expected. Levels observed in biological samples from both diets were similar. Higher betanin and isobetanin concentrations were observed in the feed samples, while serum and RB2 liver samples were high in betanidin. Decarboxylated derivatives of betanin and isobetanin were also present in the feed samples but not detected in the biological samples (serum and liver). Figure 4.1 summarises the percentage compositions of individual betalain compounds in the samples evaluated.

Data showed higher concentration of betanin than isobetanin ($P < 0.05$) and other compounds ($P < 0.001$) in RB4 diet. Decarboxylated betalain compound 17-decarboxy-betanin and 15-decarboxy-betanin were significantly different ($P < 0.05$) in diet RB2 and RB4.

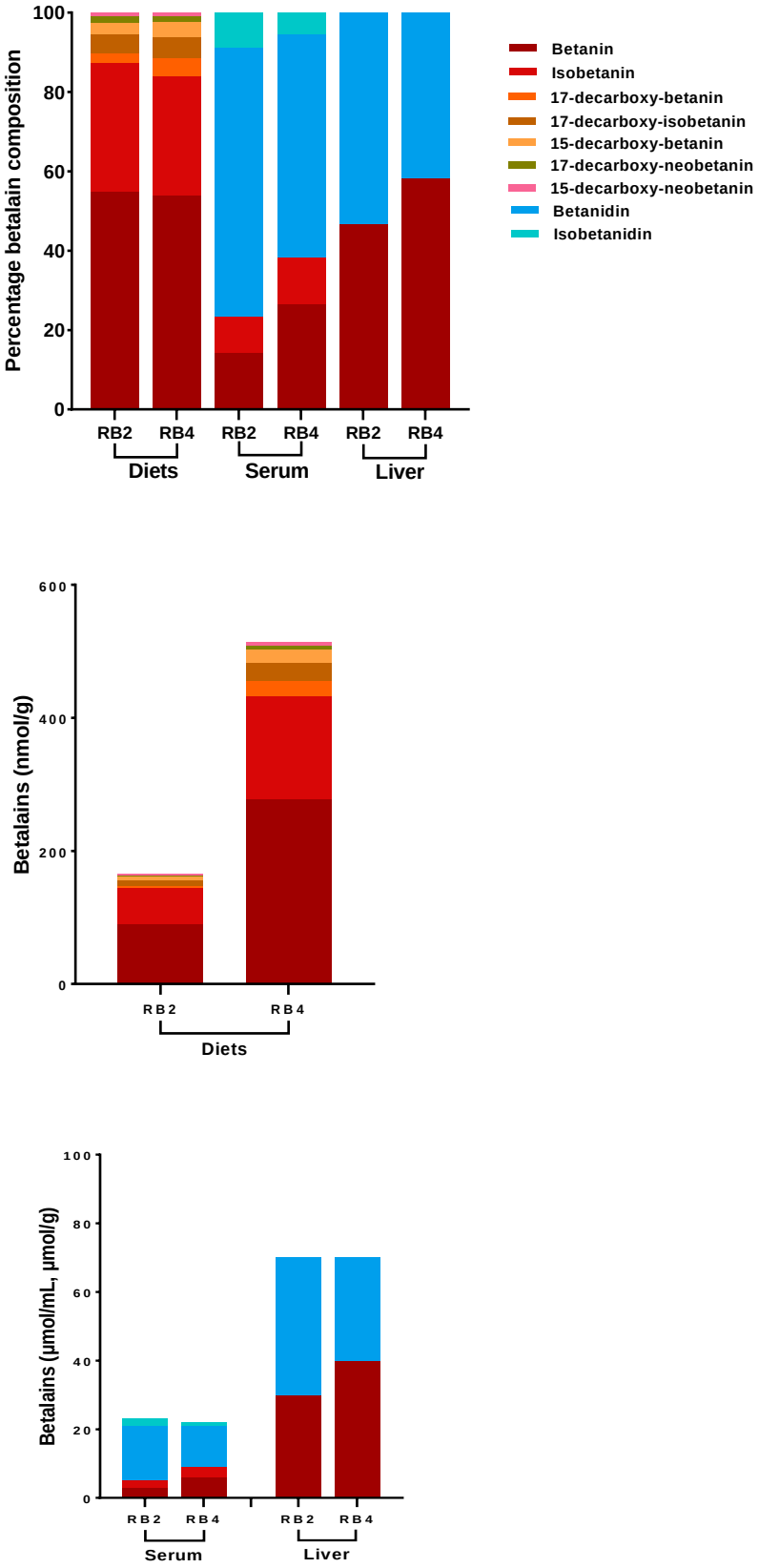


Figure 4.1: Betalains and percentage composition observed in feed, serum and liver of weaned pigs fed red beetroot supplemented diets. RB2 and RB4 represents 2% and 4% red beetroot supplemented weaned pig diets.

Table 4.2 Betalains compounds in red beetroot supplemented diets and biological samples of weaned pigs

Betalain compounds	m/z $[M + H]^+$	Feed (nmol/g)		Serum (µmol/mL)		Liver (µmol/g)	
		RB2	RB4	RB2	RB4	RB2	RB4
Betanin	551	90.3±18.10	277.4± 79.43	3.3±1.17	5.7±1.14	30.6±10.49	37.8±15.92
Isobetanin	551	53.5±11.02	154.1±47.64	2.1±0.77	2.5±0.40	ND	ND
17-decarboxy-betanin	507	4.1±1.31[a]	23.8±3.85[b]	ND	ND	ND	ND
17-decarboxy-isobetanin	507	7.8±1.36	26.9±5.91	ND	ND	ND	ND
15-decarboxy-betanin	507	4.9±0.8[a]	19.7±3.64[b]	ND	ND	ND	ND
17-decarboxy-neobetanin	505	2.6±0.58	7.1±2.78	ND	ND	ND	ND
15-decarboxy-neobetanin	505	1.7±0.26	5.4±1.93	ND	ND	ND	ND
Betanidin	389	ND	ND	15.6±5.61	12.2±8.39	35.14±12.13	27.3±9.12
Isobetanidin	389	ND	ND	2.0±0.75	1.2±0.33	ND	ND
Total betalains		**164.8±31.18**	**514.0±141.75**	**23.05±0.5**	**21.6±8.16**	**65.7±13.91**	**65.1±23.62**

Values expressed as mean ± SE. ND; not detected, means with different superscript are different (P < 0.05) for the sample type and the corresponding betalain compound. RB2 and RB4 represents; 2% and 4% red beetroot supplemented weaned pig diets respectively.

Though, comparable levels of betalain compounds were identified in the serum and liver of RB pigs, betanidin and betanin levels were high in RB2 and RB4 pigs respectively.

4.5.2 Systemic anti-inflammatory and oxidative response of weaned pigs

There was much variability in the serum cytokine levels measured in the pigs, individual pig cytokine levels are shown in Figure 4.2, while the diet group average is presented in Table 4.3. Notably, a few pigs, representative from each diet group showed high circulating serum IL10, IL1B, IL4, IL6 and TNF levels. However, all the pigs were represented in the serum IFNA and IL12p40 levels observed. On average, pigs in RB4 group showed higher serum IL1B levels compared to CON ($P < 0.01$) and RB2 ($P < 0.05$) pigs (RB4 > RB2 > CON) respectively, levels in ZNO pigs were intermediate. There was also a significant increase in the circulating IFNA in RB2 pigs compared to pigs on other diets. Though, RB pigs had high average IL6, IL8, IL10, IL12p40 and TNF levels, they were similar to levels detected in CON and ZNO pigs. IL12p40 level was significantly higher than other cytokines ($P < 0.01$) assessed, while IFNG was not detected in any of the samples.

Table 4.3: Systemic inflammatory cytokines (pg/mL) of weaned pigs

	CON	ZNO	RB2	RB4	*P* value
Cytokines					
IFNA	1.21±0.47^{b}	2.14±1.13^{b}	7.76±2.10^{a}	2.96±1.06^{b}	0.02
IL1B	0.09±0.09^{b}	49.95±49.95^{ab}	3.44±3.11^{b}	75.89±48.71^{a}	< 0.01
IL4	0.33±0.33	0.00±0.00	1.75±1.20	0.53±0.43	> 0.05
IL6	0.00±0.00	0.00±0.00	6.07±6.07	3.11±2.83	> 0.05
IL8	6.20±4.66	9.21±9.21	26.66±14.70	13.07±7.92	> 0.05
IL10	0.00±0.00	0.00±0.00	0.14±0.14	1.95±1.95	> 0.05
TNF	0.00±0.00	0.00±0.00	36.06±29.98	18.88±18.88	> 0.05
IL12p40	416.17±125.92	222.72±65.33	517.310±163.32	671.59±318.97	> 0.05

Values are mean ±standard error of serum cytokine levels in pg/mL. Significantly different diets (P< 0.05) for each cytokine are represented by different superscript across the table. CON, ZNO, RB2 and RB4 represents control diet and diets supplemented with zinc oxide, 2% and 4% red beetroot, respectively.

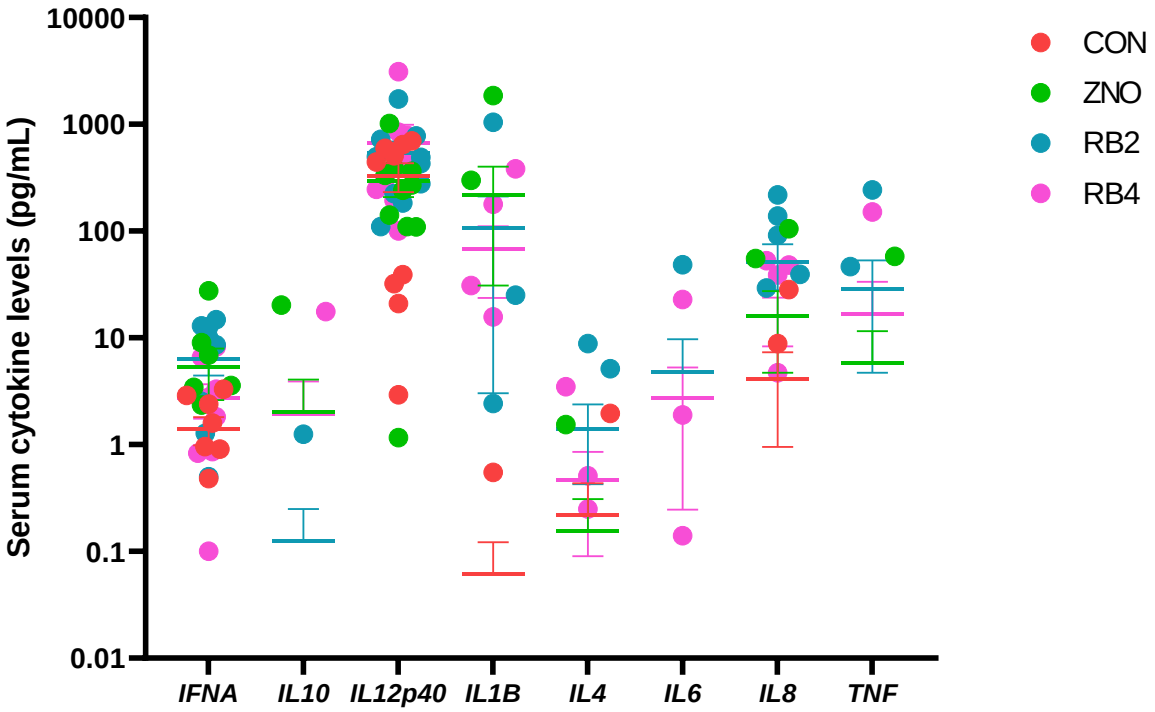


Figure 4.2: Individual serum inflammatory cytokine levels of pigs fed different experimental diet. Graph plot shows individual values, diet group mean and standard error. *. CON, ZNO, RB2 and RB4 represents control diet and diets supplemented with zinc oxide, 2% and 4% red beetroot, respectively*.

Evaluation of systemic and hepatic MDA levels showed the diets significantly influenced ($P < 0.01$) hepatic MDA levels. Hepatic MDA levels in pigs fed ZNO diet was higher compared to CON ($P \leq 0.05$) and RB2 ($P \leq 0.01$) pigs. However, levels in RB (RB2 and RB4) fed pigs, were comparable to CON pigs (Figure 4.3b). Systemic MDA levels were not different across the diet groups (Figure 4.3a), however a strong association was observed between serum isobetanin and systemic MDA levels ($R = 0.73$; $P = 0.04$).

4.5.3 Antioxidant capacity of plasma and liver homogenates

The hepatic and systemic antioxidant capacity of the pigs in this study assayed using FRAP and TEAC method is shown in Table 4.4. Though the systemic antioxidant capacity were similar in all the pigs despite the different diet, result from both methods are related ($R = 0.62$; $P < 0.001$). Thus, similarities in the systemic ability of the pigs to reduce and scavenge for reactive oxygen species.

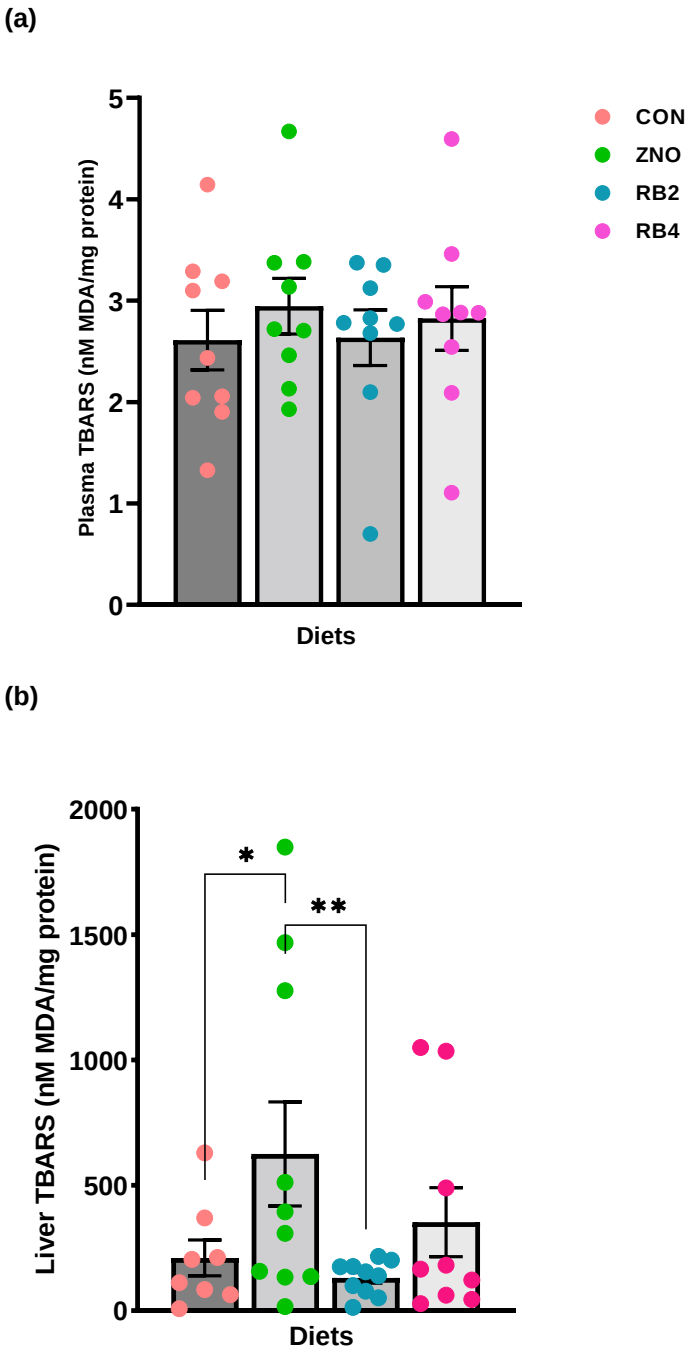


Figure 4.3: Malondialdehyde (MDA) levels in the (a) plasma and (b) liver of weaned pigs fed different diets. CON, ZNO, RB2 and RB4 represents control diet and diets supplemented with zinc oxide, 2% and 4% red beetroot, respectively.

The serum antioxidant capacity measured using the FRAP method was significantly higher ($P < 0.05$) in pigs fed RB diets than in CON and ZNO pigs, but RB2 and ZNO pigs had comparable serum TEAC measurements. The systemic antioxidant capacity determined with the TEAC method was related to the systemic MDA levels (R = 0.58;

P < 0.001). Hepatic antioxidant capacity measured with FRAP method increased significantly by 2-fold (*P* < 0.001) in ZNO pigs compared to CON and RB pigs, depicting a higher hepatic reducing ability in ZNO pigs. Hepatic antioxidant capacity in RB pigs was similar to the control for both methods, thus the hepatic radical scavenging and reducing ability of these pigs were moderated despite different diets.

Table 4.4: Systemic and hepatic total antioxidant capacity

Diets	CON	ZNO	RB2	RB4
FRAP (µg Trolox eq./mg protein)				
Plasma	30.4±2.22	32.9±3.45	35.9±3.65	33.95±2.37
Serum	38.8±3.83^{b}	41.7±3.30^{b}	54.8±6.22^{a}	46.07±2.29^{ab}
Liver	765.5±38.33^{b}	1676.0±72.26^{a}	724.0±32.50^{b}	727.9±28.25^{b}
TEAC (µg Trolox eq./mg protein)				
Plasma	1799.6±64.27	1814.4±34.40	1784.2±51.10	1757.2±68.38
Serum	1642.0±94.29^{b}	1796.3±71.09^{a}	1778.5±51.33^{a}	1631.9±73.02^{b}
Liver	1924.3±134.71	2045.1±67.26	1964.3±97.96	1943.5±82.50

Values are mean ± SE. means in a row with different superscript letter are statistically different from each other (P< 0.05). CON, ZNO, RB2 and RB4 represents control diet and diets supplemented with zinc oxide, 2% and 4% red beetroot, respectively.

Table 4.5: Systemic antioxidant enzyme activity and glutathione levels

Diets	CON	ZNO	RB2	RB4
CAT (U/mg protein)	1.67± 0.36^{b}	1.72±0.16^{b}	3.05±0.74^{a}	2.08 ± 0.41^{ab}
SOD (mU/mg protein)	43.10± 4.60^{b}	44.52±2.39^{ab}	49.86±3.82^{a}	39.36 ± 4.61^{b}
GSH (U/mg protein)	2.16± 0.31^{b}	2.44±0.16^{b}	3.21±2.01^{b}	4.63 ± 1.01^{a}

*Values are mean ± SE. means in a row with different superscript letter are statistically different from each other (P< 0.05).*CAT-catalase, SOD - superoxide dismutase, GSH – glutathione, *CON, ZNO, RB2 and RB4 represents control diet and diets supplemented with zinc oxide, 2% and 4% red beetroot, respectively.*

4.5.4 Diet effect on systemic and hepatic antioxidant enzyme activity and glutathione levels

The activity of antioxidant enzymes; catalase (CAT) and superoxide dismutase observed in pig plasma and liver homogenates are presented in Table 4.5 and 4.6 respectively. Plasma CAT and SOD enzyme activity were significantly higher ($P < 0.01$) in RB2 pigs compared to pigs fed CON and ZNO diet ($P < 0.05$). Also, plasma SOD activity was significantly increased in RB2 pigs compared to CON and RB4 ($P < 0.01$) but not different from ZNO ($P = 0.3$).

For the hepatic antioxidant defence system, the experimental diet significantly ($P = 0.05$) influenced the CAT enzyme activity. Compared to the CON pigs, reduced CAT enzyme activity was observed in pigs fed ZNO diet ($P = 0.05$), RB pigs were not different ($P > 0.05$) from CON pigs. In addition, hepatic SOD activity was not influenced ($P = 0.85$) by the different experimental diets, comparable levels were observed in the pigs, more so, hepatic SOD and TEAC were significantly related ($R = 0.79$; $P < 0.001$).

Plasma glutathione (GSH) was significantly higher in pigs fed RB4 compared to CON ($P = 0.05$) and ZNO ($P = 0.04$) and pigs, levels in RB2 pigs was midway. Similarly, hepatic glutathione level was higher ($P = 0.05$) in RB4 pigs than ZNO pigs but not different ($P > 0.05$) from CON and RB2. However, there was a correlation between serum isobetanin and plasma glutathione ($R = -0.82$; $P = 0.01$).

4.5.5 Effects of diets on hepatic antioxidant gene expression

According to table 4.7, the experimental diets significantly influenced ($P < 0.01$) the hepatic expression of the antioxidant genes. Compared to CON pigs, there was a significant increase in the hepatic expression of *GPX1a* and *SOD*, hence expression levels in the supplemented diets (ZNO, RB2 and RB4) were similar. *GCLC* levels increased in ZNO pigs compared to the CON, but levels from RB diets were intermediate between CON and ZNO. CAT expression in RB2 and ZNO pigs were high, but overall expression levels in the pigs was similar. Decreased HMOX1 expression was observed in RB4 pigs ($P = 0.02$) while others were comparable. There was a correlation between hepatic betanidin levels and *GPX1a* expression ($R= 0.71$; $P = 0.05$). Figures 4.4 and 4.5 presents a summary of the antioxidant enzyme activity and defence system of weaned pigs fed the different diets.

Table 4.6: Hepatic antioxidant enzyme activity and glutathione levels

Diets	CON	ZNO	RB2	RB4
CAT (U/mg protein)	2504.9±228.21^{a}	1612.3±216.57^{b}	2243.2±274.50^{a}	2391.5±301.79^{a}
SOD (mU/mg protein)	6152.4±730.18	5524.8±357.32	5965.1±506.311	5736.4±335.26
GSH (U/mg protein)	2614.9±550.48^{ab}	2321.1±568.67^{bc}	2879.8±572.83^{ab}	3217.1±257.66^{a}

Values are mean ± SE. Means in a row with different superscript letter are statistically different from each other (P< 0.05). CAT- catalase, SOD - superoxide dismutase, GSH - glutathione. CON, ZNO, RB2 and RB4 represents control diet and diets supplemented with zinc oxide, 2% and 4% red beetroot powder respectively.

Table 4.7: Hepatic expression of antioxidant biomarker genes[1]

Diets	CON	ZNO	RB2	RB4
CAT	1.50±0.29^{ab}	1.98±0.33^{a}	2.27±0.20^{a}	1.49±0.16^{ab}
GCLC	0.82±0.10^{b}	1.25±0.17^{a}	0.95±0.10^{ab}	0.97±0.05^{ab}
GPX1a	0.56±0.04^{b}	0.76±0.13^{a}	0.80±0.07^{a}	0.78±0.06^{a}
SOD	0.54±0.08^{b}	0.81±0.21^{a}	1.05±0.15^{a}	0.82±0.08^{a}
HMOX1	4.99±0.45^{a}	5.15±1.03^{a}	4.65±0.60^{a}	3.23±0.47^{b}

[1]*Values presented are means ±SE of square root transformed data, statistically analysed conducted using Tukey HSD for ANOVA and multiple t test (Welch's test) with Benjamini and Hochberg correction for multiple testing. P values < 0.05 are significant and values with different superscript alphabets along each row are not significantly different from each.* CAT- catalase, SOD - superoxide dismutase, GCLC *Glutamate Cysteine Ligase Catalytic*, GPx1a - Glutathione peroxidase, HMOX1 - Heme oxygenase. *CON, ZNO, RB2 and RB4 represents control diet and diets supplemented with zinc oxide, 2% and 4% red beetroot respectively.*

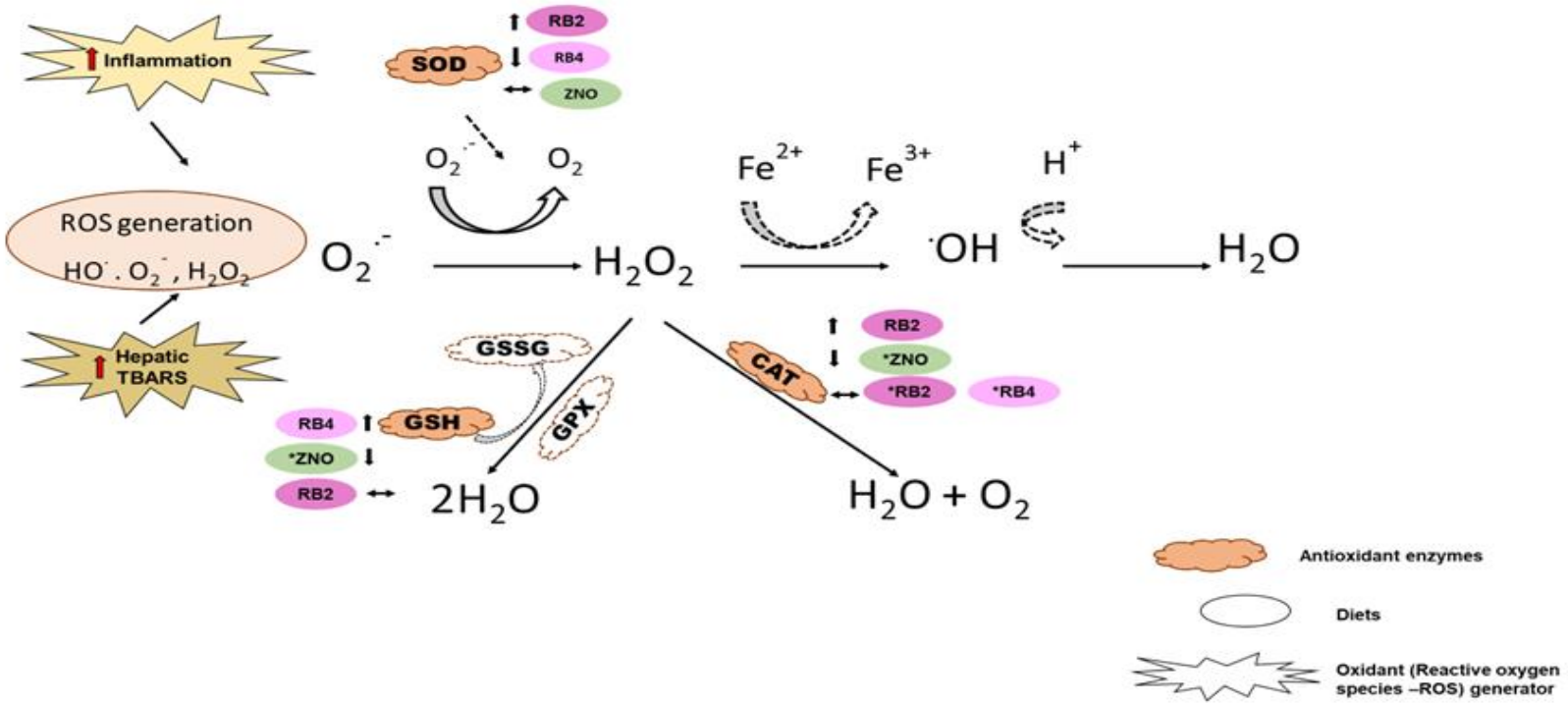


Figure 4.4 Schematic diagram of the antioxidant enzyme activity and defence system of weaned pigs fed red beetroot supplemented diets. $O_2^{\bullet-}$ - superoxide, H_2O_2 - hydrogen peroxide, •OH - hydroxyl radical, GSH – glutathione, GSSG – reduced glutathione, CAT- catalase, GPx – glutathione peroxidase, O_2 - oxygen, Fe^{2+} - ferrous, Fe^{3+} - ferric, H_20 - water, SOD – Superoxide dismutase. CON, ZNO, RB2 and RB4 represents control diet and diets supplemented with zinc oxide, 2% and 4% red beetroot, respectively. (*) represents diets influenced significant changes in the liver for the corresponding antioxidant enzymes.

4.6 Discussion

Weaning is a complex period in young pigs, presenting different clinical features and responses. Importantly, GIT changes causing gut dysbiosis and inflammation exposes weaned pigs to myriads of infections, disorder and oxidative stress (Wijtten et al., 2011; Zhu et al., 2012). Therefore, weaning plays a central role in post weaning oxidative stress and feedback mechanism regulating the expression of antioxidant genes and development of the antioxidant defence system. Although, many dietary supplements of plant origin have been proposed for use in weaned pig diet, only a few studies evaluated the antioxidant status of weaned pigs after such dietary interventions. This study assessed the anti-inflammatory and antioxidative status of weaned pigs to red beetroot supplementation.

Systemic inflammation and oxidative stress are characteristic of the post weaning transition phase of young pigs, causing increased gut permeability and dysfunctions. Oxidative stress results from high concentrations of reactive oxygen species (ROS) produced endogenously from pathogenic invasion and increased inflammation or exogenous from toxins and xenobiotic (Finkel, 2003; Beutler, 2004; Forman and Zhang, 2021). During inflammation, pro-inflammatory cytokines (e.g., IL1B, TNF, IL6) and lymphatic cytokines like IFNA and IL10 are produced, the latter been responsible for moderating systemic inflammation. Anti-inflammatory cytokines (e.g., IL10, IL4, IFNA) downregulate the production of pro-inflammatory cytokines (Opal and DePalo, 2000; Zelnickova et al., 2008) launching an immune response. Hence, an increase in systemic anti-inflammatory cytokine have been shown to depict a period of recovery from inflammation (Rousset et al., 1995).

The systemic inflammatory response of weaned pigs in this study showed a similar trend to the expression of gut inflammatory genes reported in Chapter 3. Notably is the high level of serum IL1B in RB4 pigs (4% red beetroot diet) relative to the CON and RB2 pigs. Which relates to the predisposition of increased dietary red beetroot levels (4% - RB4) to inflammation inferred from the previous chapter. However, ZNO pigs showed an inclination towards systemic inflammation, due to elevated IL1B despite downregulated TNF levels. IFNG was not detected in the serum samples, but anti-inflammatory cytokine IFNA was higher in pigs fed RB2 diet than other pigs.

In parallel with the gut IL10 expression levels (chapter 3), similar development was observed in the serum for all the pigs, more so, comparable IL6, IL4, IL8 and IL12p40 levels were detected. The similarity in the systemic levels of these cytokines could be due to transient inflammation or immune stimulation as alluded to in (Watford et al., 2003; Grilli et al., 2015). Also, the absence of an exogenous pathogen challenge that would have initiated a significant increase in pro-inflammatory cytokines production could be responsible for the effect observed. Secondly, the dietary treatments may have positively modulated inflammation by the downregulation of pro-inflammatory cytokines (IL6, IL8 and TNF) rather than enhancing the anti-inflammatory response (IL10, IL4 and IL12p40). Characteristically, anti-inflammatory effects of red beetroot have been linked to their ability to interfere with pro-inflammatory signalling cascades (Clifford et al., 2015; Calvi et al., 2022).

Thirdly, high IL12p40 levels compared to other cytokines, may be responsible for the resolution of inflammation in the pigs. IL12p40 is a heterodimer (p35 and IL-12A), bioactive cytokine interleukin (IL12 and IL23), and a pro and anti-inflammatory cytokine with an inherently agonistic nature pivotal to early initiation of the immune system (Abdi, 2002; Watford and O'Shea, 2003). IL12p40 coordinates the innate resistance and adaptive immunity thus an immune-regulatory cytokine (Trinchieri, 2003). The comparable expression of circulating pro-inflammatory cytokines reveals a more rapid immune response might have been induced by RB2 and RB4 diet, with high levels of IL12p40 although statistically similar with other diets. Consequently, it is difficult to conclude that the pigs in this study had an on-going inflammation. The pigs were relatively healthy and not exposed to any pathogenic challenge or stressful situation (transporting animals) other than weaning. However, current data suggests, the pigs had a transient inflammation and were in the recovery phase.

Although the *in vitro* and *in vivo* anti-inflammatory effects of red beetroot have been widely studied and reported in rats and human models (Clifford et al., 2015; Ninfali et al., 2017; Hadipour et al., 2020), studies involving weaned pigs are under reported. This is the first report on the *in vivo* inflammatory response of weaned pigs to red beetroot supplemented diet to the best of our knowledge. Studies investigating the anti-inflammatory role of red beetroot under pathogenic stimulation should be

considered in the future. This will aid understanding the potency of its *in vivo* anti-inflammatory response.

Meanwhile, assessment of oxidative stress via MDA levels in plasma and liver showed an absence of systemic oxidative stress in all the pigs, but a probable occurrence of hepatic stress in pigs fed ZNO diet. Contrariwise, Yin et al. (2013) and Zhu et al. (2017) reported decreased plasma MDA concentrations. A few pigs fed RB4 diet showed predisposition to increase hepatic MDA levels, suggesting pigs in these groups (RB4 and ZNO) are susceptible to inflammation (elevated IL1B) and oxidative stress (increased MDA) compared to RB2 fed pigs. Their propensity towards inflammation with increased IL1B may have led to an increased MDA levels, capable of overwhelming the antioxidant defence system, disrupting redox homeostasis and culminating in increased oxidative stress (Kohen and Nyska, 2002). This observation supports the claim that increased inflammation can result in oxidative stress and *vice versa*, thus endangering animal health (Gessner et al., 2017). Hence, low red beetroot levels (2% - RB2) reduced hepatic MDA significantly relative to ZNO diet, while high supplemental red beetroot levels (4% - RB4) may portend a risk.

Additionally, the supplemented diets (RB2, RB4 and ZNO) did not modify the plasma reducing and radical scavenging ability measured by FRAP and TEAC method respectively (Table 4.4). The hepatic antioxidant capacity was especially high in ZNO pigs, with increased reducing ability (FRAP), which could be an indirect antioxidant effect in response to the hepatic MDA levels (Velisek et al., 2011; Tkachenko et al., 2014). However, the hepatic radical scavenging ability (TEAC) of the pigs was not altered by the dietary treatments. Betalains present in red beetroot are good ROS scavengers (Vulić et al., 2013) which could explain the high hepatic radical scavenging ability (TEAC) and antioxidant enzyme activities observed. They have greater bioavailability than most flavonoids and superior stability in comparison to anthocyanin’s (Suganyadevi et al., 2010; Choo et al., 2019).

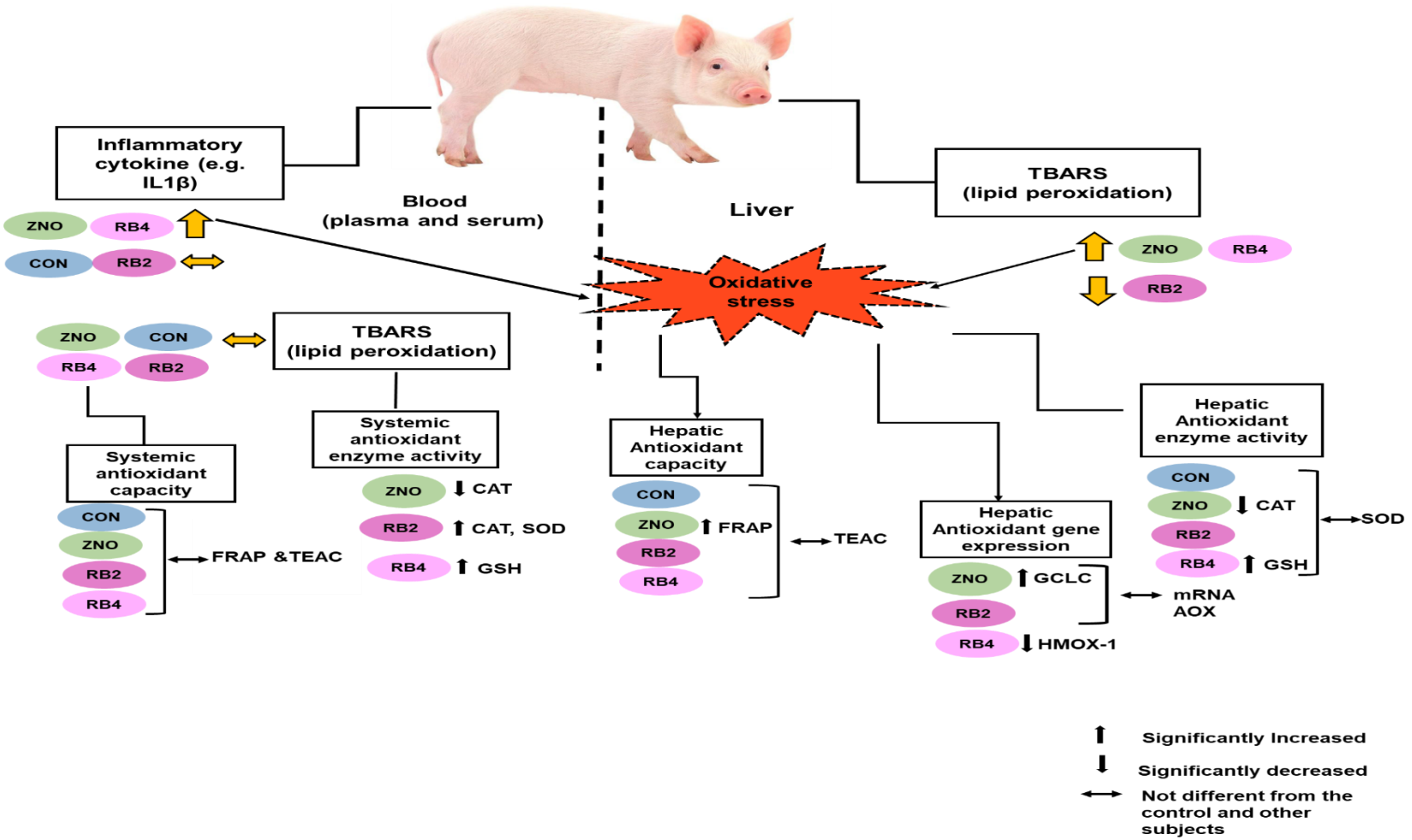


Figure 4.5: Inflammatory and antioxidative status of weaned pigs fed different diets. AOX: - Antioxidant genes, *CAT*- catalase, SOD - superoxide dismutase, *GCLC* - Glutamate Cysteine Ligase Catalytic, *GPX1a* – Glutathione peroxidase, *HMOX1* – Heme oxygenase. CON, ZNO, RB2 and RB4 represents control diet and diets supplemented with zinc oxide 2% and 4% red beet root, respectively.

These results present some notable effects of the different dietary treatments on the systemic and hepatic antioxidant defence compared to the control. Pigs fed ZNO diet had a reduced systemic and hepatic CAT enzyme activity. Reduced CAT activity indicates inadequate capacity to scavenge hydrogen peroxide in response to oxidative stress, which can be due to increased superoxide radical in the system (Tkachenko et al., 2014). RB2 (2% red beetroot) diet showed increase plasma antioxidant enzyme activity significantly for CAT and SOD. Overall, RB2 diet presented the ability to increase the systemic antioxidant defence while the hepatic antioxidant defence was maintained, signifying extra-hepatic metabolic sites for red beetroot.

Similarly, this study highlights the effect of increased red beetroot (4% in RB4) over RB2 diet (2%), with tendency for a decline in plasma SOD and CAT activity while hepatic levels were maintained. RB4 pigs had an increased plasma and hepatic glutathione (GSH) levels compared to other pigs. Increased glutathione has been recognised as an adaptive response to oxidative stress (Sagara and Schubert, 1998; Hayes and McLellan, 1999; Dickinson et al., 2003), for pigs in RB4 group, this reflects a means to prevent excessive built up of ROS that could ensue from the declining SOD and CAT enzyme activity and potentially increased MDA levels. However, a depletion in glutathione and glutathione mediated antioxidant defence system indicates oxidative stress and increased cytotoxicity (Velisek et al., 2011; Grim et al., 2013), which relates to observations in ZNO pigs. Increased ROS causes oxidative stress leading to the elevation of antioxidant enzymes as a defence mechanism to prevent cell/organ breakdown (Tkachenko et al., 2014). The first line of defence against oxidative stress consists of antioxidant enzymes; SOD, CAT and GPX that converts superoxide radicals into hydrogen peroxide, water and oxygen (Ural, 2013). Oruç (2010) reported increased SOD activity in Carp following exposure to xenobiotic. In this study, hepatic SOD activity was maintained by all the diets though RB2 diet improved systemic SOD activity significantly.

Generally, the supplemented diets (ZNO, RB2 and RB4) improved the hepatic antioxidant defence system by upregulating the expression of *GPX1a* (57.6%) and SOD (60%) antioxidant genes. Comparable transcription levels of antioxidant genes were observed in ZNO and RB pigs except for *HMOX1*. *HMOX1* is a cell protective enzyme with the capacity to reduce oxidative stress and maintain iron homeostasis.

Downregulation of *HMOX1* was observed with dietary increase of red beetroot (4% - RB4) as well as a potential decline in CAT activity. This indicates an increased beetroot (4%) may reduce the hepatic presence and activity of these antioxidant genes, predisposing weaned pigs to oxidative stress. Compared to the CON, GCLC an important antioxidant gene modulating the production of cellular glutathione was upregulated in ZNO pigs. *GCLC* is increasingly expressed during oxidative stress, glutathione depletion and exposure to toxic chemicals (Franklin et al., 2009; Lu 2009). Hence, a high pharmacological dose of ZnO such as used in this study (ZNO diet) may be hepatotoxic, causing increased MDA levels and depletion of glutathione from increased *GCLC* expression.

Due to the high betalain content in red beetroot, its *in vitro* and *in vivo* antioxidative potential have been extensively explored (Han et al., 2015; Tan et al., 2015; Tan and Hamid, 2021). However, the contributions of other constituents like the phenols (rutin, epicatechin, caffeic acid) with proven antioxidant properties (Frank et al., 2005; Georgiev et al., 2010) have been underreported. Here, the dietary treatment used was a composite and not purified betalains or betanin mostly reported (Vulić et al., 2014; Fernando et al., 2022). As a result, the undesirable effect observed for RB4 diet may be due to such unidentified components in the diet. Although, the relative contribution of these component is difficult to elaborate, their additive and synergistic effect cannot be ignored (Clifford et al., 2015; Hadipour et al., 2020). Besides, low betalain concentration have been reported to inhibit lipid peroxidation and haem decomposition (Kanner et al., 2001). However, the potential detrimental effects from increased/ prolonged intake have been under reported and under-studied.

The antioxidative effects of red beetroot have also been linked to the bioavailability of betalains and bioactive compounds in the circulation following ingestion and absorption through the gastro-intestinal tract (Toutain et al., 2004; Wootton-Beard et al., 2011). Really, the bioavailability of betalains in serum and liver samples was incredibly low (< 0.01%) and few betalains (betanin, isobetanin, betanidin and isobetanidin) were observed compared to compounds present in the feed samples. Betanin (2.1 - 5.7 µmol/mL) and betanidin (12.2 - 35.1 µmol/g) were the most abundant of all the compounds in the liver and serum, respectively.

There was a correlation between the serum isobetanin concentration, plasma MDA and glutathione levels. Hepatic betanidin was strongly associated with *GPX1a* mRNA expression (Table 6.5). The relevance of these correlations may however be inconsequential owing to the comparable concentrations of betalains observed in the serum and liver of the RB fed pigs. A higher betanidin than betanin in the serum of RB2 pigs may have given rise to increased systemic antioxidant capacity observed (in RB2 serum FRAP and TEAC). Again, in the liver, RB2 pigs had more betanidin than betanin compared to RB4 pigs which may explain the differences in their MDA levels, despite comparable hepatic antioxidant capacity and enzyme responses. Although the bioavailability of betalains have been studied comprehensively and betanin has been credited with a higher antioxidant effect compared to other betalains, there are scare reports on the antioxidative effect or health benefits of betanidin. Our study highlights that betanidin (Figure 4.6) may have a higher antioxidant capacity than betanin.

Betanidin is the aglycone form of betanin and the basic betacyanin with 5, 6 –hydroxyl moiety. It has more hydroxyl group than betanin after deglycosylation hence a stronger radical scavenging activity at pH 2 to pH 4 and high antioxidant activity than betanin (Cai et al., 2003; Gliszczyńska-Świgło et al., 2006; Khan, 2016). Concomitantly, betanidin has reduced sugar moiety than betanin, therefore may be more active and bioavailable *in vivo*. Taira et al. (2015) reported, betanidin is a potent antioxidant against peroxyl radical and nitric oxide, signifying glycosylation (to form betanin) and acylation reduces the radical scavenging ability of betacyanin. Results by Wybraniec et al. (2011) from voltammetric oxidation further support betanidin as a strong reducing agent. Hence, future studies investigating the antioxidative effect and properties of betanidin are necessary.

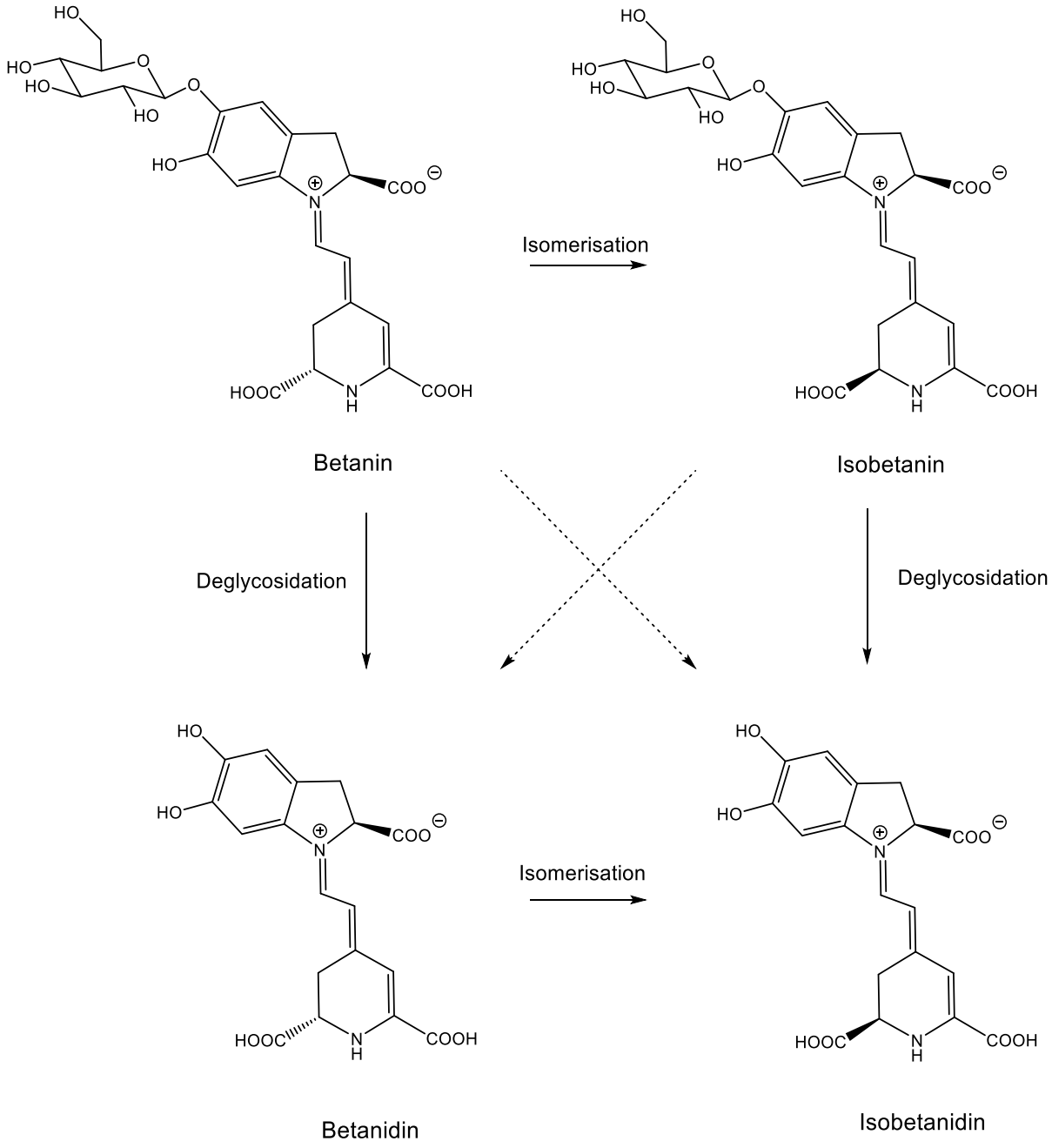


Figure 4.6: Pathways of conversion of betanin to betanidin and their isomers (Isobetanin and isobetanidin).

4.7 Conclusion

In summary, pigs fed ZNO showed a propensity towards high MDA levels and RB4 pigs presented a possibility of inflammation. Red beetroot supplementation of weaned pig diet helped maintained the hepatic antioxidant defence system (hepatoprotective) while the systemic antioxidant defence and hepatic antioxidant gene expression was improved. Particularly, pigs fed RB2 diet had better antioxidative status and response compared to other pigs. Although, pigs on ZNO and RB2 had similar ability for hepatic antioxidant gene expression, there is the danger of hepatotoxicity, reduced CAT and glutathione depletion with ZNO pigs. Increased red beetroot in the diet (RB4) revealed the danger of waning systemic antioxidant defence and heme decomposition, although compensated for by an increased systemic and hepatic glutathione levels. Red beetroot supplementation of weaned pig diet at 2% (RB2) showed an anti-inflammatory and antioxidative response, thus a potential for application in the pursuit of zero zinc oxide in weaned pig diet. Due to the unavailability of data on the polyphenols and other bioactive compounds present in the diets and biological samples, it is difficult to attribute the benefits as well as downsides of the red beetroot diets and doses to betalains alone. Future work employing pure compounds are necessary to further expand on these findings.

Chapter 5 **: General Discussion**

5.1 Introduction

Weaning is a critical phase in the production cycle of pigs, characterised with increased use of antibiotics and antimicrobials closely linked to the emergence of AMR. Pathogenic invasion of the GIT, and the subsequent diarrhoea resulting from physiological and immunological changes at weaning is a leading cause of mortality and huge economic loss during pig production. Besides improving management practice in pig farms, dietary interventions aimed at improving nutrient absorption and immune response post weaning is a viable way to optimise the health and gut health of weaned pigs.

As pig production moves into the zero-zinc oxide phase, sustainable livestock production via recycling of feed resources remains a viable means to enhance food and animal production while reducing food waste. A large amount of processed waste from agro-allied industries are currently being explored as animal feed supplement to improve and lower the cost of livestock production. Among these, red beetroot (RB) is an important source of health promoting phytochemicals with antioxidant, anti-inflammatory, and hepatoprotective properties (Clifford et al., 2015; Chhikara et al., 2019). This chapter summarises the outcomes of exploration on the impact of red beetroot on health and gut health of weaned pigs.

5.2 Impact of red beetroot on health of weaned pigs

Overall, the growth performance of the pigs did not differ among the diet groups at the end of the experimental period (Chapter 2; Table 2.1). Although, growth and performance response of the pigs was not a major objective in this research, pigs fed red beetroot (RB) supplemented diets showed potential to reduce post weaning anorexia and weight loss, which can be severe in the first week after weaning. Pigs in the RB diet group had an increased feed intake, which was significantly higher than those fed the control group (CON) or ZnO diet (ZNO) in the second week post weaning. The first week after weaning is very critical and it is important that pigs have a high feed intake for good growth performance, to prevent infections and death. This is in accordance with past reports (Manzanilla et al., 2004; Papatsiros et al., 2009).

Pigs fed control and ZnO had lower feed intake and demonstrated some weight loss during the first week unlike pigs fed RB diets. A large-scale feed trial aimed at determining growth performance difference will afford a better understanding of the weaned pig performances.

Due to comparable average faecal score across the diet groups, no adverse effect of the diet or diarrhoea was observed in the pigs. However, detailed evaluations of interaction between the diet and the GIT using histomorphometry, biochemical, molecular and genomic techniques showed the predisposition of pigs fed RB4 diet to reduced absorptive surface, diarrhoea and inflammation (Chapter 2 and 3). The reduced absorptive surface may have translated to decreased betalains (comparable systemic and hepatic levels with RB2, despite 2-4 fold increment in diet) in pigs fed high dose (4%) of red beetroot (Chapter 4; Table 4.2). Highlighting the importance of techniques employed in this study in assessing the impact of the dietary intervention, these techniques provided an in-depth evaluation of the intestinal environment than reliance on visual pen observation, faecal scores or other subjective measurements.

This study demonstrated the role of red beetroot in improving or modulating the immune response of weaned pigs rather than moderating growth, which was evident by improved gut, systemic and hepatic antioxidant status and enzyme activities observed in red beetroot fed pigs, especially RB2 diet (Chapters 3 and 4). Although, previous studies demonstrated increased weight gain with low doses (0.0125 g/kg to 0.025 g/kg) of plant bioactive supplemented diets e.g., Oregano oil, citrus peel; and chicory (Jugl-Chizzola et al., 2006; Hashemi and Davoodi, 2011). And Selim et al. (2013) reported increased growth performance with (0.5 -1%) aqueous beetroot extract following a 40-day supplementation of broiler chicken. The dose, duration and animals used in these studies may have influenced the efficacy of beetroot addition.

5.3 Oxidative Stress, inflammation and anti-oxidative status

Weaning stress is associated with oxidative processes that lead to the release of reactive oxygen species (ROS) (Zhu et al., 2017). Weaning induced oxidative balance with varying jejunal and ilea antioxidant gene expression was demonstrated in Yin et al. (2014), three days post weaning. Increased ROS have been linked to activation and upregulation of pro-inflammatory cytokines with subsequent increase in gut permeability and decreased expression of tight junction proteins (Yin et al., 2013;

Vergauwen et al., 2017). In addition, the host responds to gut inflammation by producing ROS such as nitric oxide. A nitrate rich environment favours the growth of *Enterobacteriaceae* encoding for nitrate reductase genes. Red beetroot is rich in nitrate, which could have favoured increased phylum Proteobacteria (represented by elevated ilea *Enterobacteriaceae*; Figure 6.4c) observed in pigs fed RB4 diet. Increased Proteobacteria have been linked with weaning stress and gut inflammation, which disrupts the microbiota, depleting resident gut commensal/beneficial inhabitants (Winter et al., 2013; Argüello et al., 2019). This succinctly explains the depleted production of gut metabolites (SCFA and secondary bile acids) observed in RB4 pigs compared to RB2 pigs (Chapter 2), as well as a tendency for oxidative stress and inflammation compared to CON pigs (Chapter 4). Therefore, increased dose of red beetroot was less efficient in making the health benefits of red beetroot available for the achievement of health and gut health.

Previous study by Zhu et al. (2017) demonstrated elevated plasma MDA levels, with pharmacological dose of ZnO. In this present study, a few pigs in the group had levels above the group average, which raises a concern of hepatotoxicity and /or oxidative stress with pigs fed ZnO supplemented diet (ZNO). Weaned pig diet supplemented with red beetroot at 2% inclusion (RB2) prevented hepatic oxidative stress, although comparable with control diet (CON), RB4 followed a similar trend with ZNO (Chapter 4, figure 4.3). Conversely, an increased hepatic antioxidant capacity in ZNO pigs, with increased reducing ability (FRAP method) was likely due to an increased hepatic MDA, equivalent to observations with the systemic MDA levels and antioxidant capacity.

The total antioxidant capacity (TAC) is a viable tool for assessing nutritional interventions with antioxidant rich foods. It entails measuring the antioxidant capacity of all antioxidants in a sample, mostly with the FRAP and TEAC method (Miller et al., 1993; Szeto et al., 2002). These methods measure different antioxidants, because a standard and officially recognised method to evaluate TAC is unavailable. Hence, TAC is not a standalone method, interpretation of the antioxidant capacity of any antioxidants containing diet must include other antioxidant defence biomarkers for a rounded reflection of the health status. Interestingly in this study, results from both methods were similar.

Furthermore, results from Chapter 4 showed RB2 diet increased plasma CAT and SOD activity, with a slight decline with increased RB level to 4%. Hepatic SOD enzyme activity was not affected by the dietary supplement (RB and ZnO), but CAT activity was reduced in ZNO pigs. This demonstrates the ability of RB to increase the systemic antioxidant defence and preserve the hepatic antioxidant defence (hepatoprotective). Also, ZNO and RB2 diets had comparable hepatic antioxidant gene expression but RB4 downregulated *HMOX1* expression. Many studies have shown that phytochemicals act through several antioxidant defence mechanisms to improve redox balance and prevent oxidative stress damages (Rahimi et al., 2019; Hadipour et al., 2020). Reduction in antioxidant enzyme (e.g., CAT) activity indicates a disruption in the redox state, and increased lipid peroxidation (Drummond et al., 2011), as apparent in ZNO pigs (Chapter 4, Tables 4.6, 4.7).

Again, this study showed the propensity of ZNO diet to deplete hepatic glutathione and increased hepatic *GCLC* expression. Increased cell injury and ROS from increased lipid peroxidation (MDA levels) has been linked to glutathione depletion, further confirming the risk of hepatotoxicity with pharmacological doses (3000mg/kg) of in ZnO (ZNO diet) highlighted earlier. Sales (2013) described the possibility of zinc toxicity in diets supplemented with levels above 200mg/Kg from zinc carbonate and 100 mg/Kg from zinc lactate. Burrough et al. (2019) reported hepatotoxicity in pigs fed ZnO supplemented diet up to (3000 - 6000 mg/kg).

However, the outcome of this study supports the hepatoprotective benefit of red beetroot reported in existing literature. In an *in vivo* study conducted on rats, Krajka-Kuźniak et al. (2012) attributed the hepatoprotective activity of RB to its betanin content. Betanin is responsible for inducing detoxification and expression of antioxidant enzymes (Krajka-Kuźniak et al., 2013). In another study, rats exposed to high lipid diet and purified betanin intake, da Silva et al. (2019) observed reduced hepatic MDA levels and increased SOD, CAT and GPx activity. Betanin acts through induction of antioxidant defence mechanism and free radical scavenging mechanism (Esatbeyoglu et al., 2015). They exert antioxidant effect by regulating the expression of transcription factor erythroid 2- related factor 2 (*Nrf2*) and phase II enzymes (e.g., Heme-oxygenase 1, Glutathione transferase) involved in detoxification and elimination of ROS. Betanin also activates *GPx, CAT* and *SOD* genes thus enhancing availability

of enzyme molecules to exert antioxidant activity (da Silva et al., 2019). Hence, red beetroot-betanin (RB2; 90.3 ± 18.10, RB4; 277.4 ± 79.43 in the diet used in our study could be responsible for the antioxidant effect observed in this thesis.

5.4 Impact of red beetroot on the gut health of weaned pigs

Further, the thesis assessed the impact of the diets on the gut health of the weaned pigs. Gut health involves the complex interaction between diet, gut mucosa and the gut microbial environment. At weaning, the GIT undergoes alterations in its functionality with changes ranging from increased epithelia turn over, to change in structure, microbiota composition and diversity, which can affect the gut health positively or negatively (Kim et al., 2012; Belkaid and Hand, 2014; Fouhse et al., 2016). Consequently, the gut health constitutes an important factor in the alleviation of weaning induced GIT dysfunctionality and post weaning diarrhoea. The impact of red beetroot supplemented diet on the gut health of weaned pigs was assessed with different biomarkers categorised in; gut structure, gut inflammation and immune response, and gut microbiota and metabolites, as discussed below.

5.4.1 Gut structure

The effect of diet on the small intestinal morphology have been investigated in weaned pigs, however, evidence from existing literature and this thesis suggests that diet components may contribute to the breakdown of the gut structure (van Beers-Schreurs et al., 1998; Ngoc et al., 2013). Though red beetroot supplemented diet alleviated post weaning anorexia, observations from pigs fed an increased RB inclusion (4%) red showed a reduction in jejunal and ileal measurements (VH, CD and VH:CD) (Chapter 3; Figure 3.1). Which portends a reduction in the intestinal absorptive capacity alluded to previously. Although, diarrhoea was not observed in the animal from the faecal score evaluations, the tendency for shortened intestinal absorption surface in RB4 pigs signifies a risk to the gut morphology.

The reasons behind this gut presentation in weaned pigs is not particularly clear, however, an increase in dietary fibre levels can be implied. Dietary crude fibre analyses of experimental diet used in this thesis showed high levels for RB4 (Table 6.1). Data from sugar beet fibre showed a higher presence of soluble fibre (up to 62%) than insoluble fibre (Knudsen, 1997; Fadel et al., 2000). Hedemann et al. (2006) earlier

demonstrated better intestinal morphology in pigs fed diets with high insoluble fibre than those offered diets with high soluble fibre (e.g., pectin). Accordingly, in Montagne et al. (2003), soluble fibre increases digesta viscosity and sloughing of the villus epithelium leading to villus atrophy. This resonates with changes observed in the gut morphology of pigs fed RB4 (Chapter 3, Figure 3.1).

Additionally, increased dietary soluble fibre decreases digesta retention time, exposes young pigs to diarrhoea and exacerbates pathogenic invasion of the gut (Kim et al., 2012; Heo et al., 2013). This fits into the increased abundance of phylum Proteobacteria (family; *Enterobacteriaceae*) and caecal *E. coli* in pigs fed RB4 diet (Chapter 2 and 3). Therefore, increasing red beetroot (RB) supplementation from 2% to 4% in the diet of weaned pigs may have deleterious effect on the gut morphology.

5.4.2 Gut inflammation and immune response

Gut health also entails the absence of inflammation and adequate immune response to GIT changes induced by weaning. Weaning induced alterations in the gut structure, microbial composition and functions activates the gut and systemic immune response. These changes moderate inflammation and other immune pathways by the secretion of pro and anti-inflammatory cytokines, immunoglobulins, antimicrobial peptides, tight junction proteins and mucins to regulate on-going changes in the GIT and the body system (Celi et al., 2019).

Coupled with exposure to a new diet post-weaning, the inflammatory and immune responses as well as proteins regulating gut permeability and barrier function can be severely impacted (Yin et al., 2019; Kaminsky et al., 2021). The choice of red beetroot used this study was informed by its anti-inflammatory property reported in the literature. Also, the mechanism of action of ZnO and its anti-inflammatory role in weaned pig diet has been reported in many studies (Li et al., 2010; Sargeant et al., 2010).This novel report, utilised RB in weaned pig diet hence the need to evaluate its *in-vivo* anti-inflammatory effect amidst claims of success in other animal species e.g., humans and rats (Pietrzkowski et al., 2010; El Gamal et al., 2014; Tan et al., 2015).

First, there were similarities in the gut and systemic response of the pigs (Chapter 3 and 4), diet group with increased expression of inflammatory genes in the gut tissues (RB4) had high serum inflammatory cytokines. Also, due to variation in the levels

observed within each diet group, only serum IL1B and IFNA levels were significant. This suggests the pigs had a short-lived systemic inflammation or they were in different inflammatory-recovery phase at the time of sampling. Pié et al. (2004), reported transient upregulation of inflammatory cytokines after weaning. Additionally, inflammation occurs during the first week after weaning and is expected to resolve as the animals adjust to a new diet and environment (Bian et al., 2016; Chong et al., 2018). In this study, samples were collected on day-14, hence the pigs had passed an active inflammatory phase, which could be responsible for this outcome. Again, as stated earlier, this re-emphasises the need for non-invasive methods or biomarkers for time-point and periodic evaluations of inflammation during the post-weaning period in pig production.

The faecal calprotectin is a viable and accurate non-invasive marker of gut inflammation and immunity. During gut inflammation, activated neutrophils infiltrate the gut mucosa, thereby secreting proteins that are detectable in the faeces. Faecal calprotectin levels provide insights into ongoing intestinal enterocytes changes from inflammation or dietary interventions (Fagerhol, 2000; Šplíchal et al., 2005; Alibrahim et al., 2015). As highlighted previously, the faecal calprotectin results support inflammation in RB4 pigs (Chapter 3, Figure 3.2c).

RB2 diet was closely associated with the CON diet in terms of gut (jejunum and ileum) expression of inflammatory cytokines (Chapter 3; Figure 3.2a, 3.2b). Notably, RB2 diet may have prevented exacerbation of weaning induced changes observed in the pigs fed the control diet, however, an increase in RB level - RB4 diet resulted in increased expression of pro- inflammatory cytokine (*IL1B*). The red beetroot diets may have modulated inflammation by downregulating the expression of pro-inflammatory cytokines. Baker et al. (2011) and Calvi et al. (2022) stated that red beetroot exhibits anti-inflammatory effects by interfering with pro-inflammatory signalling cascades especially the nuclear factor kappa B (NF-κB) pathway. Nevertheless, this was not dose sensitive as an increased RB inclusion from 2% to 4%, increased the expression of pro inflammatory genes (*IL1B, TNF*).

Importantly, the pigs were not challenged, the absence of a pathogenic stimulation may have presented a weak anti-inflammatory response. Many *in vitro* studies have demonstrated the anti-inflammatory effect of red beet in the presence of

lipopolysaccharides, pathogens and stimulations by inflammatory cytokines (Reddy et al., 2005; Vidal et al., 2014; Verhelst et al., 2014). While *in vitro* outcomes do not translate directly to *in vivo* effects, understanding the responses of pathogenically challenged weaned pigs to red beetroot supplemented diets is worth exploring in future red beetroot research. The anti-inflammatory response of the pigs provided a glimpse into the sites and mechanism of action for ZnO and red beet. Pro-inflammatory cytokines were highly expressed in the jejunum of ZNO pigs but resolved in the ileum. Evident from this thesis is the ability of ZnO to downregulate gut and systemic TNF levels, which could be its mechanism of mitigating intestinal inflammation in weaned pigs. Clearly, RB at 2% mediated inflammation by downregulating the secretion of pro-inflammatory cytokines in the gut and systemic circulation. Although, RB4 showed this prospect in the systemic circulation, there was increased gut and systemic IL1B depicting inflammation.

5.4.3 Gut barrier and permeability function

Tight junction (TJ) and fatty acid binding (FAB) proteins protect the gut barrier integrity, regulating the paracellular flow of ions, electrolytes, solutes and water in and out of the gut lumen. However, a breakdown in the gut barrier induced by weaning and dietary changes can impair the gut barrier function causing increased permeability. Here, the experimental diets mediated expression of the TJ and FAB proteins in response to the inflammatory cytokines. Remarkably, the trend observed aligns with previous reports of TJ modulatory actions from pro-inflammatory cytokines levels (IL1B, TNF and IFNG) during intestinal inflammation (Kaminsky et al., 2021). Previous studies have shown that, increased *IL1B* and *TNF* causes decreased gut TJ expression and consequently an increased gut permeability (Ma et al., 2004; Al-Sadi and Ma, 2007). However, it is uncertain this is the case in this study, due to comparable expression of jejunal *CLDN3* and *ZO1* in all the pigs in spite of high *IL1B* in pigs fed ZNO and RB4 diet (Chapter 3, Figure 3.4a). Moreover, there *OCLN* levels in the ileum were comparable and a high *ZO1* level with increased *IL1B* in RB4 pigs (Chapter 3, figure 3.4b) was observed.

Several inconsistencies have trailed the influence of *IL1B* on TJ permeability from past studies. Zhu et al. (2019) reported *IL1B* had no effect on CLDN proteins. Also, Haines et al. (2016) observed downregulation of *CLDN3* while *ZO1* remained unchanged,

Wang et al. (2017) revealed *IL1B* decreased *ZO1* and *OCLN* expression and Al-Sadi et al. (2008) suggested that barrier defects might also occur independently of inflammation. Hence, it is difficult to conclude if the pigs' gut permeability was compromised, due to individual variability and other related factors viz inflammatory cytokines, gut microbiota composition and antimicrobial secretions (peptides) which may have masked expression of the TJ proteins (Suzuki and Hara, 2010; Ulluwishewa et al., 2011; Xiong et al., 2019). More so, increased *IL1B* in ZNO and RB4 pigs did not induce downregulation of the gut TJ proteins.

Given that, *IL1B* increases TJ permeability via activation of the NF-kB pathway (Al-Sadi and Ma, 2007). There is the possibility of an antagonistic reaction with red beetroot downregulating pro-inflammatory signalling cascade via the NF-κB pathway and in the same pathway *IL1B* mediating a decreased expression of TJ proteins. As a result, NF-κB stimulation may be insufficient to affect an increased TJ permeability, thus the gut permeability is likely moderated or undisturbed. Data available from digesta L-lactate levels infer an undisrupted gut permeability and barrier function in ZNO and RB2 pigs due to reduced ilea L-lactate levels compared to CON and RB4 pigs. Considering that existing studies in this regard were mostly *in vitro*, current interpretation of this result is limited.

5.4.4 Gut microbiota composition and metabolites

This thesis reports on novel outcomes of red beetroot supplementation of weaned pig diet on the gut microbiota. Generally, RB2 and ZNO diets were different from the CON in modulating changes in the alpha and beta diversity of the gut microbiota. In addition, the caecal microbiota was richer and more diverse than the jejunum and ileum, while the jejunal and ilea microbiota are similar (Chapter 2, Table 2.3). CON diet mediated a significantly higher Actinobacteriota in the gut microbiota (represented by Olsenella *and Bifidobacterium*) which has been linked to lactate accumulation from increased abundance of lactic acid producing bacteria in the gut. Equally, predicted function of the gut microbiota showed upregulated pathways mediating stress, endotoxin production and inflammation. Observations from pigs fed ZNO diet in this study aligns with earlier reports of reduced jejunal and ileal species in pigs fed diet containing pharmacological levels of ZnO (Namkung et al., 2006; Shen et al., 2014; Yu et al., 2017).

The total SCFA and bile acid levels in RB2 pigs was similar to CON pigs, unlike reduced levels observed in ZNO and RB4 pigs (Chapter 2, Tables 2.2, 2.3). Which signifies the ability of RB2 diet to maintain/mediate microbiota metabolite production. Some studies have inferred correlations between the gut health and microbiome richness (Patterson et al., 2014; Singh et al., 2015), in this case, pigs fed RB2 had an improved gut health compared to other pigs.

This thesis highlights the implications of differentially abundant genera mediated by the experimental diets. RB fed pigs had an increased caecal *Anaerovibrio* abundance. *Anaerovibrio* has strong correlations with SCFA levels especially butyrate, which is an important gut microbiota metabolite recognised for its effects on the gut health (Morrison and Preston, 2016). Meanwhile, RB4 fed pigs showed differentially increased Proteobacteria (Ileum and caecum), which has been linked to stress and gut barrier disruption in weaned pigs. Again, this aligns with increased caecal *E. coli* expression (Chapter 3; Figure 3.3b) as well as upregulation of microbiota mediated pathogenic *Escherichia coli* infection and *Shigellosis* pathway (Figure 6.8c). This is in sharp contrast to the benefits afforded by RB2 diets. According to Rivera-Chávez *et al.* (2016), reduced SCFA levels lead to increase facultative anaerobic genus e.g., *Enterobacteriaceae*, which aligns with observations in RB4 pigs. Also, gut inflammation in pigs fed RB4 diet can constitute a gut microbiota shift and vice-versa according to Pickard et al. (2017), owing to these risks, red beetroot levels up to 4% in weaned pig diet is not advised.

Another major finding from this research is the increased abundance *Erysipelotrichaceae unclassified* in pigs fed of ZNO diet. There is evidence linking *Erysipelotrichaceae* to host immunogenicity and high lipid profiles (Etxeberria et al., 2015; Kaakoush, 2015), which aligns with significantly high hepatic lipid peroxidation observed in ZNO pigs (Chapter 4; Figure 4.1), and down regulated pathways mediating lipid metabolism (Chapter 2). This describes the complex role of the gut microbiota, linking the gut health to health and mediating not only the gut function but also host metabolism and homeostasis (Brown et al., 2012; Tuddenham and Sears, 2015; Nie et al., 2015). While the gut microbiota mediated functions are similar for diet ZNO and RB2, weaned pigs fed diet RB2 showed improved gut microbiota richness

and diversity, metabolite production and mediated functional pathways promoting health via stress reduction, anti-oxidation and anti-inflammation.

5.5 Red beetroot dose effect and betalain bioavailability

This research add-up to the body of evidence on the *in vivo* health benefits of red beetroot in weaned pigs. The study recounts on the ability of red beetroot supplemented diet to alleviate post weaning induced GIT changes and dysfunction. However, there was no dose effect observed on the biomarkers evaluated, which negate our expectation of proportional response with increase in RB dose. While the reasons for this is unclear, below are a few clarifications within the scope of this study and from past literature.

Red beetroot has high betalain content, recognised for its health benefits as reported in many literature. It contains a variety of phytochemicals and bioactive compounds including polyphenols (e.g., flavonoids), vitamins (ascorbic acid, vitamin B, thiamine, and niacin), carotenoids, phenols, organic acid and betanin (Kujala et al., 2002; Nemzer et al., 2011; Baião et al., 2017), exerting different or similar functions. These compounds may act synergistically with betalains influencing the overall biological effect observed. Past studies on the *in vivo* benefits of betalains (e.g., betanin) from red beetroot were conducted using commercially available whole red beetroot extract (Han et al., 2015; Dhananjayan et al., 2017). Obviously, the beneficial effect observed from these studies cannot be accrued to betalains alone. Though, it is difficult to access the biological functions of individual component in red beetroot, investigations using pure betalains are necessary for better understanding and confirmation of their exact effects (Rahimi et al., 2019).

Additionally, the red beetroot diets (RB2 and RB4) were composite, containing other dietary components (e.g., fibre) and probable anti-nutritional components (e.g., tannins, saponins and oxalates). Doubling the inclusion level of red beetroot means a direct increase in these components with resultant adverse effect (e.g., gut dysbiosis, diarrhoea and oxidative stress) earlier mentioned. Observation from *in vitro* simulated gastric digestion of betalains by Tesoriere et al. (2013), reveals the food matrix can cause degradation of red beetroot in the gastric environment, affecting host accessibility to inherent bioactive compounds. Inability to assess the bioactive ingredients in the diet will result impair availability and absorption of the compounds

(betalains). Some studies claim polyphenols are more bio-accessible than betalains, however most studies evaluated betalain bioavailability (Tesoriere et al., 2004a; Wiczkowski et al., 2018; Sawicki et al., 2020).

An important concern about the *in vivo* activity of plant containing antioxidant properties such as red beetroot, have always been the bioavailability of bioactive compound in the diet after digestion. Oral administration of betanin may lead to poor absorption from extensive pigment metabolism in the GIT wall. In addition, the bioavailability of betalains is indeed very low (< 0.01% observed in this study) and entails systemic elimination involving renal clearance (Moreno et al., 2008). According to Frank et al. (2005) measurement of bioavailable betalains after dietary intervention should include unchanged compounds and metabolites in the plasma, urine and bile. This thesis reports on betalains bioavailability in serum and liver samples, hence limited information on the presence of other compounds and metabolites that could be present.

Balance between oxidation and anti-oxidation is crucial for optimal health and a minimum level of oxidative stress is required to maintain cell integrity, superoxide and biological system. Consequently, most dietary interventions designed to address oxidative stress with increased antioxidant supplementation have failed to improve health and are usually not advised (Alía et al., 2003; Mueller et al., 2012). An increased antioxidant in the system can function as pro-oxidant, creating a level of oxidative stress (oxidants) to balance the excessive circulating antioxidants (Celi and Gabai, 2015). For instance, excessive amounts of vitamin- antioxidants and vitamin E can trigger oxidative stress, cell DNA damage and increase mortality in humans (Donnelly et al., 1999; Miller III et al., 2005). This may be responsible for declining antioxidant enzyme activity with increased antioxidants despite increased diet supplementation with antioxidant compounds (e.g., with increased red beetroot RB4).

Again, alluding to the study conducted by da Silva et al. (2019), reduced hepatic MDA and increased SOD, CAT and GPx activity in rats exposed to high lipid diets and purified (20 mg /kg) betanin for 20 days. The dose was determined from a previous experiment with 10, 20 and 40 mg/kg dose betanin, where more effectiveness and bioactivity was observed with 20 mg/kg betanin compared to 40 mg/kg dose (Dhananjayan et al., 2017).

5.6 Application and thesis contributions to zero-zinc oxide diet

The reason behind zero ZnO policy is to avert the detrimental effect (antimicrobial resistance and environmental pollution) of pharmacological doses of ZnO in weaned pig diets. The focus in pig production is to optimise the gut health, improve pig health and production. Many alternatives are under investigation to replace ZnO in weaned pig diet, towards improving the gut health. Findings from this thesis have described the health benefits of red beetroot in weaned pigs, as well as gut health benefits. Red beetroot supplementation accounted for the anti-inflammatory, anti-oxidative, gut microbiota modulation, antimicrobial and hepatoprotective benefits observed in this study. Betalains (especially betanidin; an aglycone of betanin) present in red beetroot also emerged as phytochemicals with ability to downregulate the expression of inflammatory cytokines and enhance the expression of hepatic antioxidant genes.

Along with other managerial and husbandry requirement for pig production, red beetroot is safe for use in pig nutrition to replace in-feed ZnO and for the achievement of zero ZnO diet. Present findings, support supplementation of weaned pig diet with 2% red beetroot without any adverse effect to growth and for a healthy gut. Red beetroot is commercially available for purchase in pulverized form without any restrictions or prescription. The initial setback for industrial application of red beetroot in weaned pig diet was due to the red pigment sticking or staining feed conveyors and troughs. However, at the required dose (2%) this very unlikely to happen.

5.7 Limitations of the study

- The thesis was proposed to explore the components of red beetroot *in vitro*, to assess the active compounds and dose before the feeding trial. However, due to the COVID-19 pandemic, the scope and scale of this research was curtailed.
- This study evaluated whole red beetroot and briefly assessed the bioavailability of betalains in feed, serum and liver homogenates. Other bioactive constituents and polyphenols in red beetroot were not covered in this thesis. Consequently, the study did not decipher the red beetroot constituent responsible for the effects observed.
- In addition, due to limited available data on red beetroot in pigs and especially in weaned pigs, it was difficult to compare the observations in this study to past

literature. Hence, this report serves as a novel study of the effect of red beetroot in weaned pigs.

- This thesis is a pilot study, exploring red beetroot supplementation in weaned pig diet, hence the animal feeding trial was conducted with limited number of replicates. Thus, data collected only suggests the effect of red beetroot on growth and diarrhoea incidence of weaned pigs.
- The insufficient sample numbers, could have marred the outcomes the parameters measured (e.g. extreme effect of hepatic oxidative stress and inflammation was observed in some animals in the ZNO group) and much individual variability observed.
- The colon metabolites could not be correlated with the colon microbiota due to absence of 16S rRNA data from the colon digesta. Hence gut metabolites like secondary bile acids and betalain transformation in the colon could not be verified.
- The nitrate levels in the RB diets were not quantified, hence it was difficult to correlate with increased lactate level in RB4 or phylum Proteobacteria.
- Moreover, too close inclusion levels did not allow for evaluation of likely responses from intermediate doses. The research did not consider the bio-accessibility and bioavailability of red beetroot to a large extent, hence could not infer reasons for lack of dose response in the result observed.

5.8 Future work

- Future work should, involve an *in vitro* experiment, that will enable the trial of the different (isolated and purified) constituent of red beetroot. The supplementation of weaned pig diet with pure compounds/betalains and bioactives from red beetroot will enable proper elucidation of the health benefits/ properties (antioxidative, anti-inflammatory etc.) attributed to red beetroot in weaned pigs.
- A large-scaled (complete randomized block design) feeding trial involving sufficient number of replicates (8 -12 replicate; 8 to 10 pigs per replicate) employing the most effective component of red beetroot should be considered in the future. This will allow for comparison of growth responses between diet groups and enable understanding the role of red beetroot on growth, as well as

the most essential part of the plant to concentrate on to maximise benefit for animal production.

- This study should incorporate different sampling time especially at the acute phase of post weaning changes (3 - 5 days), the adaptive phase (7-14 days) as well as the growers' phase (15 days and beyond). This will aid tracking the most effective time and duration for feeding red beetroot to weaned pigs and a clue into the likely adverse effect it may present from short and long-term consumption. Also, the metabolism, bio-accessibility and availability of the bioactive compounds (betalains and polyphenols) in red beetroot can be studied efficiently.
- Additionally, a wide range of doses should be tested before narrowing-in on most likely effective doses. Most studies have reported healthier benefits with low doses (e.g., 0.5 - 1.5%), hence future work could provide better elucidation towards decision on an effective dose.
- There is dearth of information on the in vivo inflammatory response of pigs to red beetroot. Although, this was somewhat covered in this thesis, the transient nature of post weaning inflammation and the individual variability of the data flawed adequate elucidation of this response. The anti-inflammatory effect of red beetroot and betalain compounds have been reported in studies involving pathogenic challenge and oxidative stress induced by toxicant. Similarly, future studies in weaned pig should entail comparative response in pathogenic challenge and unchallenged animals/pigs to simulate the therapeutic role of red beetroot especially in contaminated or infection laden farms.

5.9 Conclusion

Red beetroot supplementation of weaned pig diet at 2% has the potential to replace pharmacological doses of ZnO in weaned pig diet without adverse effect to health or gut health. The gut health response observed with 2% red beetroot in weaned pig diet compares with in-feed ZnO (3000 mg/kg). There is also an added benefit of an improved anti-inflammatory and antioxidant response in weaned pigs undergoing post weaning stress and GIT dysfunction. In addition, the use of red beetroot in weaned pigs will contribute to sustainability of pig production, recycling of red beetroot waste and utilisation of natural compounds to tackle antimicrobial resistance and emerging antibiotics resistant species from animal production. Finally, this thesis provides first-

hand information on the impact of red beetroot on the gut microbiota diversity and metabolite profile, gut structure integrity and barrier function, gut inflammation and immune response of weaned pigs.

Chapter 6 : **Appendix A**

Proposal to use animals for non-regulated scientific procedures at he registered premises of the University of Leeds

No. 070519HM

Section 1 Personal Details

Title and name	Professor Helen M Miller
Department	School of Biology, FBS
Ext	0113 34 32842
E-mail	H.M.Miller@leeds.ac.uk
Title of Project	The effects of red beetroot fibre on gut health of weaner pigs
Site where study to be undertaken	University of *Leeds Spen Farm,* Spen Common Lane, Bramham, Tadcaster LS24 9NS
Person/s who will be carrying out the work	Opeyemi Adekolurejo
Do you, or the person who will perform the study hold a personal licence under the Animals (Scientific Procedures) Act 1986?	Yes
If so please give details	Applicant PIL number Researcher PIL Number 60/6544

Section 2 Project Details

Description of work (include all intended manipulative procedures).

Background

Weaning is a critical period in a pig's life and is often associated with decreased feed intake, poor growth rates and diarrhoea (Funderburke and Seerley, 1990; Lecce et al., 1979). In UK commercial practice pigs undergo an abrupt change from a liquid diet to a solid diet at weaning. The digestive system is ill prepared for this change which along with a reduction in feed intake may compromise small intestine structure and function. In addition to this many other stressors are imposed upon the pig such as; removal from the sow into a new environment and mixing with unfamiliar pigs (Lecce et al., 1979). Weaner diets are therefore formulated to try and promote optimal intake and growth performance during this stressful time. Traditionally in-feed antibiotics were included in weaner diets to help improve intake and performance, however due to concerns over antibiotic-resistance the European Union banned the use of in-feed antibiotics in livestock diets in January 2006. Currently inorganic Zn in the form of ZnO is a common recommendation to reduce post-weaning diarrhoea and improve growth performance immediately post weaning, however the addition of ZnO at pharmacological levels (2,000 to 4,000 mg/kg) will be banned in the European Union from 2022 owing to environmental concerns due to the accumulation of zinc (Liu et al., 2018).

Beetroot is a good source of antioxidants as well as containing other health promoting compounds (including nitrates and soluble fibre). It is often produced as whole beetroot powder or beetroot juice-powder, creating a more convenient way for humans to consume the vegetable and its health promoting compounds (Wootton-Beard and Ryan, 2011). Beetroot fibre, a by-product of beetroot juice may be of interest to the pig industry as an inexpensive source of fibre with some antioxidant activity (Shyamala and Jamuna, 2010). Minimal levels of fibre can have a positive effect on gut health, supporting normal physiological activity, reducing the incidence of diarrhoea and consequently increasing post weaning performance and thus may be an alternative additive to ZnO.

Objectives:

To investigate the effects of beetroot fibre supplementation in weaner pig diets on post weaning performance and gut health.

Hypothesis

The addition of beetroot fibre will improve post weaning growth performance and gut health when compared to a control diet without pharmacological levels of ZnO.

MATERIALS AND METHODS

Description of the research products

During the trial we will study the impact of whole beetroot powder (20% total fibre) added to weaner pig diets at 2% and 4% level of inclusion.

Experimental design and diets

The trial is a four-treatment design. The replicate is the individual pig; there will be 12 pigs per treatment and 48 pigs in total. Pigs will be housed in pens of 6 with 2 pens per treatment generating a total of 8 treatment pens.

At weaning, pigs will be offered one of four diets: 1) Control diet, 2) Control diet + ZnO, 3) Control + 2% whole beetroot powder 4) Control + 4% whole beetroot powder. The chosen percentages were selected based on the fibre level and commonly used levels of sugar beet pulp (Wang, L.F. et al., 2016; Yan, C.L. et al., 2017b). The control (diet 1) will be prepared (by a commercial diet manufacturer) in meal form and will provide enough nutrient to satisfy minimum requirements and normal growth performance. Diet 2 will be the

same as for the control diet plus ZnO at 3,100 mg/kg. The control diet will be mixed with 2% or 4% whole beetroot powder to produce diets 3 and 4. Pigs will be fed and provided water *ad libitum*.

Individually tagged pigs will be weighed and allocated to trial at approximately 28 days of age. Pigs will remain on trial for 14 days. Pigs will be allocated to one of four treatments (as above). Within replicate each treatment will be balanced for:

•Group size (6 pigs per pen) • Group total weight •Gender profile •Sow

Growth performance

Daily pen feed intake will be measured throughout the trial for 14 days. Pigs will be individually weighed at trial start and on day 7 and day 14. Feed will be sampled weekly for analysis. Faecal scores will be assessed and recorded daily in accordance with established protocol at the trial site. Pig health will be assessed and recorded daily in accordance with established protocol at the trial site. A record will be kept of any veterinary interventions; this to include the date, reason for administration, medication used, and the identification of the individual(s) treated. Any mortality will be recorded.

At the end of the trial (day 14/15) all pigs will be euthanised by schedule 1 procedure. Pigs will be stunned using captive-bolt and then exsanguinated in accordance with the Animals (Scientific Procedures) Act 1986. Immediately following death, blood samples will be collected (from both portal and peripheral circulations into heparinized tubes). The gastrointestinal tract will be removed, and the pH of each section recorded. Mucosa samples will be collected for microbiome analysis. Tissue samples from the liver, kidney, spleen, lung and heart will also be collected and snap frozen.

Give details of animal housing and husbandry procedures which apply to your work. E.g., does the cage/pen space allocation (and other husbandry procedures) comply with the appropriate Home Office Code of Practice for the species and number of animals to be used?

The study will be conducted at Spen Farm, University of Leeds Farms Ltd., Leeds, for 14 days. The pigs will be kept under commercial conditions on fully slatted floor pens (6 pigs per pen) throughout the trial with 4 pens per room. Each pen measures 135 cm x 135 cm. Heating and ventilation will always be controlled reducing temperature over time from 30°C at weaning to 26°C at day14. Each pen is equipped with an open trough and 2 nipple drinkers. Food and water will be provided *ad libitum* throughout the trial.

Has confirmation been obtained from the Home Office that the above protocol, as described, would not be considered to include any regulated procedure that would require it to be licensed under the Animals (Scientific Procedures) Act 1986.

~~YES/~~NO delete as appropriate

Date confirmation received from the Home Office	
Name of individual from whom has confirmation been received	
Signed	
Date	

The Animal Welfare and Ethical Review Committee has considered the proposal

Entitled: **The effects of red beetroot fibre on gut health of weaner pigs**

Submitted by: **Professor Helen M. Miller**

and accepts that, as written, it describes work which is not regulated under the Animals (Scientific Procedures) Act 1986 (as amended).

This work may commence subject to the understanding that:

1. Before any further changes are made to the protocol as written such changes must, in the first instance, be discussed with the NACWO and Named Veterinary Surgeon.

2. The Animal Welfare and Ethical Review Committee will require the work to be stopped should it become known that changes have been made to the protocol without prior discussion.

3. Any adverse signs shown by any animal on the study, irrespective of whether the study is considered to be or not to be the cause, must be reported to the NACWO and Named Veterinary Surgeon.

4. No procedures other than husbandry procedures normal for the species may be applied.

Signed .....S.K. Abbas..... Date .....7 May 2019.....

NVS & NTCO

Signed..... Date.....

Named Animal Care and Welfare Officer

Signed..... Date.....

Applicant

Appendix B

Table 6.1: Composition of experimental diets

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 raw whole meal	15.57	15.57	15.26	14.95
Wheat meal bulk	12.50	12.50	12.25	12.00
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 powder	0.00	0.00	2.00	4.00
Soya oil	3.40	3.40	3.33	3.26
Calculated nutrient composition				
Dry Matter (%)	89.93	89.65	89.47	89.01
Oil B (%)	6.55	6.55	6.41	6.28
Crude Protein (%)	22.12	22.12	21.92	21.71
Crude Fibre (%)	2.22	2.22	2.58	2.93
Ash (%)	0.85	0.85	0.83	0.82
Calcium (%)	0.71	0.71	0.69	0.68

Phosphorus (%)	0.45	0.45	0.44	0.43
Sodium (%)	0.20	0.20	0.20	0.19
Copper (mg/kg)	0.46	0.46	0.45	0.44
Selenium (mg/kg)	12500.00	12500.00	12250.00	12000.00
Vitamin A (IU/kg)	2000.00	2000.00	1960.00	1920.00
Vitamin D3 (IU/kg)	300.00	300.00	294.00	288.00
Vitamin E (IU/kg)	1.50	1.50	1.47	1.44
Total Lysine (%)	1.35	1.35	1.32	1.30
SID Lysine (%)	0.58	0.58	0.57	0.56
Total Methionine (%)	0.54	0.54	0.53	0.52
SID Methionine (%)	10.16	10.16	9.95	9.75
NE Piglet (MJ/kg)	10.19	10.19	10.19	10.19
Analysed nutrient Composition				
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	187

SID; standardised ileal digestibility

Table 6.1b: Growth performance response and faecal score of weaned pigs

Parameters	**CON**	**ZNO**	**RB2**	**RB4**	**SEM**	***F***	**P value**
Initial BW (kg)	7.90	7.64	7.69	8.02	0.186	0.021	0.883
Final BW (kg)	9.11	8.67	9.37	9.59	0.259	0.57	0.640
Weight gain (kg)	1.21	1.04	1.67	1.57	0.214	0.48	0.700
ADFI (kg/day)	0.184	0.171	0.251	0.257	0.016	4.08	0.104
ADG (kg/day)	0.086	0.074	0.119	0.112	0.016	0.49	0.684
FCR	2.14	2.31	2.11	2.29	0.089	0.42	0.747
Faecal score							
Day 0	2.5	3.5	2.0	2.0	0.354	0.42	0.108
Day 7	2.5	2.0	2.5	2.5	0.125	1.05	0.804
Day 14	2.0	2.0	2.0	2.0	0.000	0.73	1.000

Values presented in mean, SEM- standard error of mean, CON, ZNO, RB2 and RB4 represents; control diet, diet supplemented with zinc oxide, 2% and 4% red beetroot respectively. ADFI; Average daily feed intake, ADG; Average daily gain, FCR; Feed conversion ratio.

Table 6.2a: Short chain fatty acid levels (mM) from experimental diet groups

SCFA/ gut locations	Diets				SEM	*P* value		
	CON	ZNO	RB2	RB4		L	D	L*D
Acetate								
Plasma	1.59	1.48	1.86	1.37	0.148			
Jejunum	6.27	6.34	9.66	5.92	0.524			
Ileum	7.58	6.23	7.56	6.63	0.407			
Caecum	14.86	14.83	15.17	13.93	0.682			
Colon	14.97	12.62	11.12	13.95	0.608			
Faeces	23.87	13.26	18.44	14.84	1.618			
Total Acetate	**69.14^{a}**	**54.76^{b}**	**63.81^{ab}**	**56.64^{b}**	**3.317**	**< 0.01**	**< 0.05**	**> 0.05**
Propionate								
Plasma	0.38	0.38	0.43	0.24	0.087			
Jejunum	1.13	1.32	1.63	1.16	0.077			
Ileum	1.57	1.52	1.74	1.58	0.050			
Caecum	6.57	4.96	5.56	5.75	0.317			
Colon	6.02	4.16	4.01	5.43	0.288			
Faeces	8.20	4.69	6.96	5.00	0.598			
Total Propionate	**23.83^{a}**	**17.04^{b}**	**20.34^{ab}**	**19.16^{b}**	**1.421**	**< 0.05**	**< 0.01**	**< 0.01**
Butyrate								
Plasma	0.00	0.00	0.00	0.00	0.000			
Jejunum	0.00	0.21	0.00	0.00	0.052			
Ileum	0.07	0.16	0.46	0.24	0.060			
Caecum	2.86	2.22	2.50	2.28	0.140			
Colon	2.68	1.75	1.77	2.34	0.171			
Faeces	3.87	2.43	3.35	2.24	0.290			
Total Butyrate	**9.48^{a}**	**6.76^{b}**	**8.08^{ab}**	**7.09^{b}**	**0.610**	**< 0.05**	**< 0.05**	**> 0.05**
Isobutyrate								
Plasma	0.00	0.00	0.00	0.00	0.000			
Jejunum	0.00	0.00	0.00	0.00	0.000			
Ileum	0.00	0.00	0.05	0.00	0.014			
Caecum	0.29	0.16	0.35	0.08	0.040			
Colon	0.55	0.57	0.33	0.35	0.056			
Faeces	1.75	0.88	1.06	0.43	0.242			
Total Isobutyrate	**2.59^{a}**	**1.60^{b}**	**1.79^{b}**	**0.86^{c}**	**0.355**	**< 0.02**	**< 0.02**	**< 0.02**
Isovalerate								
Plasma	0.00	0.00	0.00	0.00	0.000			
Jejunum	0.00	0.00	0.00	0.00	0.000			
Ileum	0.00	0.00	0.00	0.00	0.000			
Caecum	0.03	0.02	0.05	0.00	0.010			
Colon	0.03	0.02	0.01	0.01	0.008			
Faeces	1.05	0.68	0.61	0.43	0.149			
Total Isovalerate	**1.11^{a}**	**0.71^{b}**	**0.67^{b}**	**0.44^{c}**	**0.139**	**< 0.02**	**< 0.02**	**< 0.05**
Valerate								
Plasma	0.02	0.01	0.02	0.00	0.005			
Jejunum	0.00	0.00	0.00	0.00	0.000			
Ileum	0.00	0.00	0.00	0.00	0.000			
Caecum	0.35	0.18	0.08	0.09	0.036			
Colon	0.34	0.13	0.07	0.22	0.040			
Faeces	0.86	0.48	0.42	0.32	0.091			
Total valerate	**1.55^{a}**	**0.82^{ab}**	**0.58^{b}**	**0.63^{b}**	**0.225**	**< 0.02**	**< 0.02**	**< 0.05**
Overall total SCFA	**107.70^{a}**	**81.69^{b}**	**95.28^{a}**	**84.82^{b}**	**5.876**	**< 0.05**	**< 0.05**	**< 0.05**

Data expressed in mean for each diet and location; significant differences indicated by different letters along the column for the location and across the row for diets. Statistics computed in R using GLM for normalized data and negative binomial - link function for non-parametric and zero inflated data. CON, ZNO, RB2 and RB4 represents control diet, diets supplemented with zinc oxide, 2% and 4% red beetroot, respectively. [d] overall mean for each location per SCFA, [e] *p- value* for effect of location, [e] *p- value* for significant effect of diets, [g] *p- value* interaction between location and diet.

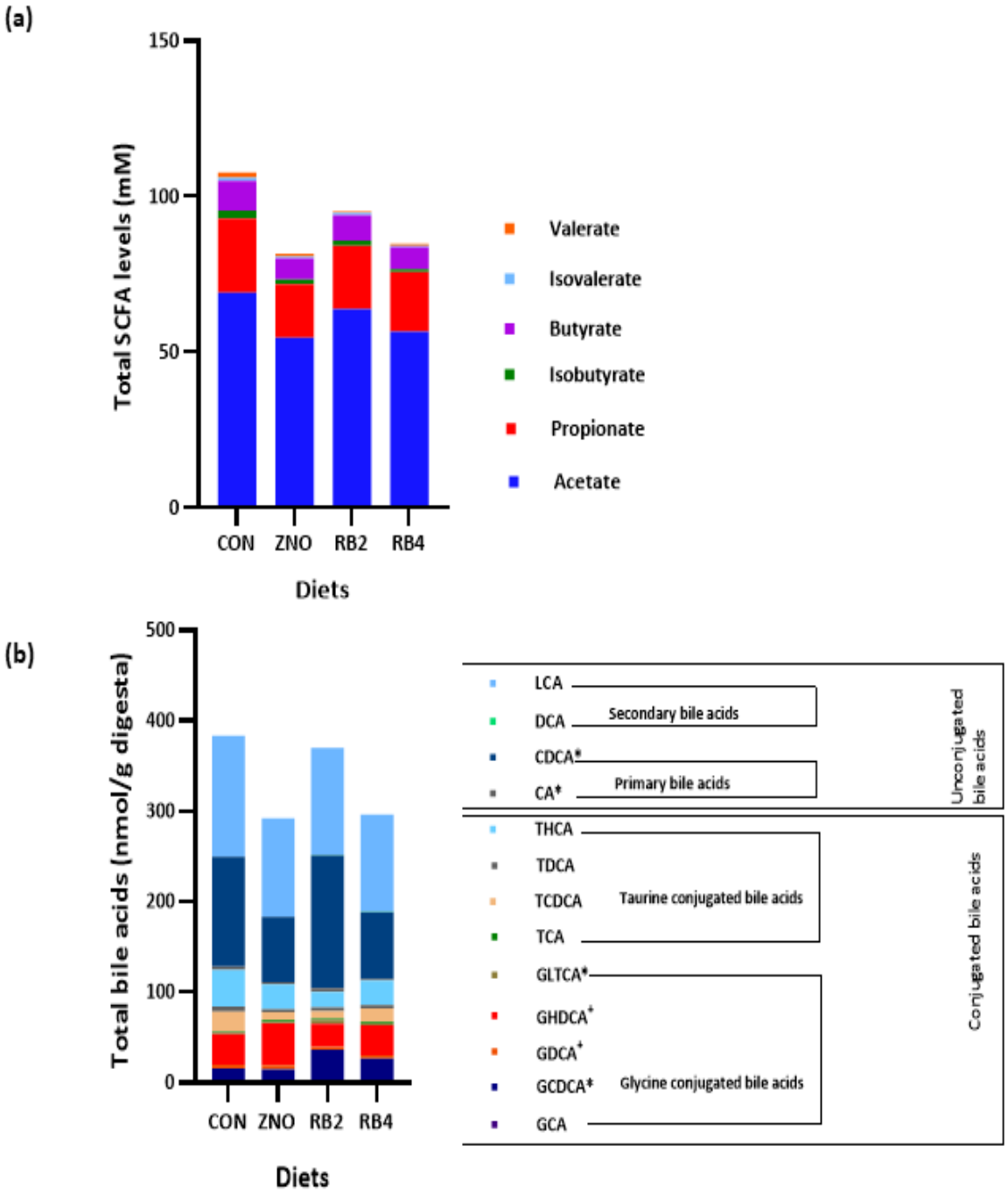


Figure 6.2: Gut microbiota metabolite profile of weaned pigs (a) total short chain fatty acids (b) total bile acid in weaned pigs fed the experimental diets. (*, +) represents bile acid significantly higher ($P < 0.001$) in RB2 and ZNO fed pigs, respectively. THCA, taurohyodeoxycholic acid; GHDCA, glycohyodeoxycholic acid; TCA, taurocholic acid; GCA, glycocholic acid; TCDCA, taurochenodeoxycholic acid; TDCA, taurodeoxycholic acid; GCDCA, glycochenodeoxycholic acid; GDCA, glycodeoxycholic acid; CA, cholic acid; GLTHCA, glycolithocholic acid; CDCA, chenodeoxycholic acid; DCA, deoxycholic acid and LCA, lithocholic acid. CON, ZNO, RB2 and RB4 represents; control, zinc oxide, 2% and 4% red beetroot supplemented diets, respectively.

Table 6.3a: Bile acid levels (nmol/g digesta) from diet groups and different locations evaluated

Bile acids/total	Diets				Location					Statistics	*P* value		
	CON	ZNO	RB2	RB4	Jejunum	Ileum	Caecum	Colon	Faeces		[e]L	[f]D	[g]L*D
THCA	40.74[a]	27.29[b]	16.88[c]	26.86[b]	64.91[a]	39.50[b]	2.63[c]	2.38[c]	2.35[c]		0.01	0.01	0.75
GHDCA	33.78[b]	46.08[a]	25.76[c]	33.45[b]	80.67[a]	56.71[b]	0.58[c]	0.69[c]	0.40[c]	ZNO * Jejunum (W = 2.03, P =0.02) RB2* ileum (Z = -1.97, P = 0.05)	0.001	0.05	0.62
TCA	1.50[b]	1.56[b]	1.96[a]	1.30[b]	1.49[b]	2.07[a]	0.72[c]	1.09[c]	0.96[c]		0.001	0.001	0.001
GCA	0.40b	0.47b	0.45b	1.37a	1.02[a]	0.97[a]	0.00[c]	0.09[c]	0.60[b]	ZNO * Colon (Z = 0.00, P =1.00), RB4 *faeces (Z = -0.656, P = 0.511)	0.05	0.05	0.05
TCDCA	21.90[a]	8.89[c]	8.30[c]	15.09[b]	33.91[a]	16.25[b]	0.99[c]	1.07[c]	1.95[c]		0.001	0.001	0.001
TDCA	5.11[a]	3.42[b]	4.07[ab]	3.90[b]	5.61[a]	2.51[b]	2.48[b]	2.60[b]	3.30[b]	ZNO *Jejunum (t = -2.30, P =0.02). ZNO vs Jejunum (t value = -2.55, P = 0.01) RB2 *Ile. (W = 1.60, P= 0.04)	0.02	0.05	0.05
GCDCA	15.42[c]	13.88[c]	35.91[a]	25.40[b]	46.83[a]	31.78[a]	4.04[c]	4.49[c]	3.47[c]	RB2*Jejunum (Z = 2.19, P = 0.03).	0.001	0.001	0.25
GDCA	3.57[b]	4.91[a]	2.95[c]	3.26[b]	3.79[a]	1.79[c]	2.51[b]	2.77[b]	3.84[a]		0.001	0.001	0.05
CA	3.80[b]	2.59[c]	4.23[a]	1.63[d]	3.85[a]	1.47[b]	1.69[b]	1.49[b]	3.75[a]		0.001	0.001	0.05
GLTCA	2.11[ab]	1.99[b]	3.60[a]	1.96[b]	1.56[c]	1.31[c]	0.97[d]	2.19[b]	3.62[a]	RB2* Faeces levels (Z = 2.910, P = 0.003).	0.042	0.001	0.18
CDCA	121.16[b]	72.28[c]	147.09[a]	74.32[c]	358.91[a]	44.68[b]	2.82[d]	4.80[c]	3.63[c]		0.001	0.001	.001
DCA	0.10[b]	0.14[b]	0.31[a]	0.12[b]	0.00[c]	0.00[c]	0.00[c]	0.09[b]	0.58[a]		0.002	0.05	0.001
LCA	134.25[a]	108.90[b]	118.20[b]	107.66[b]	10.18[e]	15.55[d]	50.37[c]	132.09[b]	260.81[a]		0.001	0.05	0.05
Total bile acid/diet	**383.82[a]**	**292.391[b]**	**369.71[a]**	**296.33[b]**						RB2 (Z = -1.70, P = 0.09), ZNO (Z =-1.99, P = 0.05), RB4 (Z = -2.34, P = 0.02)	0.001	0.05	0.001

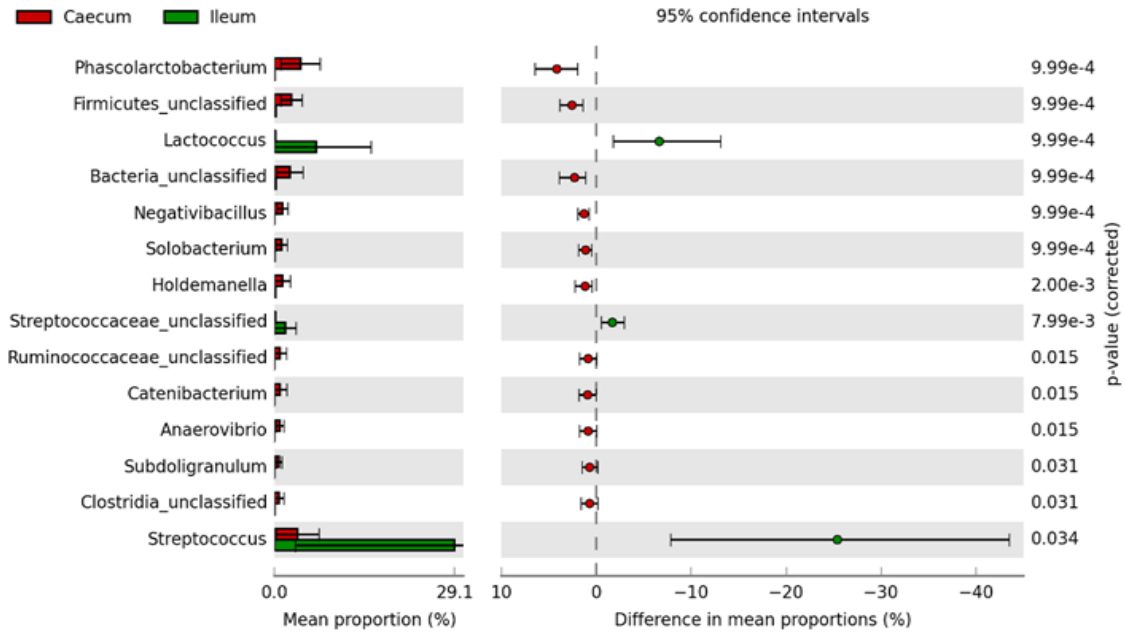
Data expressed in mean for each diet and location; significant differences indicated by superscripts along the column within each diet group and location evaluated. Statistics computed in R using GLM for normalized data and negative binomial - link function for non-parametric and zero inflated data. CON, ZNO, RB2 and RB4 represents control diet, diets supplemented with zinc oxide, 2% and 4% red beetroot, respectively. [e]*p - value for effect of location,* [f] *p- value for significant effect of diets,* [g] *p- value interaction between gut 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), glycolithocholic acid (GLTHCA), chenodeoxycholic acid (CDCA), deoxycholic acid (DCA) and lithocholic acid*.

Table 6.4: Alpha diversity measure in pig gut locations from the different diet groups

Indices/ Gut locations	Diets					*P* value		
	CON	ZNO	RB2	RB4	[e]SEM	[f]L	[g]D	[h]L*D
Chao 1 Index						< 0.001	0.01	0.12
Jejunum	1552.62[b]	1271.76[b]	2198.48[a]	1470.22[b]	100.66			
Ileum	1399.89[b]	1278.95[b]	1530.21[b]	1186.38[b]	72.21			
Caecum	2108.29[a]	2228.54[a]	2253.18[a]	2089.51[a]	68.70			
Shannon Index						< 0.001	0.07	0.08
Jejunum	2.44[a]	2.71[b]	2.55[b]	2.66[a]	0.10			
Ileum	2.84[a]	2.69[b]	2.82[b]	2.56[a]	0.10			
Caecum	2.92[a]	4.11[a]	3.67[a]	2.83[a]	0.18			
Simpson Index						0.58	0.16	0.18
Jejunum	0.73	0.76	0.76	0.77	0.02			
Ileum	0.81	0.78	0.79	0.73	0.02			
Caecum	0.70	0.89	0.85	0.71	0.03			

Data presented as mean of species richness and or evenness for each diet and gut location evaluated. Means with different letters along the columns are not significantly different. Diets: CON, ZNO, RB2, and RB4 represents; the control diet, diet supplemented with zinc oxide, 2% and 4% red beetroot supplemented diets respectively. [d] *overall mean for each gut location (jejunum, ileum and caecum) and all diet-mean (CON, ZNO, RB2 RB4),* [e] *Standard error of mean for each gut location and all diets,* [f] *P value for gut location,* [g] *P value for Diets,* [h] *P value of interaction between gut location and diets.*

CON (a)



CON (b)

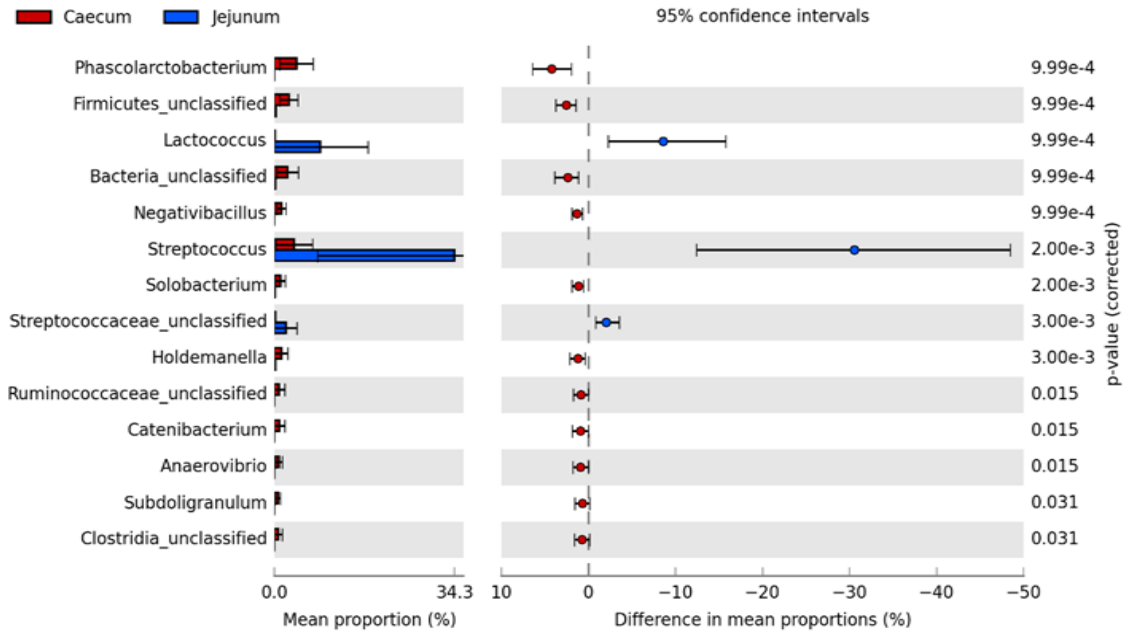
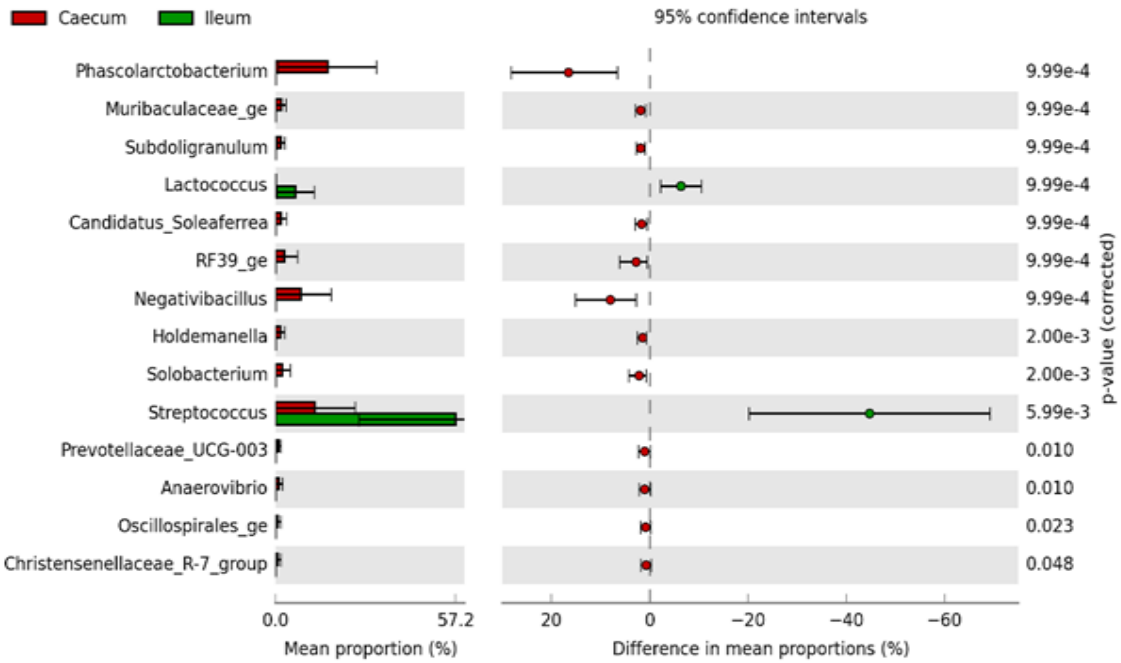


Figure 6.1: Differentially increased genera with CON diet between gut locations CON (a) caecum vs ileum; CON (b) caecum vs jejunum.

ZNO (a)



ZNO (b)

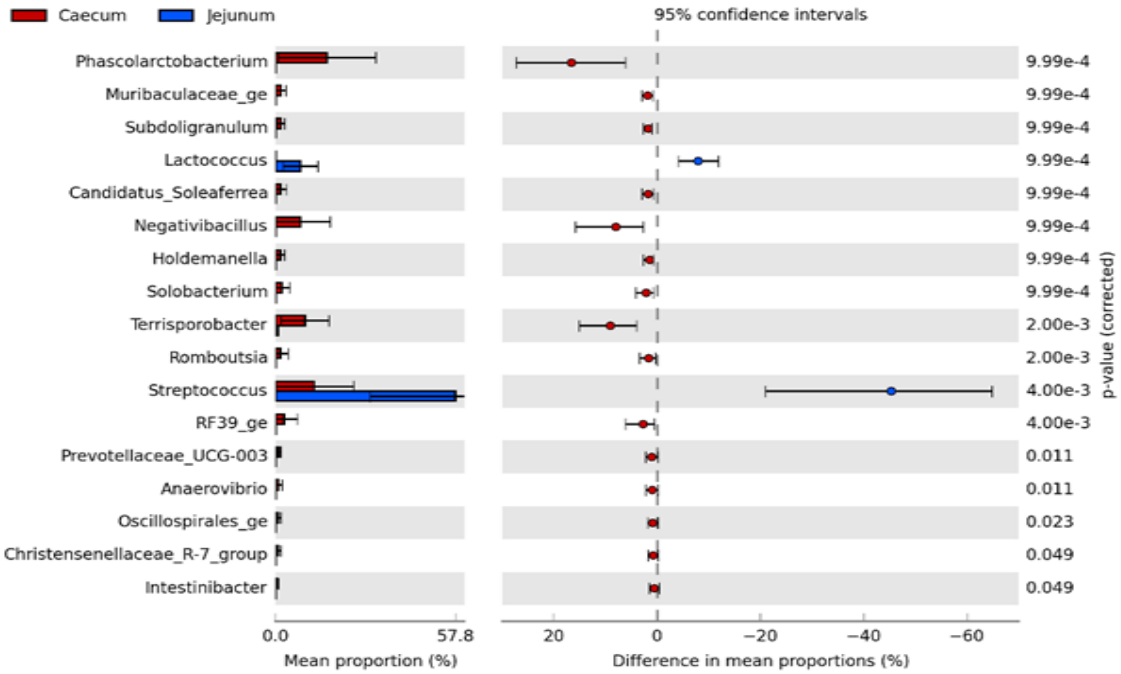
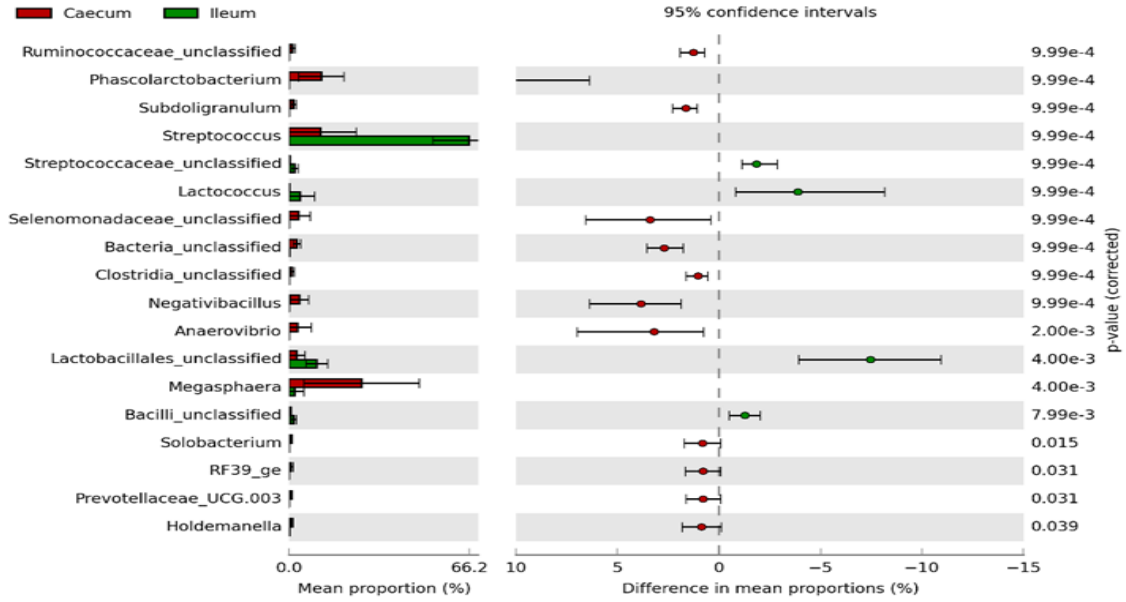
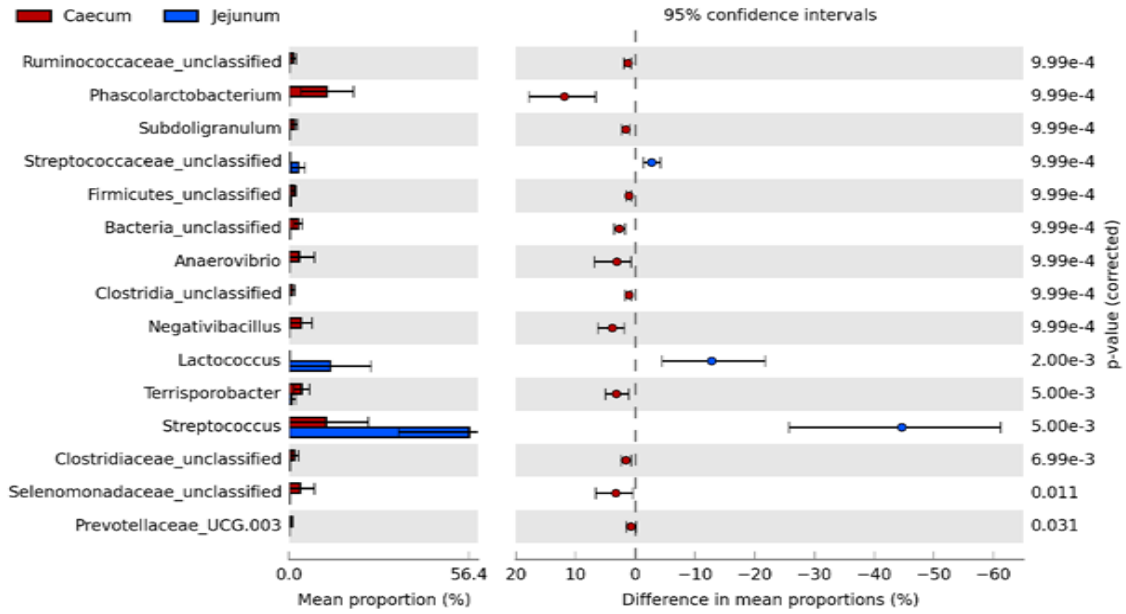


Figure 6.2: Differentially increased genera with ZNO diet between the gut locations ZNO (a) caecum vs ileum; ZNO (b) caecum vs jejunum.

RB2 (a)



RB2 (b)



RB2(c)

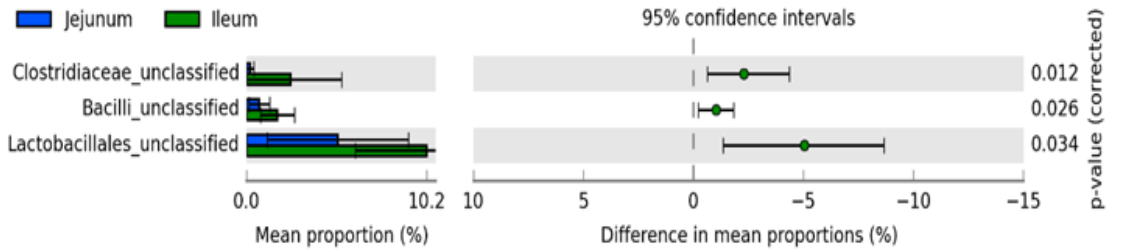
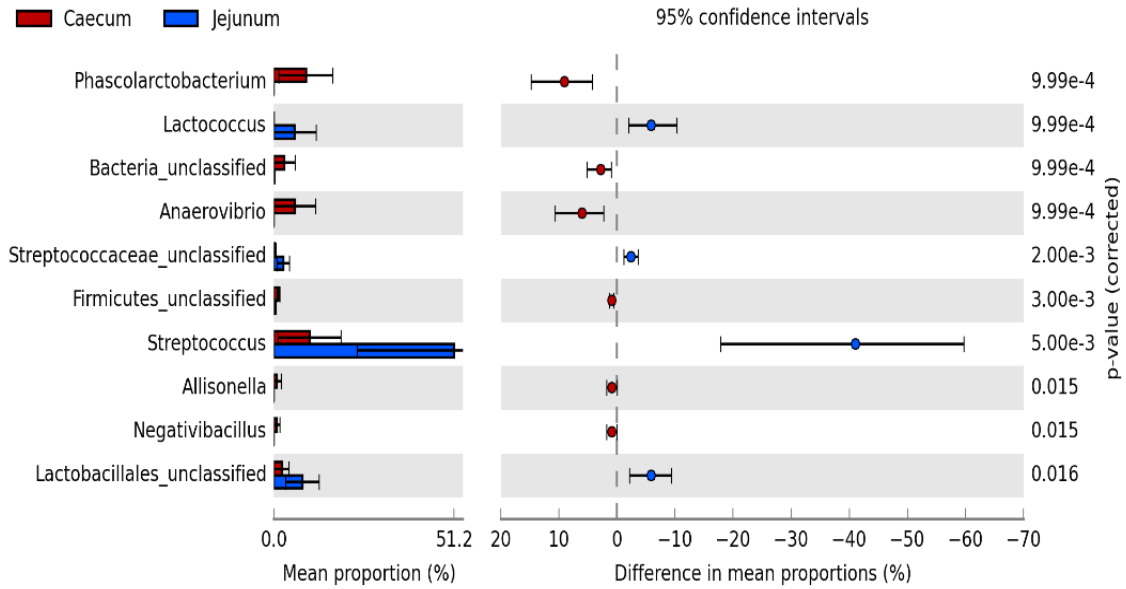
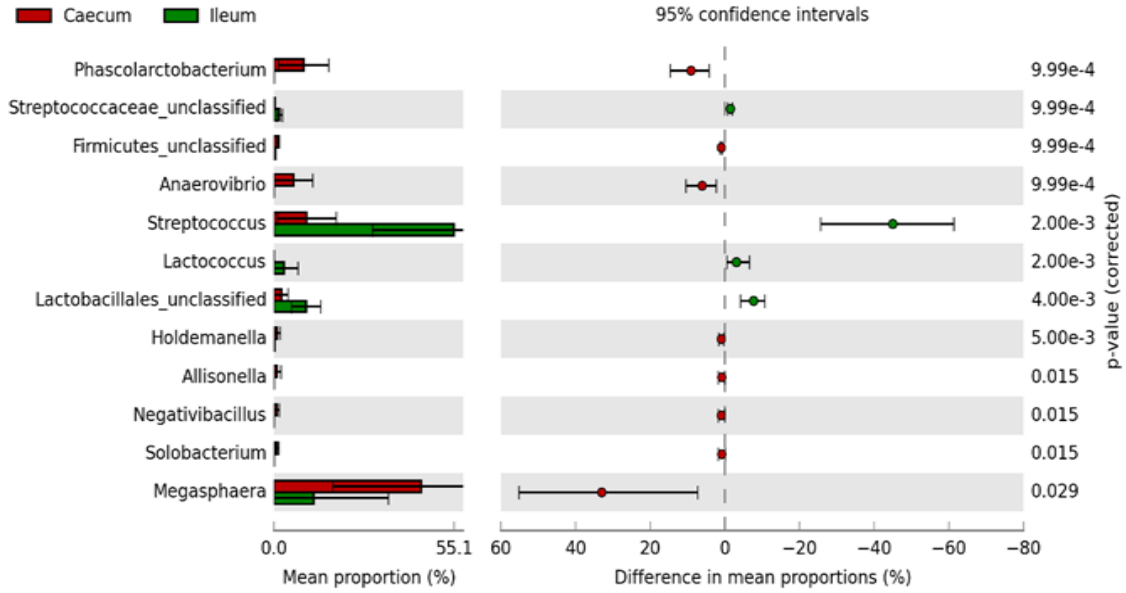


Figure 6.3: Differentially increased genera with RB2 between gut locations RB2 (a) caecum versus ileum; (b) caecum versus jejunum (c) jejunum versus ileum.

RB4 (a)



RB4 (b)



RB4(c)

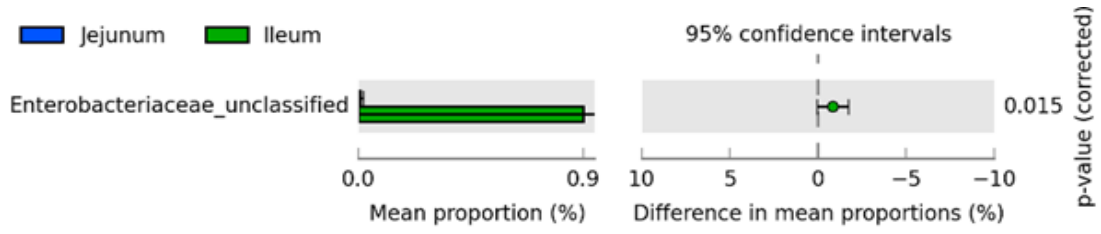


Figure 6.4: Differentially increased genera with RB4 diet between gut locations RB4 (a) caecum versus ileum; (b) caecum versus jejunum (c) jejunum versus ileum.

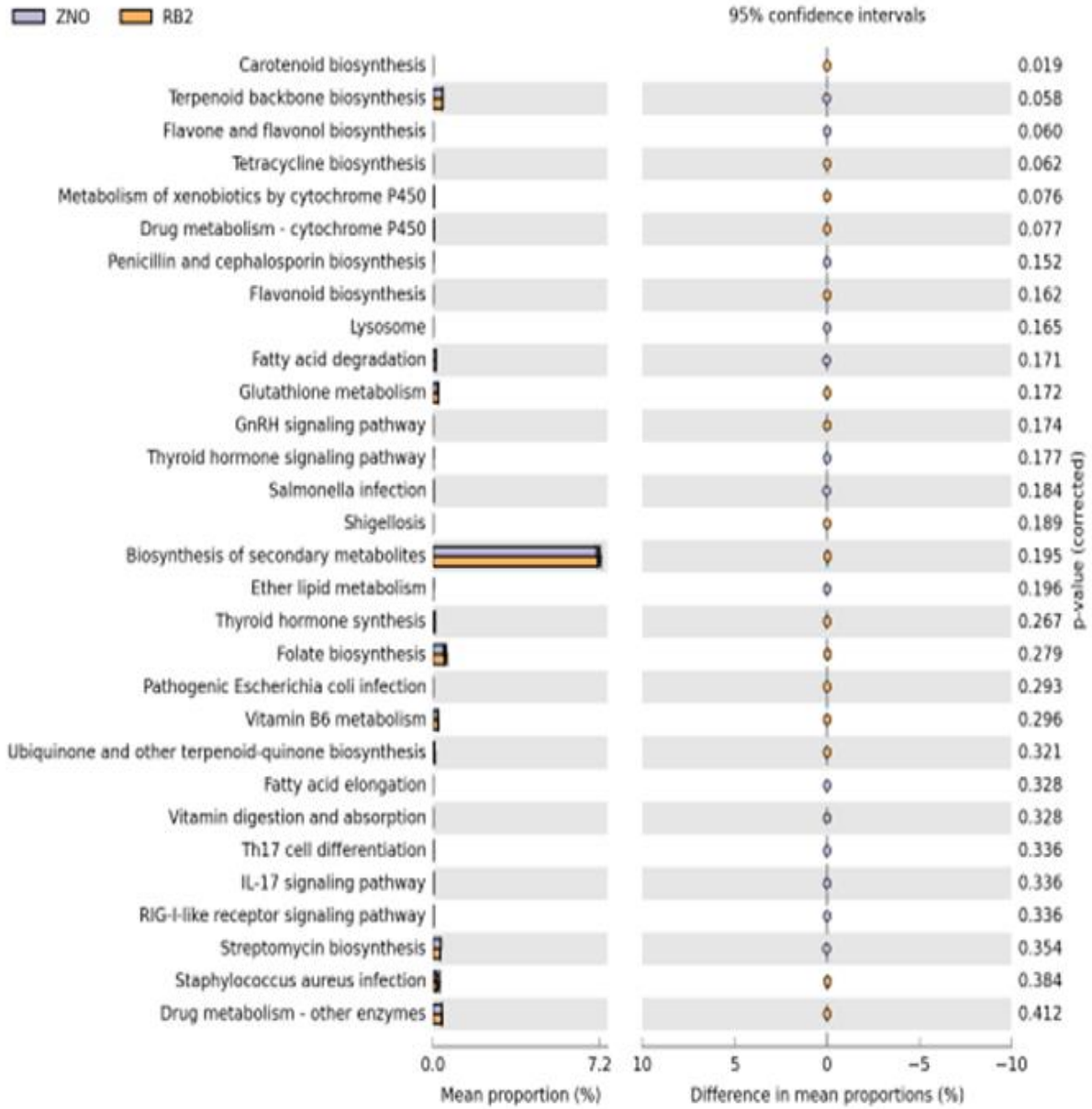


Figure 6.5: Comparison of predicted gut microbial functions in pigs fed ZNO and RB2 diet. No significant difference (P > 0.05) observed between functional pathways influenced by the diets.

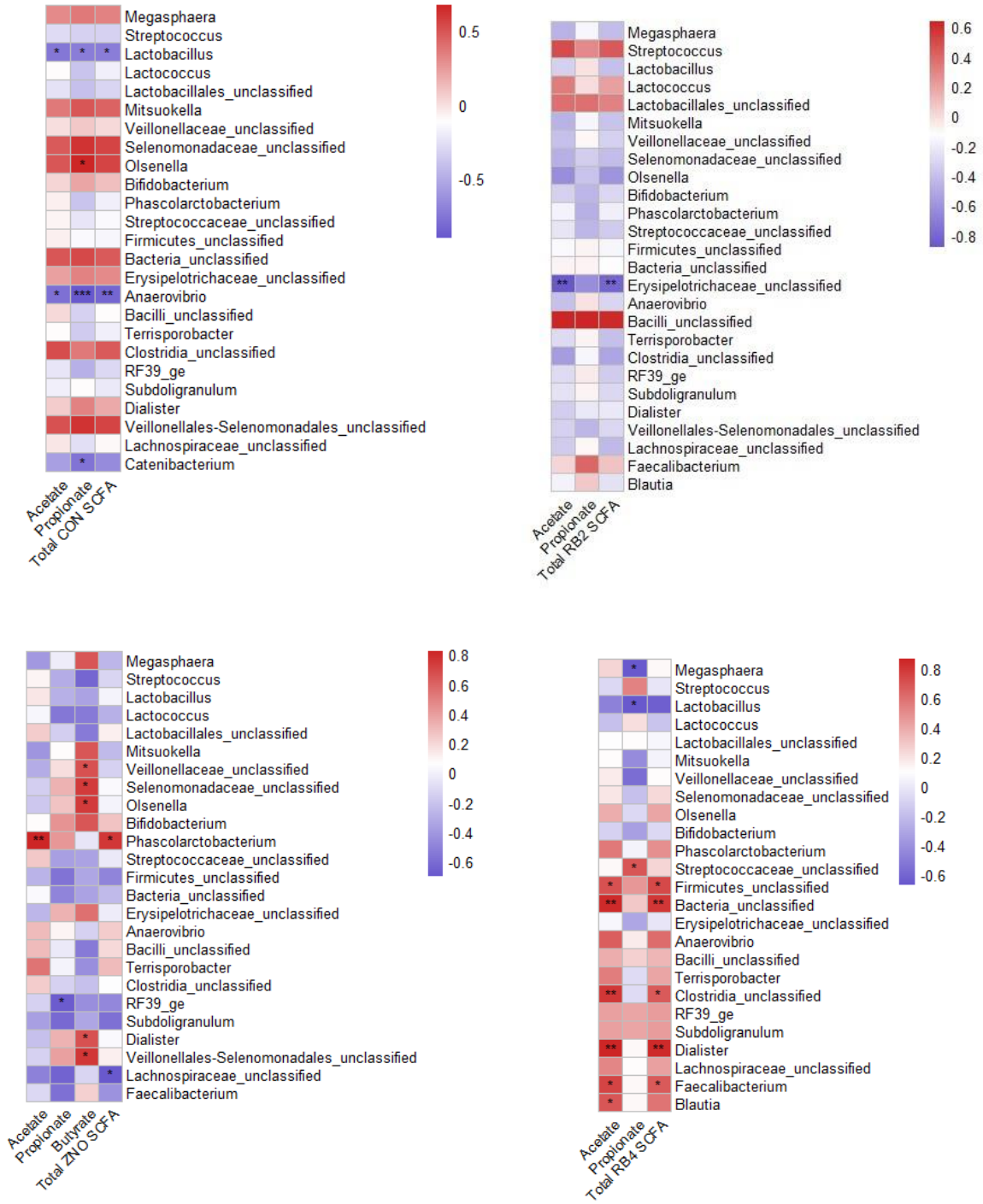


Figure 6.6a: Spearman correlation analyses between top abundant bacteria genera and jejunal short chain fatty acids. Fatty acids omitted were not detected in the corresponding location hence not shown, color depth depicts correlation between genera and gut metabolite where red color denotes a positive correlation and blue color a negative correlation. The strength of association between the subjects is indicated by the color intensity, *** P ≤ 0.001, **P ≤ 0.01, *P ≤ 0.05). CON, ZNO, RB2 and RB4 represent control diet and diets supplemented with zinc oxide, 2% and 4% red beetroot respectively.

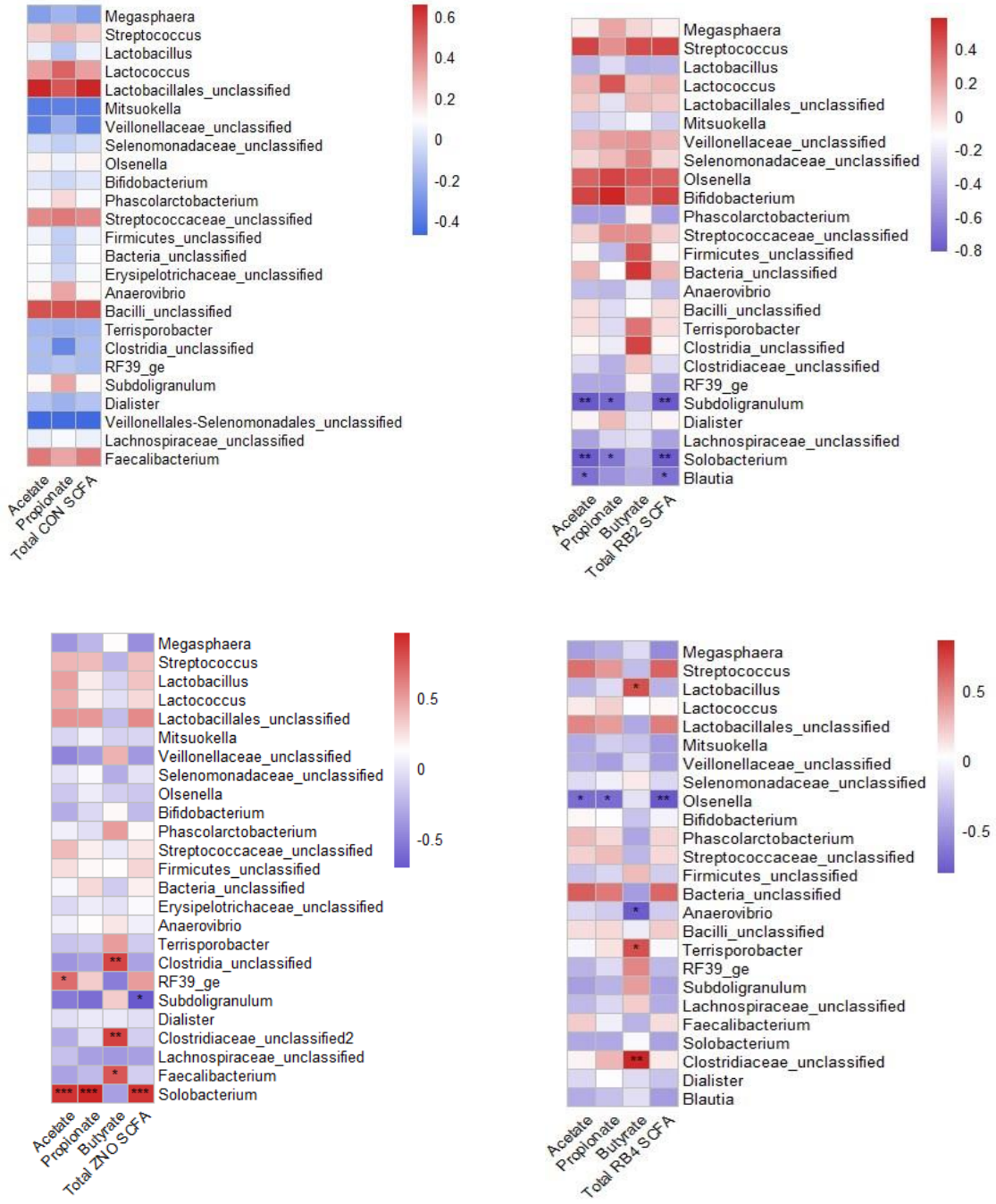


Figure 6.6b: Spearman correlation analyses between top abundant bacteria genera and ileal short chain fatty acids. Fatty acids omitted fatty acids were undetected in the corresponding location hence not shown, colour depth depicts correlation between genera and gut metabolite where red colour denotes a positive correlation and blue colour a negative correlation. The strength of association between the subjects is indicated by the colour intensity, *** P ≤ 0.001, **P ≤ 0.01, *P ≤ 0.05). CON, ZNO, RB2 and RB4 represent control diet and diets supplemented with zinc oxide, 2% and 4% red beetroot respectively.

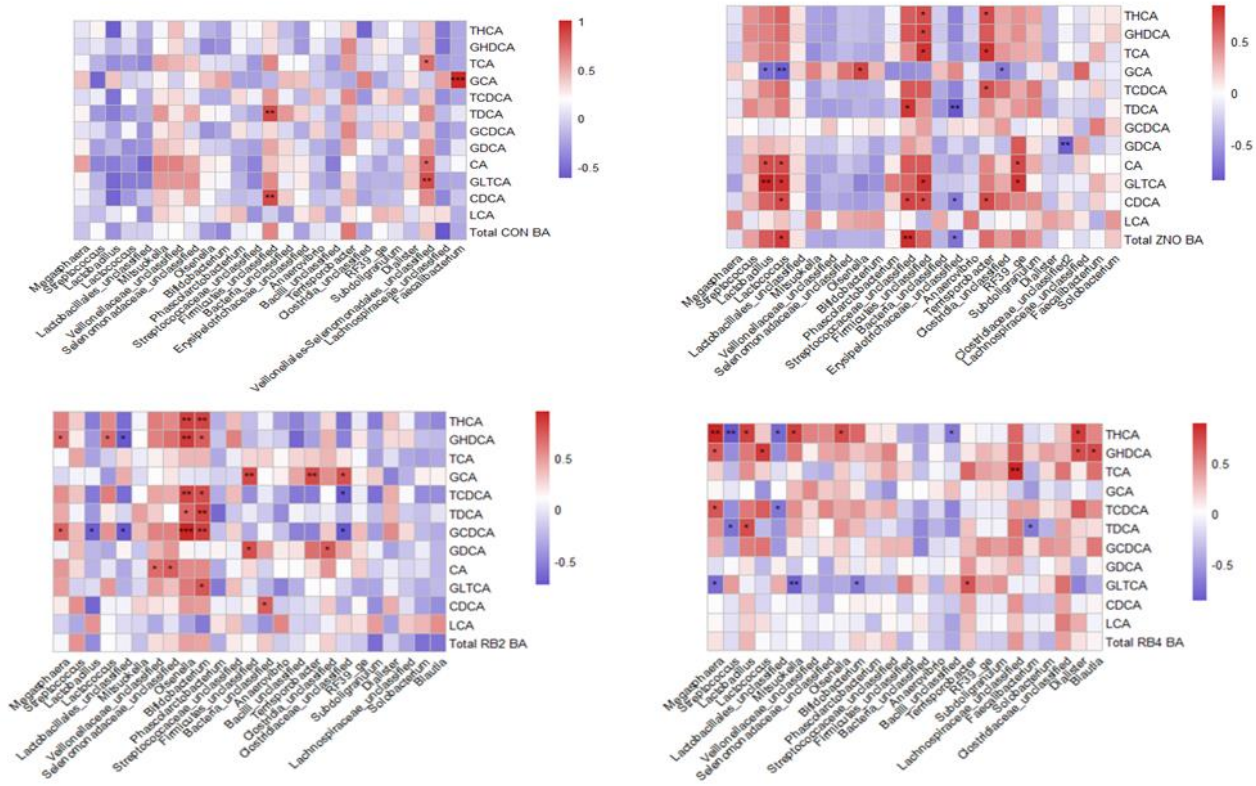


Figure 6.7a: Spearman correlation matrices between ilea genera abundance and bile acid levels. Omitted bile acid (deoxycholic acid - DCA) was not detected in the jejunum for the pigs hence not shown. Correlation depicted by color depth, where red color denotes a positive and blue color a negative correlation. The strength of association between the subjects is indicated by the color intensity and *** P ≤ 0.001, **P ≤ 0.01, *P ≤ 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.

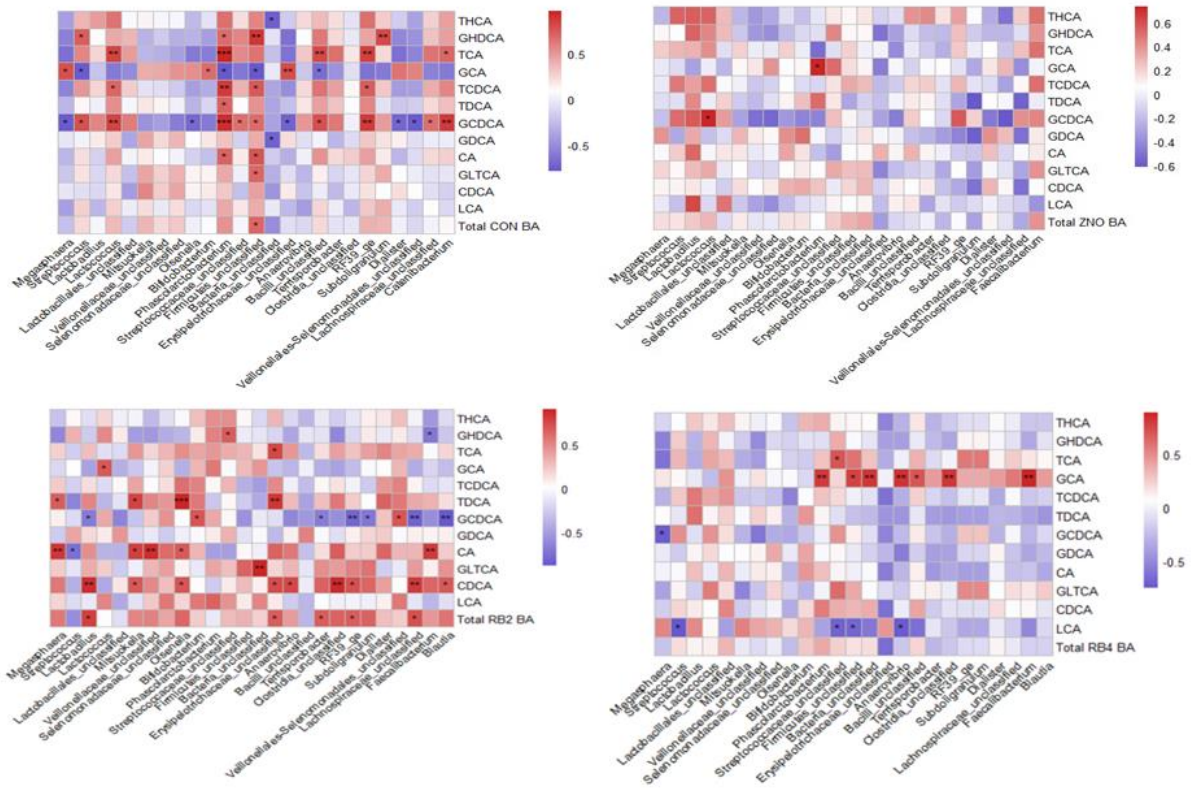


Figure 6.7b: Spearman correlation matrices between jejunal genera abundance and bile acid levels. Bile acid (deoxycholic acid - DCA) omitted was not detected in the jejunum for the pigs hence not shown. Correlation depicted by color depth, where red color denotes a positive and blue color a negative correlation. The strength of association between the subjects is indicated by the color intensity and *** P ≤ 0.001, **P ≤ 0.01, *P ≤ 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.

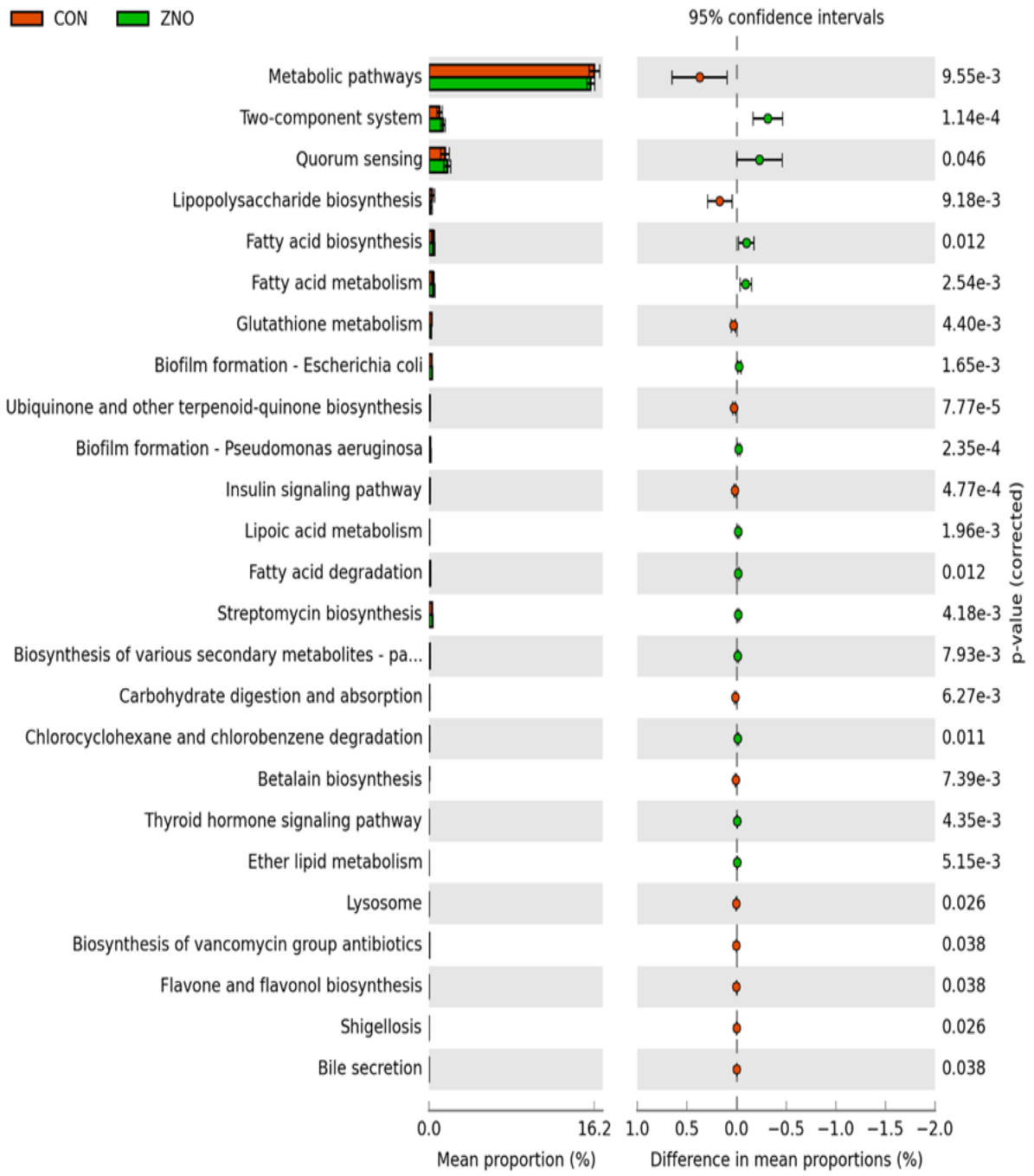


Figure 6.8a: Predicted gut microbiota functions of weaned pig, comparisons between the diet CON and ZNO.

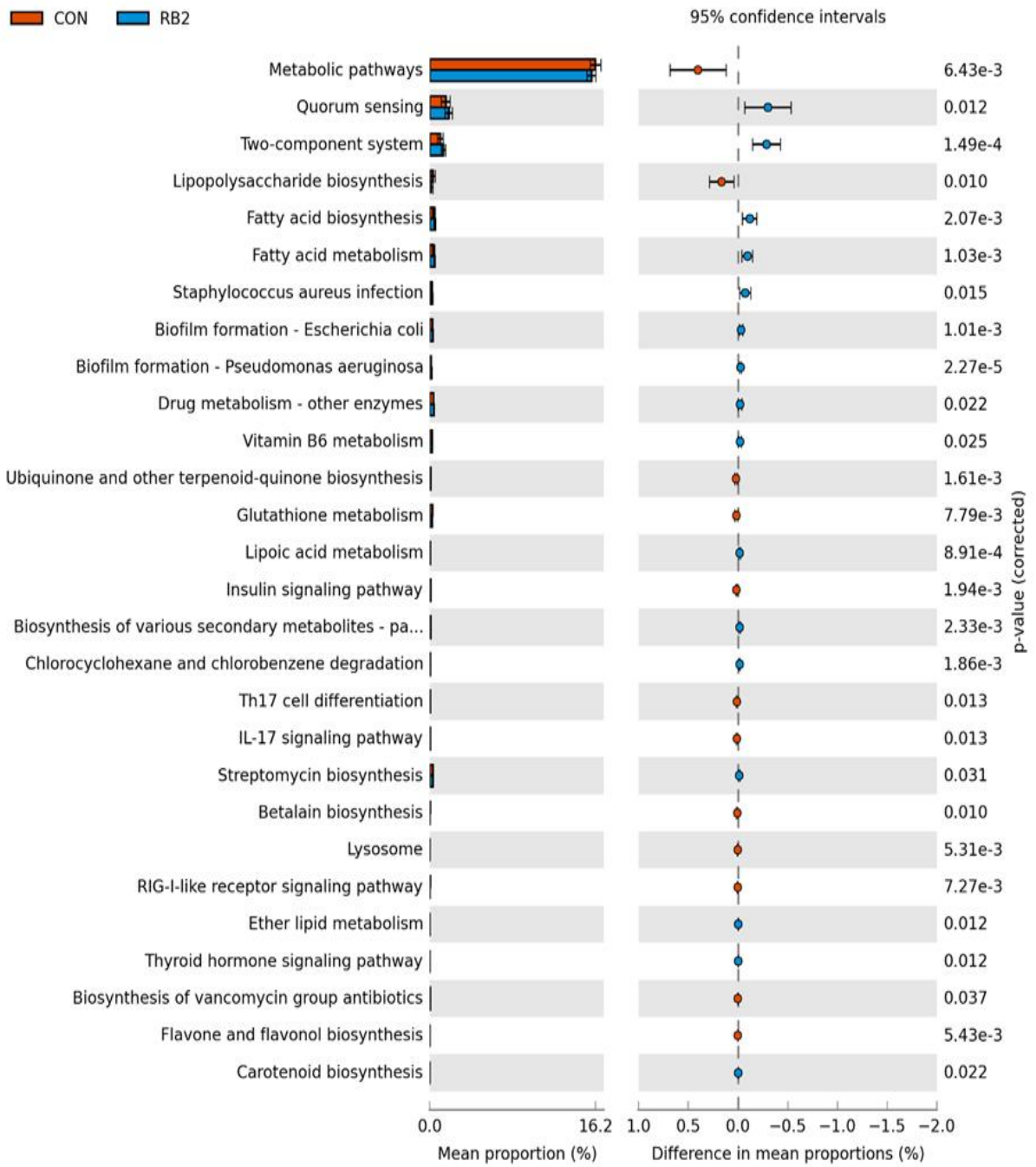


Figure 6.8b: Predicted gut microbiota functions of weaned pig, comparisons between the diet CON and RB2.

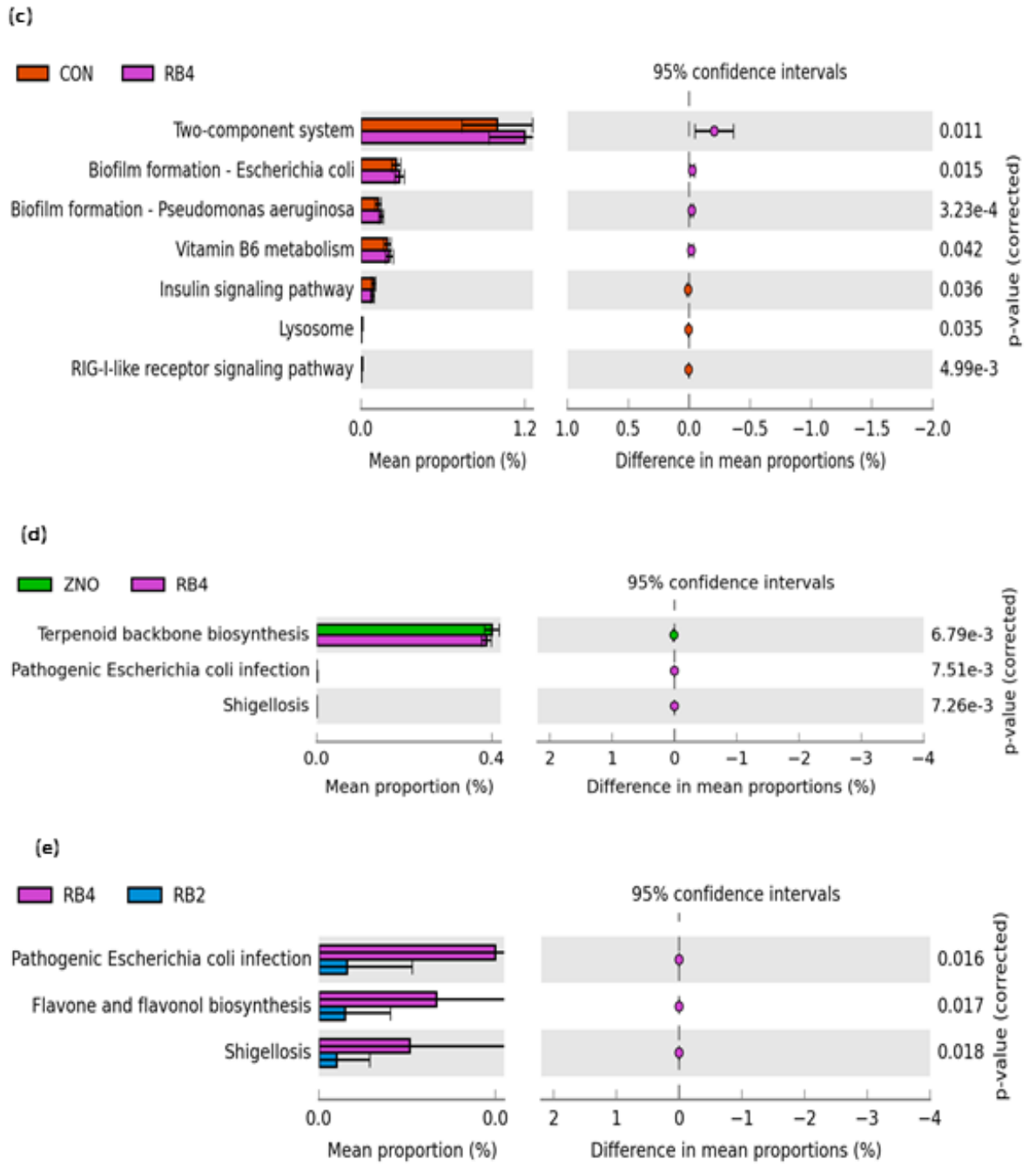
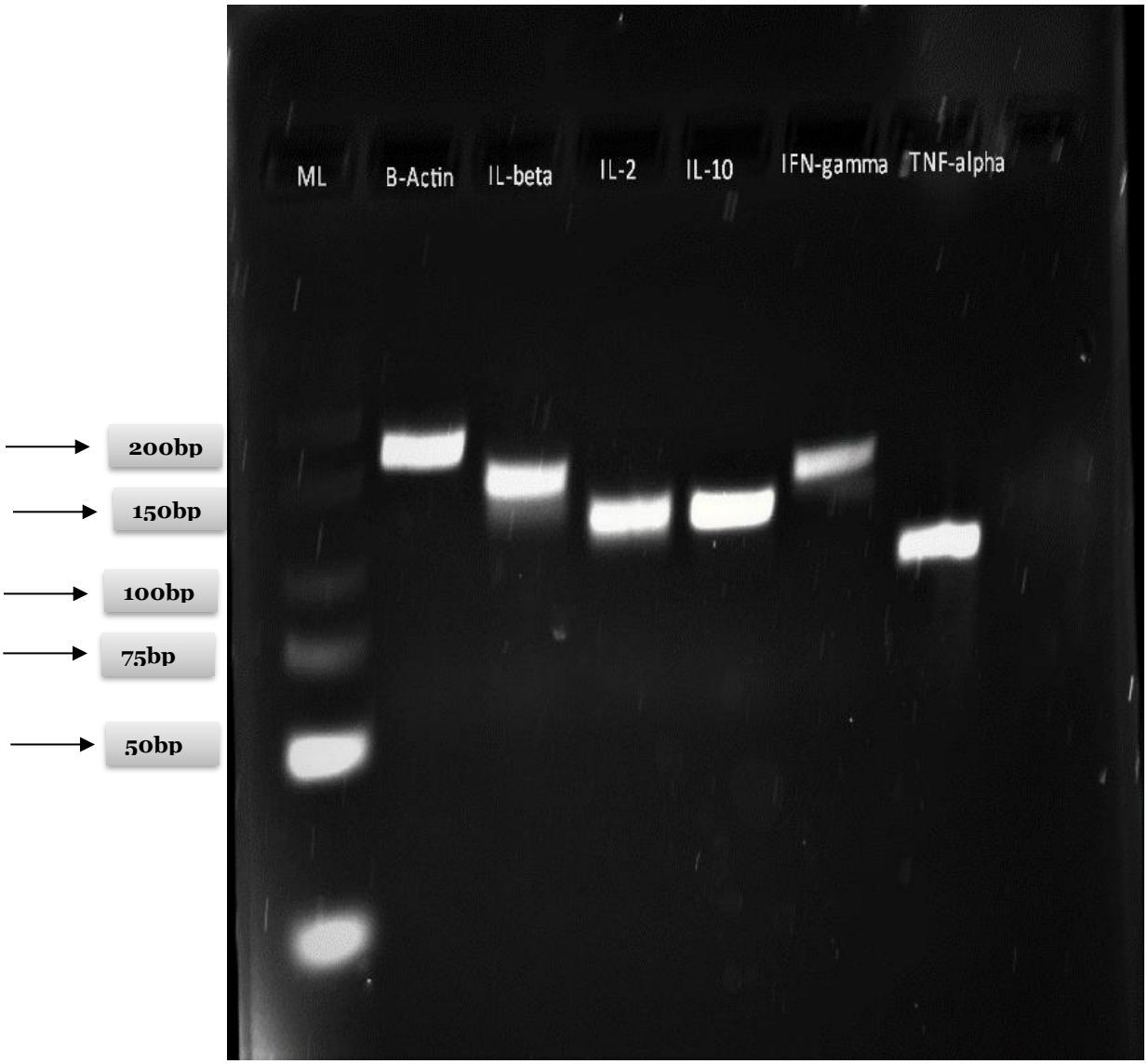
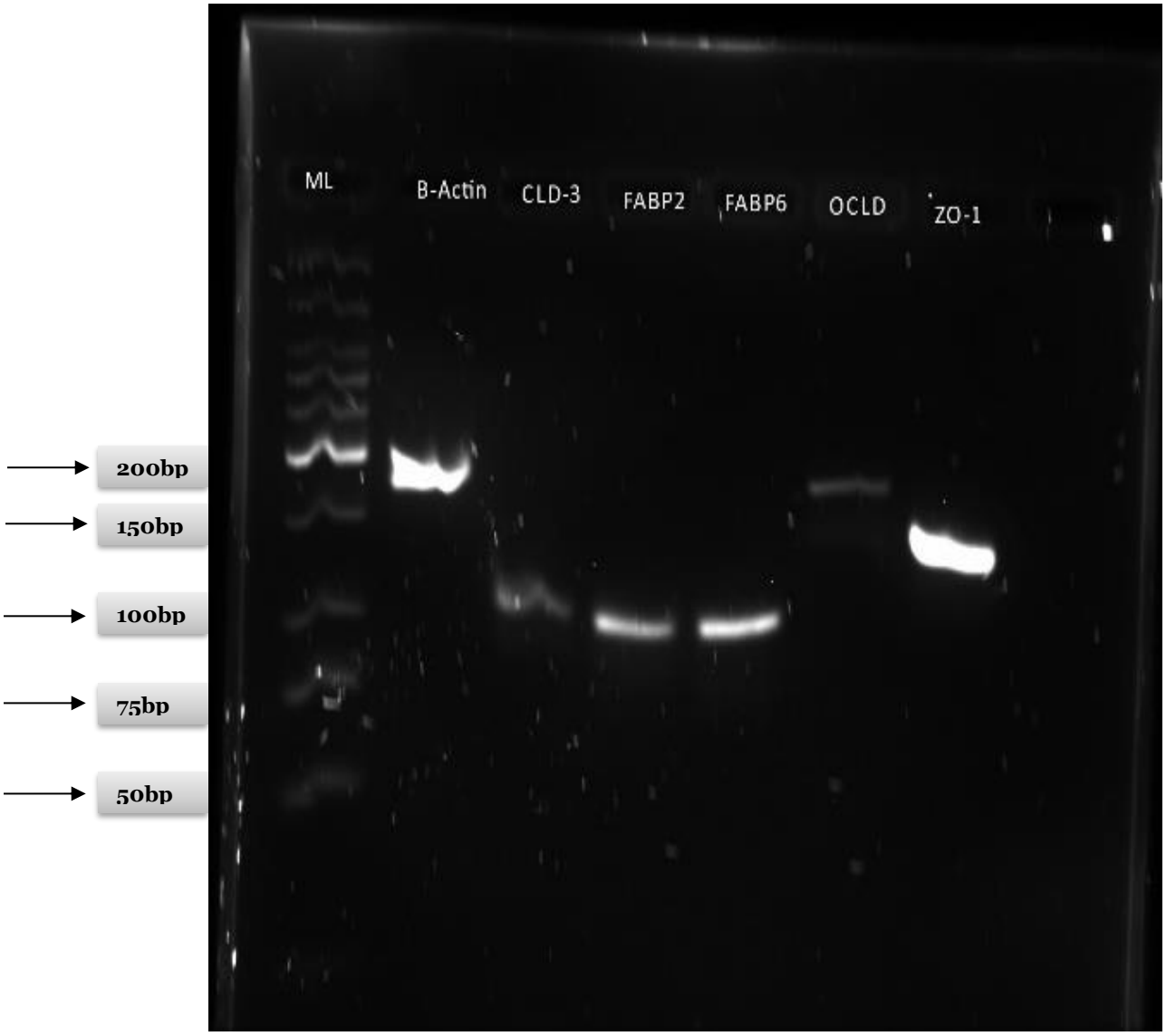


Figure 6.8 c, d and e: Predicted gut microbiota functions of weaned pig, comparisons between the diet CON and RB4, ZNO and RB4, RB4 and RB2.

Appendix C


ML
B-Actin
IL-beta
IL-2
IL-10
IFN-gamma
TNF-alpha
200bp
150bp
100bp
75bp
50bp


ML
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ZO-1
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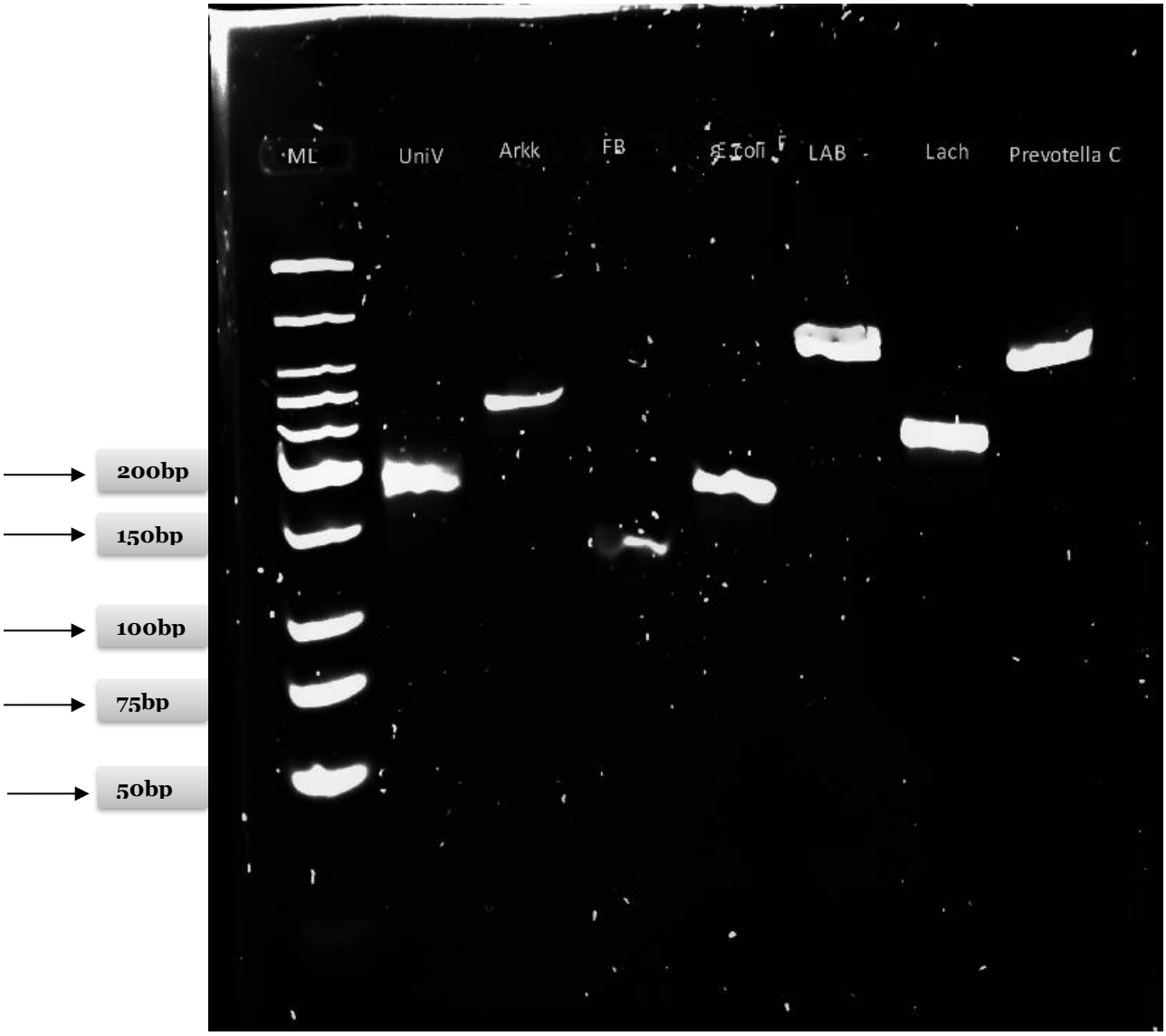

Figure 6.9: Agarose gel electrophoresis of qPCR product.

ML; (Molecular ladder), β-Actin; (ACTB), IL1β; (Interleukin 1B), IL2; (Interleukin 2), IL10; (Interleukin 10), TNF-α; (Tissue necrosis factor), IFN-ɣ;- (INFG), FABP2; FABP6 - (Fatty acid binding protein 2 and 6), CLDN3; (Claudin 3), OCLN; (Occludin), ZO-1; (Zona occludens/ tight junction protein 1), Univ; (Universal Primer), Arkk; (Akkermansia muciniphila), FB; (Faecalibacterium prausnitzii), LAB; (Lactobacillus spp), Lach; (Lachnospiraceae spp).

Table 6.5: Correlation analyses between betalain compounds and antioxidant biomarkers in pigs fed red beetroot diets

Correlation analyses	Serum isobetanin	Serum betanidin	Plasma MDA	Plasma GSH	Hepatic CAT	Hepatic SOD	Hepatic GPX1a
Serum betanin							
Correlation coefficient				-0.71			
Sig.				0.05			
Serum isobetanin							
Correlation coefficient			0.73	-0.82	-0.84	- 0.87	
Sig.			0.04	0.01	0.01	0.01	
Serum betanidin							
Correlation coefficient	0.72						
Sig.	0.04						
Serum isobetanidin							
Correlation coefficient		0.72					
Sig.		0.04					
Liver betanidin							
Correlation coefficient							0.73
Sig.							0.05

CAT- catalase, *SOD* - superoxide dismutase, *GPX1a* – Glutathione peroxidase, GSH – Glutathione, MDA - malondialdehyde.

List of References

Aarestrup, F.M. 2005. Veterinary drug usage and antimicrobial resistance in bacteria of animal origin. *Basic Clincal Pharmacology Toxicology.* **96**(4), pp.271-281.

Abdi, K. 2002. IL-12: the role of p40 versus p75. *Scandinavian Journal of Immunology.* **56**(1), pp.1-11.

Achilonu, M., Shale, K., Arthur, G., Naidoo, K. and Mbatha, M. 2018. Phytochemical benefits of agroresidues as alternative nutritive dietary resource for pig and poultry farming. *Journal of Chemistry.* (2018), p15.

Akan, S., Tuna Gunes, N. and Erkan, M. 2021. Red beetroot: Health benefits, production techniques, and quality maintaining for food industry. *Journal of Food Processing Preservation* **45**(10), p15781.

Akerboom, T.P. and Sies, H. 1981. [48] Assay of glutathione, glutathione disulfide, and glutathione mixed disulfides in biological samples. *Methods in Enzymology.* Elsevier, pp.373-382.

Al-Sadi, R., Boivin, M., Ma, T.J.F. 2009. Mechanism of cytokine modulation of epithelial tight junction barrier. *Frontiers in Bioscience* **14**, p2765.

Al-Sadi, R., Khatib, K., Guo, S., Ye, D., Youssef, M. and Ma, T. 2011. Occludin regulates macromolecule flux across the intestinal epithelial tight junction barrier. *American Journal of Physiology-Gastrointestinal Liver Physiology* **300**(6), pp.G1054-G1064.

Al-Sadi, R., Ye, D., Dokladny, K. and Ma, T.Y. 2008. Mechanism of IL-1β-induced increase in intestinal epithelial tight junction permeability. *Journal of Immunology.* **180**(8), pp.5653-5661.

Al-Sadi, R.M. and Ma, T.Y. 2007. IL-1β causes an increase in intestinal epithelial tight junction permeability. *Journal of Immunology* **178**(7), pp.4641-4649.

Alexa, P., Stouracova, K., Hamrik, J. and Rychlik, I. 2001. Gene typing of the colonisation factors K88 (F4) in enterotoxigenic Escherichia coli strains isolated from diarrhoeic piglets. *Veterinarni Medicina.* **46**(2), pp.46-49.

Alía, M., Horcajo, C., Bravo, L. and Goya, L. 2003. Effect of grape antioxidant dietary fiber on the total antioxidant capacity and the activity of liver antioxidant enzymes in rats. *Nutrition Research* **23**(9), pp.1251-1267.

Alibrahim, B., Aljasser, M.I. and Salh, B. 2015. Fecal calprotectin use in inflammatory bowel disease and beyond: a mini-review. *Canadian Journal of Gastroenterology Hepatology.* **29**(3), pp.157-163.

Alkadi, H. 2020. A review on free radicals and antioxidants. *Infectious Disorders-Drug Targets* **20**(1), pp.16-26.

Almeida, V., Nuñez, A., Schinckel, A., Alvarenga, P., Castelini, F., Silva-Guillen, Y. and Thomaz, M. 2017. Interactive effect of dietary protein and dried citrus pulp levels on growth performance, small intestinal morphology, and hindgut fermentation of weanling pigs. *Journal of Animal Science.* **95**(1), pp.257-269.

Alou, M.T., Lagier, J.C. and Raoult, D. 2016. Diet influence on the gut microbiota and dysbiosis related to nutritional disorders. *Human Microbiome Journal* **1**, pp.3-11.

Amachawadi, R., Scott, H., Aperce, C., Vinasco, J., Drouillard, J. and Nagaraja, T. 2015. Effects of in-feed copper and tylosin supplementations on copper and antimicrobial resistance in faecal enterococci of feedlot cattle. *Applied Microbiology.* **118**(6), pp.1287-1297.

Amarakoon, A.M.S.B.K. 2017. *Regulation of oxidative stress in weaned piglets challenged with Escherichia coli*. thesis.

Amezcua, R., Friendship, R.M., Dewey, C.E., Gyles, C. and Fairbrother, J.M. 2002. Presentation of postweaning Escherichia coli diarrhea in southern Ontario, prevalence of hemolytic E-coli serogroups involved, and their antimicrobial resistance patterns. *Canadian Journal of Veterinary Research.* **66**(2), pp.73-78.

Anadón, A. 2006. WS14 The EU ban of antibiotics as feed additives (2006): alternatives and consumer safety. *Veterinary Pharmacology Therapeutics.* **29**, pp.41-44.

Anguita, M., Gasa, J., Nofrarias, M., Martín-Orúe, S. and Pérez, J. 2007. Effect of coarse ground corn, sugar beet pulp and wheat bran on the voluntary intake and physicochemical characteristics of digesta of growing pigs. *Livestock Science.* **107**(2-3), pp.182-191.

Ao, W., Cheng, M., Chen, Y., Sun, J., Zhang, C., Zhao, X., Liu, M. and Zhou, B. 2022. Fermented Apple Pomace Improves Plasma Biochemical and Antioxidant Indicators and Fecal Microbiota of Weaned Pigs. *Agriculture.* **12**(10), p1603.

Apajalahti, J. and Vienola, K. 2016. Interaction between chicken intestinal microbiota and protein digestion. *Animal Feed Science Technology.* **221**, pp.323-330.

Apak, R.a. 2019. Current issues in antioxidant measurement. *Journal of Agricultural Food Chemistry* .**67**(33), pp.9187-9202.

Argüello, H., Estellé, J., Leonard, F.C., Crispie, F., Cotter, P.D., O'sullivan, O., Lynch, H., Walia, K., Duffy, G. and Lawlor, P.G. 2019. Influence of the intestinal microbiota on colonization resistance to Salmonella and the shedding pattern of naturally exposed pigs. *Msystems.* **4**(2), pp.e00021-00019.

Arjin, C., Souphannavong, C., Sartsook, A., Seel-Audom, M., Mekchay, S. and Sringarm, K.J.V.I.S. 2021. Efficiency of Fresh and Fermented Banana Stem in Low Protein Diet on Nutrient Digestibility, Productive Performance and Intestinal Morphology of Crossbred Pig ((Thai native x Meishan) x Duroc):*Veterinary Integrative Sciences.* **19**(1), pp.51-64.

Arpaia, N., Campbell, C., Fan, X., Dikiy, S., Van Der Veeken, J., Deroos, P., Liu, H., Cross, J.R., Pfeffer, K. and Coffer, P.J. 2013. Metabolites produced by commensal bacteria promote peripheral regulatory T-cell generation. *Nature.* **504**(7480), pp.451-455.

Arrieta, M.-C., Stiemsma, L.T., Amenyogbe, N., Brown, E.M. and Finlay, B. 2014. The intestinal microbiome in early life: health and disease. *Frontiers in Immunology* **5**, p427.

Arts, M.J., Dallinga, J.S., Voss, H.-P., Haenen, G.R. and Bast, A. 2004. A new approach to assess the total antioxidant capacity using the TEAC assay. *Food Chemistry.* **88**(4), pp.567-570.

Awati, A., Williams, B., Bosch, M., Gerrits, W. and Verstegen, M. 2006. Effect of inclusion of fermentable carbohydrates in the diet on fermentation end-product profile in feces of weanling piglets. *Journal of Animal Science.* **84**(8), pp.2133-2140.

Ayala-Zavala, J., Vega-Vega, V., Rosas-Domínguez, C., Palafox-Carlos, H., Villa-Rodriguez, J., Siddiqui, M.W., Dávila-Aviña, J. and González-Aguilar, G. 2011. Agro-industrial potential of exotic fruit byproducts as a source of food additives. *Food Research International.* **44**(7), pp.1866-1874.

Azeredo, H.M. 2009. Betalains: properties, sources, applications, and stability–a review. *International Journal of Food Science Technology* **44**(12), pp.2365-2376.

Bäckhed, F., Ley, R.E., Sonnenburg, J.L., Peterson, D.A. and Gordon, J.I. 2005. Host-bacterial mutualism in the human intestine. *Science.* pp.1915-1920.

Baholet, D., Skalickova, S., Batik, A., Malyugina, S., Skladanka, J. and Horky, P. 2022. Importance of Zinc Nanoparticles for the Intestinal Microbiome of Weaned Piglets. *Frontiers in Veterinary Science.* **9**, 852085.

Baião, D., da Silva, D.V. and Paschoalin, V.M. 2020. Beetroot, a remarkable vegetable: Its nitrate and phytochemical contents can be adjusted in novel formulations to benefit health and support cardiovascular disease therapies. *Antioxidants.* **9**(10), p960.

Baião, D.d.S., da Silva, D.V., Del Aguila, E.M. and Paschoalin, V.M.F. 2017. Nutritional, bioactive and physicochemical characteristics of different beetroot formulations. *Food Additives.* **6**(6).

Baker, A., Davis, E., Spencer, J., Moser, R. and Rehberger, T. 2013. The effect of a Bacillus-based direct-fed microbial supplemented to sows on the gastrointestinal microbiota of their neonatal piglets. *Journal of Animal Science.* **91**(7), pp.3390-3399.

Baker, R.G., Hayden, M.S. and Ghosh, S. 2011. NF-κB, inflammation, and metabolic disease. *Cell Metabolism* **13**(1), pp.11-22.

Bakshi, M., Wadhwa, M. and Makkar, H.P. 2016. Waste to worth: vegetable wastes as animal feed. *CABI Reviews* (2016), pp.1-26.

Barbosa, J.A., Rodrigues, L.A., Columbus, D.A., Aguirre, J.C., Harding, J., Cantarelli, V.S. and Costa, M.d.O. 2021. Experimental infectious challenge in pigs leads to elevated fecal calprotectin levels following colitis, but not enteritis. *Porcine Health Management.* **7**(1), pp.1-9.

Barszcz, M. and Skomial, J. 2011. The development of the small intestine of piglets - chosen aspects. *Journal of Animal and Feed Sciences.* **20**(1), pp.3-15.

Batson, K.L., Neujahr, A.C., Burkey, T., Fernando, S.C., Tokach, M.D., Woodworth, J.C., Goodband, R.D., DeRouchey, J.M., Gebhardt, J.T. and Calderón, H.I. 2021. Effect of fiber source and crude protein level on nursery pig performance and fecal microbial communities. *Journal of Animal Science.* **99**(12), p343.

Bauchart-Thevret, C., Stoll, B. and Burrin, D.G. 2009. Intestinal metabolism of sulfur amino acids. *Nutrition Research Reviews.* **22**(2), pp.175-187.

Bauer, E., Williams, B.A., Smidt, H., Verstegen, M.W. and Mosenthin, R. 2006. Influence of the gastrointestinal microbiota on development of the immune system in young animals. *Current issues in Intestinal Microbiology.* **7**(2), pp.35-52.

Bäumler, A.J. and Sperandio, V. 2016. Interactions between the microbiota and pathogenic bacteria in the gut. *Nature.* **535**(7610), pp.85-93.

Beaurepaire, C., Smyth, D. and McKay, D.M. 2009. Interferon-γ regulation of intestinal epithelial permeability. *Journal of Interferon Cytokine Research* **29**(3), pp.133-144.

Becattini, S., Taur, Y. and Pamer, E.G. 2016. Antibiotic-induced changes in the intestinal microbiota and disease. *Trends in Molecular Medicine.* **22**(6), pp.458-478.

Bedford, A. and Gong, J. 2018. Implications of butyrate and its derivatives for gut health and animal production. *Animal Nutrition.* **4**(2), pp.151-159.

Bednorz, C., Oelgeschlager, K., Kinnemann, B., Hartmann, S., Neumann, K., Pieper, R., Bethe, A., Semmler, T., Tedin, K., Schierack, P., Wieler, L.H. and Guenther, S. 2013. The broader context of antibiotic resistance: zinc feed supplementation of piglets increases the proportion of multi-resistant Escherichia coli in vivo. *International Journal of Medical Microbiology.* **303**(6-7), pp.396-403.

Begley, M., Gahan, C.G. and Hill, C. 2005. The interaction between bacteria and bile. *FEMS Microbiol Reviews.* **29**(4), pp.625-651.

Belfiore, C., Castellano, P. and Vignolo, G. 2007. Reduction of Escherichia coli population following treatment with bacteriocins from lactic acid bacteria and chelators. *Food Microbiology.* **24**(3), pp.223-229.

Belkaid, Y. and Hand, T.W. 2014. Role of the microbiota in immunity and inflammation. *Cell.* **157**(1), pp.121-141.

Bengtsson, B. and Greko, C. 2014. Antibiotic resistance—consequences for animal health, welfare, and food production. *Upsala Journal of Medical Sciences.* **119**(2), pp.96-102.

Berkeveld, M., Langendijk, P., Verheijden, J., Taverne, M., Van Nes, A., Van Haard, P. and Koets, A. 2008. Citrulline and intestinal fatty acid-binding protein: Longitudinal markers of postweaning small intestinal function in pigs? *Animal Science* **86**(12), pp.3440-3449.

Berrocoso, J., Menoyo, D., Guzmán, P., Saldaña, B., Cámara, L. and Mateos, G. 2015. Effects of fiber inclusion on growth performance and nutrient digestibility of piglets reared under optimal or poor hygienic conditions. *Animal Science.* **93**(8), pp.3919-3931.

Beutler, B. 2004. Innate immunity: an overview. *Molecular Immunology.* **40**(12), pp.845-859.

Bian, G., Ma, S., Zhu, Z., Su, Y., Zoetendal, E.G., Mackie, R., Liu, J., Mu, C., Huang, R. and Smidt, H. 2016. Age, introduction of solid feed and weaning are more important determinants of gut bacterial succession in piglets than breed and nursing mother as revealed by a reciprocal cross-fostering model. *Environment and Microbiology.* **18**(5), pp.1566-1577.

Bischoff, S.C. 2011. 'Gut health': a new objective in medicine? *BMC Medicine.* **9**(1), pp.1-14.

Bischoff, S.C., Barbara, G., Buurman, W., Ockhuizen, T., Schulzke, J.-D., Serino, M., Tilg, H., Watson, A. and Wells, J.M. 2014. Intestinal permeability–a new target for disease prevention and therapy. *BMC Gastroenterology.* **14**(1), pp.1-25.

Blake, A.B. 2017. *Fecal Lactate Concentrations in Dogs with Gastrointestinal Disease*. thesis.

Boivin, M.A., Roy, P.K., Bradley, A., Kennedy, J.C., Rihani, T. and Ma, T.Y. 2009. Mechanism of interferon-γ–induced increase in T84 intestinal epithelial tight junction. *Interferon Cytokine Research.* **29**(1), pp.45-54.

Bomba, L., Minuti, A., Moisá, S.J., Trevisi, E., Eufemi, E., Lizier, M., Chegdani, F., Lucchini, F., Rzepus, M. and Prandini, A. 2014. Gut response induced by weaning in piglet features marked changes in immune and inflammatory response. *Functional Integrative Genomics.* **14**(4), pp.657-671.

Bonetti, A., Tugnoli, B., Piva, A. and Grilli, E. 2021. Towards Zero Zinc Oxide: Feeding Strategies to Manage Post-Weaning Diarrhea in Piglets. *Animals (Basel).* **11**(3), p642.

Bontempo, V., Jiang, X., Cheli, F., Verso, L.L., Mantovani, G., Vitari, F., Domeneghini, C. and Agazzi, A. 2014. Administration of a novel plant extract product via drinking water to post-weaning piglets: effects on performance and gut health. *Animal.* **8**(5), pp.721-730.

Boqvist, S., Soderqvist, K. and Vagsholm, I. 2018. Food safety challenges and One Health within Europe. *Acta Veterinaria Scandinavica.* **60**(1), p1.

Bottery, M.J., Wood, A.J. and Brockhurst, M.A. 2017. Adaptive modulation of antibiotic resistance through intragenomic coevolution. *Nature Ecology Evolution.* **1**(9), pp.1364-1369.

Boudry, G., Péron, V., Le Huërou-Luron, I., Lallès, J.P. and Sève, B. 2004. Weaning induces both transient and long-lasting modifications of absorptive, secretory, and barrier properties of piglet intestine. *Journal of Nutrition.***134**(9), pp.2256-2262.

Brestenský, M., Nitrayová, S., Bomba, A., Patráš, P., Strojný, L., Szabadošová, V., Pramuková, B. and Bertková, I. 2017. The content of short chain fatty acids in the jejunal digesta, caecal digesta and faeces of growing pigs. *Livestock Science.* **205**, pp.106-110.

Broom, L., Miller, H., Kerr, K. and Knapp, J. 2006. Effects of zinc oxide and Enterococcus faecium SF68 dietary supplementation on the performance, intestinal microbiota and immune status of weaned piglets. *Research in Veterinary Science.* **80**(1), pp.45-54.

Brown, K., DeCoffe, D., Molcan, E. and Gibson, D.L. 2012. Diet-induced dysbiosis of the intestinal microbiota and the effects on immunity and disease. *Nutrients.* **4**(8), pp.1095-1119.

Bruewer, M., Luegering, A., Kucharzik, T., Parkos, C.A., Madara, J.L., Hopkins, A.M. and Nusrat, A. 2003. Proinflammatory cytokines disrupt epithelial barrier function by apoptosis-independent mechanisms. *Journal of Immunology.***171**(11), pp.6164-6172.

Burger-van Paassen, N., Loonen, L.M., Witte-Bouma, J., Korteland-van Male, A.M., De Bruijn, A.C., van der Sluis, M., Lu, P., Van Goudoever, J.B., Wells, J.M. and Dekker, J. 2012. Mucin Muc2 deficiency and weaning influences the expression of the innate defense genes Reg3β, Reg3γ and angiogenin-4. *PloS One.* **7**(6), pe38798.

Burger-van Paassen, N., Vincent, A., Puiman, P.J., van der Sluis, M., Bouma, J., Boehm, G., Van Goudoever, J.B., Van Seuningen, I. and Renes, I.B. 2009. The regulation of intestinal mucin MUC2 expression by short-chain fatty acids: implications for epithelial protection. *Biochemical Journal.* **420**(2), pp.211-219.

Burrin, D. and Stoll, B. 2003. 12 Intestinal nutrient requirements in weanling pigs. *Weaning the pig: concepts and consequences.* p301.

Burrough, E.R., De Mille, C. and Gabler, N.K. 2019. Zinc overload in weaned pigs: tissue accumulation, pathology, and growth impacts. *Veterinary Diagnostic Investigation.* **31**(4), pp.537-545.

Cai, Y., Sun, M. and Corke, H. 2003. Antioxidant activity of betalains from plants of the Amaranthaceae. *Journal of Agricultural Food Chemistry.* **51**(8), pp.2288-2294.

Calixto, J.B., Campos, M.M., Otuki, M.F. and Santos, A.R. 2004. Anti-inflammatory compounds of plant origin. Part II. Modulation of pro-inflammatory cytokines, chemokines and adhesion molecules. *Planta Medica.* **70**(02), pp.93-103.

Callens, B., Persoons, D., Maes, D., Laanen, M., Postma, M., Boyen, F., Haesebrouck, F., Butaye, P., Catry, B. and Dewulf, J. 2012. Prophylactic and metaphylactic antimicrobial use in Belgian fattening pig herds. *Preventive Veterinary Medicine.* **106**(1), pp.53-62.

Callesen, J., Halas, D., Thorup, F., Knudsen, K.B., Kim, J., Mullan, B., Hampson, D., Wilson, R. and Pluske, J. 2007. The effects of weaning age, diet composition, and categorisation of creep feed intake by piglets on diarrhoea and performance after weaning. *Livestock Science.* **108**(1-3), pp.120-123.

Calvi, P., Terzo, S. and Amato, A. 2022. Betalains: colours for human health. *Natural Product Research.* pp.1-20.

Campbell, J.M., Crenshaw, J.D., González-Esquerra, R. and Polo, J. 2019. Impact of spray-dried plasma on intestinal health and broiler performance. *Microorganisms.* **7**(8), p219.

Campbell, J.M., Crenshaw, J.D. and Polo, J. 2013. The biological stress of early weaned piglets. *Animal Science Biotechnology.* **4**(1), pp.1-4.

Capaldo, C.T. and Nusrat, A.J.B.e.B.A.-B. 2009. Cytokine regulation of tight junctions. *Biochimica et Biophysica Acta -Biomembranes.* **1788**(4), pp.864-871.

Carrillo, C., Rey, R., Hendrickx, M., Del Mar Cavia, M. and Alonso-Torre, S. 2017. Antioxidant Capacity of Beetroot: Traditional vs Novel Approaches. *Plant Foods Hum Nutr.* **72**(3), pp.266-273.

Casal, J., Mateu, E., Mejía, W. and Martín, M. 2007. Factors associated with routine mass antimicrobial usage in fattening pig units in a high pig-density area. *Veterinary Research.* **38**(3), pp.481-492.

Cash, H.L. 2006. *The Ligand and Function of the RegIII Family of Bactericidal C-Type Lectins*. thesis.

Celi, P., Cowieson, A., Fru-Nji, F., Steinert, R., Kluenter, A.-M. and Verlhac, V. 2017. Gastrointestinal functionality in animal nutrition and health: new opportunities for sustainable animal production. *Animal Feed Science Technology.* **234**, pp.88-100.

Celi, P. and Gabai, G. 2015. Oxidant/antioxidant balance in animal nutrition and health: the role of protein oxidation. *Frontiers in Veterinary Science.* **2**, p48.

Celi, P., Verlhac, V., Calvo, E.P., Schmeisser, J. and Kluenter, A.-M. 2019. Biomarkers of gastrointestinal functionality in animal nutrition and health. *Animal Feed Science and Technology.* **250**, pp.9-31.

Celli, G.B. and Brooks, M.S.-L. 2017. Impact of extraction and processing conditions on betalains and comparison of properties with anthocyanins—A current review. *Food Research International.* **100**, pp.501-509.

Cerezal, P. and Duarte, G. 2005. Some characteristics of cactus pear (Opuntia ficus-indica (L.) Miller) harvested in the Andean altiplane of region 2 of Chile. *Journal of the Professional Association for Cactus Development.* **7**, pp.34-60.

Cerisuelo, A., Castelló, L., Moset, V., Martínez, M., Hernández, P., Piquer, O., Gómez, E., Gasa, J. and Lainez, M. 2010. The inclusion of ensiled citrus pulp in diets for growing pigs: Effects on voluntary intake, growth performance, gut microbiology and meat quality. *Livestock Science.* **134**(1-3), pp.180-182.

Chan, C.L., Gan, R.-y., Shah, N.P. and Corke, H. 2018. Enhancing antioxidant capacity of Lactobacillus acidophilus-fermented milk fortified with pomegranate peel extracts. *Food Bioscience.* **26**, pp.185-192.

Chan, E.W.C., Lim, Y.Y., Wong, L., Lianto, F.S., Wong, S., Lim, K., Joe, C. and Lim, T. 2008. Antioxidant and tyrosinase inhibition properties of leaves and rhizomes of ginger species. *Food Chemistry.* **109**(3), pp.477-483.

Chang, J.I., Tsai, J. and Wu, K.J.W.m. 2006. Composting of vegetable waste. *Waste Management Research* **24**(4), pp.354-362.

Chauvin, C., Beloeil, P.-A., Orand, J.P., Sanders, P. and Madec, F. 2002. A survey of group-level antibiotic prescriptions in pig production in France. *Preventive Veterinary Medicine.* **55**(2), pp.109-120.

Cheminet, G., Kapel, N., Bleibtreu, A., Yayé, H.S., Bellanger, A., Duval, X., Joly, F., Fantin, B. and de Lastours, V. 2018. Faecal microbiota transplantation with frozen capsules for

relapsing Clostridium difficile infections: the first experience from 15 consecutive patients in France. *Journal of Hospital Infection.* **100**(2), pp.148-151.

Chen, J., Tellez, G., Richards, J.D. and Escobar, J.e. 2015. Identification of potential biomarkers for gut barrier failure in broiler chickens. *Frontiers in Veterinary Sciences.* **2**, p14.

Chen, K., Zhao, H., Shu, L., Xing, H., Wang, C., Lu, C. and Song, G. 2020. Effect of resveratrol on intestinal tight junction proteins and the gut microbiome in high-fat diet-fed insulin resistant mice. *International Journal of Food Sciences and Nutrition.* **71**(8), pp.965-978.

Chen, L., Xu, Y., Chen, X., Fang, C., Zhao, L. and Chen, F. 2017. The Maturing Development of Gut Microbiota in Commercial Piglets during the Weaning Transition. *Frontiers in Microbiology.* **8**, p1688.

Chen, L., Zhu, Y., Hu, Z., Wu, S. and Jin, C. 2021. Beetroot as a functional food with huge health benefits: Antioxidant, antitumor, physical function, and chronic metabolomics activity. *Food Science Nutrition.* **9**(11), pp.6406-6420.

Chen, X., Zheng, R., Liu, R. and Li, L. 2020. Goat milk fermented by lactic acid bacteria modulates small intestinal microbiota and immune responses. *Journal of Functional Foods.* **65**, p103744.

Cheng, C., Wei, H., Xu, C., Xie, X., Jiang, S. and Peng, J. 2018. Maternal soluble fiber diet during pregnancy changes the intestinal microbiota, improves growth performance, and reduces intestinal permeability in piglets. *Applied Environmental Microbiology* **84**(17), pp.e01047-01018.

Cheng, G., Hao, H., Xie, S., Wang, X., Dai, M., Huang, L. and Yuan, Z. 2014. Antibiotic alternatives: the substitution of antibiotics in animal husbandry? *Frontiers in Microbiology.* **5**, p217.

Cheng, P.H., Wang, Y., Liang, J., Wu, Y.B., Wright, A. and Liao, X.D. 2018. Exploratory Analysis of the Microbiological Potential for Efficient Utilization of Fiber Between Lantang and Duroc Pigs. *Frontiers in Microbiology.* **9**.

Cheng, S., Ma, X., Geng, S., Jiang, X., Li, Y., Hu, L., Li, J., Wang, Y. and Han, X. 2018. Fecal microbiota transplantation beneficially regulates intestinal mucosal autophagy and alleviates gut barrier injury. *Msystems.* **3**(5), pp.e00137-00118.

Chhikara, N., Kushwaha, K., Sharma, P., Gat, Y. and Panghal, A. 2019. Bioactive compounds of beetroot and utilization in food processing industry: A critical review. *Food Chemistry.* **272**, pp.192-200.

Cholewa, J.M., Hudson, A., Cicholski, T., Cervenka, A., Barreno, K., Broom, K., Barch, M. and Craig, S.A. 2018. The effects of chronic betaine supplementation on body composition and performance in collegiate females: a double-blind, randomized, placebo controlled trial. *Journal of the International Society of Sports Nutrition.* **15**(1), p37.

Cholewa, J.M., Wyszczelska-Rokiel, M., Glowacki, R., Jakubowski, H., Matthews, T., Wood, R., Craig, S.A. and Paolone, V. 2013. Effects of betaine on body composition,

performance, and homocysteine thiolactone. *Journal of the International Society of Sports Nutrition.* **10**(1), p39.

Chong, C.Y.L., Bloomfield, F.H. and O'Sullivan, J.M. 2018. Factors Affecting Gastrointestinal Microbiome Development in Neonates. *Nutrients.* **10**(3), p274.

Choo, K.Y., Ong, Y.Y., Lim, R.L.H., Tan, C.P. and Ho, C.W. 2019. Study on bioaccessibility of betacyanins from red dragon fruit (Hylocereus polyrhizus). *Food Science Biotechnology* **28**(4), pp.1163-1169.

Choudhury, R., Middelkoop, A., Boekhorst, J., Gerrits, W., Kemp, B., Bolhuis, J. and Kleerebezem, M. 2021. Early life feeding accelerates gut microbiome maturation and suppresses acute post-weaning stress in piglets. *Environmental Microbiology* **23**(11), pp.7201-7213.

Cicero, A.F. and Colletti, A. 2016. Role of phytochemicals in the management of metabolic syndrome. *Phytomedicine.* **23**(11), pp.1134-1144.

Ciesinski, L., Guenther, S., Pieper, R., Kalisch, M., Bednorz, C. and Wieler, L.H. 2018. High dietary zinc feeding promotes persistence of multi-resistant E. coli in the swine gut. *PLoS One.* **13**(1), pe0191660.

Clifford, T., Constantinou, C.M., Keane, K.M., West, D.J., Howatson, G. and Stevenson, E.J. 2017. The plasma bioavailability of nitrate and betanin from Beta vulgaris rubra in humans. *European Journal of Nutrition.* **56**, pp.1245-1254.

Clifford, T., Howatson, G., West, D.J. and Stevenson, E.J. 2015. The potential benefits of red beetroot supplementation in health and disease. *Nutrients.* **7**(4), pp.2801-2822.

Commission, E.U. 2003. *Commission regulation (EC) No 1334/2003 of 25 July 2003 amending the conditions for authorisation of a number of additives in feedingstuffs belonging to the group of trace elements.*

Commission, E.U. 2005. *Commission Regulation (EC) No 378/2005 of 4 March 2005 on detailed rules for the implementation of Regulation (EC) No 1831/2003 of the European Parliament and of the Council of 22 September 2003 on additives for use in animal nutrition.*

Cong, C., Yuan, X., Hu, Y., Chen, W., Wang, Y. and Tao, L. 2021. Sinigrin attenuates angiotensin II-induced kidney injury by inactivating nuclear factor-κB and extracellular signal-regulated kinase signaling in vivo and in vitro. *International Journal of Molecular Medicine.* **48**(2), pp.1-10.

Costa, M.M., Alfaia, C.M., Lopes, P.A., Pestana, J.M. and Prates, J.A. 2022. Grape By-Products as Feedstuff for Pig and Poultry Production. *Animals.* **12**(17), p2239.

Costa, M.O., Chaban, B., Harding, J.C.S. and Hill, J.E. 2014. Characterization of the fecal microbiota of pigs before and after inoculation with “Brachyspira hampsonii”. *PLoS One.* **9**(8), pe106399.

Cuevas-Tena, M., del Pulgar, E.M.G., Benitez-Paez, A., Sanz, Y., Alegria, A. and Lagarda, M.J. 2018. Plant sterols and human gut microbiota relationship: An in vitro colonic fermentation study. *Functional Foods.* **44**, pp.322-329.

Cuny, C., Wieler, L.H. and Witte, W. 2015. Livestock-Associated MRSA: The Impact on Humans. *Antibiotics (Basel).* **4**(4), pp.521-543.

D'Angelo, F., Felley, C. and Frossard, J.L. 2017. Calprotectin in daily practice: where do we stand in 2017? *Digestion.* **95**(4), pp.293-301.

da Silva, D.V.T., Pereira, A.D., Boaventura, G.T., Ribeiro, R.S.A., Vericimo, M.A., Carvalho-Pinto, C.E., Baiao, D.D.S., Del Aguila, E.M. and Paschoalin, V.M.F. 2019. Short-Term Betanin Intake Reduces Oxidative Stress in Wistar Rats. *Nutrients.* **11**(9).

Dalile, B., Van Oudenhove, L., Vervliet, B. and Verbeke, K. 2019. The role of short-chain fatty acids in microbiota–gut–brain communication. *Nature reviews GastroenterologyHepatology.* **16**(8), pp.461-478.

Dang, D.X., Choi, S.Y., Choi, Y.J., Lee, J.H., Castex, M., Chevaux, E., Saornil, D., de Laguna, F.B., Jimenez, G. and Kim, I.H. 2023. Probiotic, Paraprobiotic, and Hydrolyzed Yeast Mixture Supplementation Has Comparable Effects to Zinc Oxide in Improving Growth Performance and Ameliorating Post-weaning Diarrhea in Weaned Piglets. *Probiotics Antimicrobial Proteins.* pp.1-10.

Danneskiold-Samsøe, N.B., Barros, H.D.d.F.Q., Santos, R., Bicas, J.L., Cazarin, C.B.B., Madsen, L., Kristiansen, K., Pastore, G.M., Brix, S. and Júnior, M.R.M. 2019. Interplay between food and gut microbiota in health and disease. *Food Research International.* **115**, pp.23-31.

Darwish, W.S., Eldaly, E.A., El-Abbasy, M.T., Ikenaka, Y., Nakayama, S. and Ishizuka, M. 2013. Antibiotic residues in food: the African scenario. *Veterinary Resources* **61 Suppl**, pp.S13-22.

Davila, A.-M., Blachier, F., Gotteland, M., Andriamihaja, M., Benetti, P.-H., Sanz, Y. and Tomé, D. 2013. Re-print of “Intestinal luminal nitrogen metabolism: Role of the gut microbiota and consequences for the host”. *Pharmacological Research.* **69**(1), pp.114-126.

de Araujo, G.H., Ferreira, L.F.M., Leal, I.F., Araujo, G.A., de Oliveira Carvalho, P.L., Toledo, J.B., Andrade, M.P.C., Pozza, P.C. and Castilha, L.D. 2022. Dehydrated citrus pulp reduces plasma cholesterol of weaned piglets, and an exogenous enzyme complex improves plasma calcium and performance. *Livestock Science.* **260**, p104933.

de Been, M., Lanza, V.F., de Toro, M., Scharringa, J., Dohmen, W., Du, Y., Hu, J., Lei, Y., Li, N. and Tooming-Klunderud, A. 2014. Dissemination of cephalosporin resistance genes between Escherichia coli strains from farm animals and humans by specific plasmid lineages. *PLoS Genetics.* **10**(12), pe1004776.

de Moraes Crizel, T., Jablonski, A., de Oliveira Rios, A., Rech, R. and Flôres, S.H. 2013. Dietary fiber from orange byproducts as a potential fat replacer. *LWT-Food Science Technology.* **53**(1), pp.9-14.

de Oliveira, S.P.A., do Nascimento, H.M.A., Sampaio, K.B. and de Souza, E.L. 2021. A review on bioactive compounds of beet (Beta vulgaris L. subsp. vulgaris) with special emphasis on their beneficial effects on gut microbiota and gastrointestinal health. *Critical Reviews in Food Science and Nutrition.* **61**(12), pp.2022-2033.

De Rosa, V., Galgani, M., Porcellini, A., Colamatteo, A., Santopaolo, M., Zuchegna, C., Romano, A., De Simone, S., Procaccini, C. and La Rocca, C. 2015. Glycolysis controls the induction of human regulatory T cells by modulating the expression of FOXP3 exon 2 splicing variants. *Nature Immunology.* **16**(11), pp.1174-1184.

De Vries, H. and Smidt, H. 2020. Microbiota development in piglets. *The suckling weaned piglet.* p95.

Delisle, B., Calinescu, C., Mateescu, M.A., Fairbrother, J.M. and Nadeau, E. 2012. Oral immunization with F4 fimbriae and CpG formulated with carboxymethyl starch enhances F4-specific mucosal immune response and modulates Th1 and Th2 cytokines in weaned pigs. *Journal of Pharmacy Pharmaceutical Sciences.* **15**(5), pp.642-656.

Denev, P., Kratchanova, M., Ciz, M., Lojek, A., Vasicek, O., Nedelcheva, P., Blazheva, D., Toshkova, R., Gardeva, E. and Yossifova, L. 2014. Biological activities of selected polyphenol-rich fruits related to immunity and gastrointestinal health. *Food Chemistry.* **157**, pp.37-44.

Devkota, S. and Chang, E.B. 2015. Interactions between diet, bile acid metabolism, gut microbiota, and inflammatory bowel diseases. *Digestive diseases.* **33**(3), pp.351-356.

Dhananjayan, I., Kathiroli, S., Subramani, S. and Veerasamy, V.J.B. 2017. Ameliorating effect of betanin, a natural chromoalkaloid by modulating hepatic carbohydrate metabolic enzyme activities and glycogen content in streptozotocin–nicotinamide induced experimental rats. *Biomedicine Pharmacotherapy.* **88**, pp.1069-1079.

Diao, H., Jiao, A., Yu, B., Mao, X. and Chen, D. 2019. Gastric infusion of short-chain fatty acids can improve intestinal barrier function in weaned piglets. *Genes Nutrition.* **14**(1), pp.1-16.

Dickinson, D.A., Moellering, D.R., Iles, K.E., Patel, R.P., Levonen, A.L., Wigley, A., Darley-Usmar, V.M. and Forman, H.J. 2003. Cytoprotection against oxidative stress and the regulation of glutathione synthesis. *Biololgical Chemistry.* **384**(4), pp.527-537.

Dietert, R.R. and Silbergeld, E.K. 2015. Biomarkers for the 21st century: listening to the microbiome. *Toxicological Sciences.* **144**(2), pp.208-216.

Dingeo, G., Brito, A., Samouda, H., Iddir, M., La Frano, M.R. and Bohn, T. 2020. Phytochemicals as modifiers of gut microbial communities. *Food Functional Integrative Genomics.* **11**(10), pp.8444-8471.

Dohm, J.C., Minoche, A.E., Holtgräwe, D., Capella-Gutiérrez, S., Zakrzewski, F., Tafer, H., Rupp, O., Sörensen, T.R., Stracke, R. and Reinhardt, R. 2014. The genome of the recently domesticated crop plant sugar beet (Beta vulgaris). *Nature.* **505**(7484), pp.546-549.

Dong, X., Xu, Q., Wang, C., Zou, X. and Lu, J. 2019. Supplemental-coated zinc oxide relieves diarrhoea by decreasing intestinal permeability in weanling pigs. *Applied Animal Research.* **47**(1), pp.362-368.

Donnelly, E.T., McClure, N. and Lewis, S.E. 1999. The effect of ascorbate and α-tocopherol supplementation in vitro on DNA integrity and hydrogen peroxide-induced DNA damage in human spermatozoa. *Mutagenesis.* **14**(5), pp.505-512.

Dou, S., Gadonna-Widehem, P., Rome, V., Hamoudi, D., Rhazi, L., Lakhal, L., Larcher, T., Bahi-Jaber, N., Pinon-Quintana, A., Guyonvarch, A., Huerou-Luron, I.L. and Abdennebi-Najar, L. 2017. Characterisation of Early-Life Fecal Microbiota in Susceptible and Healthy Pigs to Post-Weaning Diarrhoea. *PLoS One.* **12**(1), pe0169851.

Dowarah, R., Verma, A., Agarwal, N., Patel, B. and Singh, P. 2017. Effect of swine based probiotic on performance, diarrhoea scores, intestinal microbiota and gut health of grower-finisher crossbred pigs. *Livestock Science.* **195**, pp.74-79.

Drummond, G.R., Selemidis, S., Griendling, K.K. and Sobey, C.G. 2011. Combating oxidative stress in vascular disease: NADPH oxidases as therapeutic targets. *Nature Reviews Drug Discovery.* **10**(6), pp.453-471.

Duan, Q., Lee, J., Liu, Y., Chen, H. and Hu, H. 2016. Distribution of heavy metal pollution in surface soil samples in China: a graphical review. *Bulletin of Environmental Contamination Toxicology.* **97**(3), pp.303-309.

Duarte, M.E. and Kim, S.W. 2022. Intestinal microbiota and its interaction to intestinal health in nursery pigs. *Animal Nutrition.* **8**, pp.169-184.

Dubreuil, J.D. 2017. Enterotoxigenic Escherichia coli targeting intestinal epithelial tight junctions: An effective way to alter the barrier integrity. *Microbial Pathogenesis* **113**, pp.129-134.

Dubreuil, J.D., Isaacson, R.E. and Schifferli, D.M. 2016. Animal enterotoxigenic Escherichia coli. *EcoSal Plus.* **7**(1).

Dufourny, S., Antoine, N., Pitchugina, E., Delcenserie, V., Godbout, S., Douny, C., Scippo, M.-L., Froidmont, E., Rondia, P. and Wavreille, J. 2021. Apple Pomace and Performance, Intestinal Morphology and Microbiota of Weaned Piglets—A Weaning Strategy for Gut Health? *Microorganisms.* **9**(3), p572.

Duncan, S.H., Louis, P. and Flint, H.J. 2004. Lactate-utilizing bacteria, isolated from human feces, that produce butyrate as a major fermentation product. *Applied Environmental Microbiology* **70**(10), pp.5810-5817.

Dunne-Castagna, V.P., Mills, D.A. and Lönnerdal, B. 2020. Effects of milk secretory immunoglobulin A on the commensal microbiota. *Milk, Mucosal Immunity the Microbiome: Impact on the Neonate.* **94**, pp.158-168.

EFSA. 2017. Safety and efficacy of zinc chelate of methionine sulfate for all animal species. *EFSA Journal.* **15**(6), pe04859.

EFSA. 2020. Scientific Opinion on the update of the list of QPS-recommended biological agents intentionally added to food or feed as notified to EFSA (2017–2019). *EFSA Journal.* **18**(2), pe05966.

El Gamal, A.A., AlSaid, M.S., Raish, M., Al-Sohaibani, M., Al-Massarani, S.M., Ahmad, A., Hefnawy, M., Al-Yahya, M., Basoudan, O.A. and Rafatullah, S. 2014. Beetroot (Beta vulgaris L.) extract ameliorates gentamicin-induced nephrotoxicity associated oxidative stress, inflammation, and apoptosis in rodent model. *Mediators of Inflammation.* **2014**.

Endersen, L. and Coffey, A. 2020. The use of bacteriophages for food safety. *Current Opinion in Food Science.* **36**, pp.1-8.

Engels, C., Gänzle, M.G. and Schieber, A. 2010. Fractionation of gallotannins from mango (Mangifera indica L.) kernels by high-speed counter-current chromatography and determination of their antibacterial activity. *Journal of Agricultural Food Chemistry.* **58**(2), pp.775-780.

Eriksen, E.Ø., Kudirkiene, E., Christensen, A.E., Agerlin, M.V., Weber, N.R., Nødtvedt, A., Nielsen, J.P., Hartmann, K.T., Skade, L. and Larsen, L.E. 2021. Post-weaning diarrhea in pigs weaned without medicinal zinc: risk factors, pathogen dynamics, and association to growth rate. *Porcine Health Management.* **7**(1), pp.1-19.

Erinle, T.J., Oladokun, S., MacIsaac, J., Rathgeber, B. and Adewole, D. 2022. Dietary grape pomace–effects on growth performance, intestinal health, blood parameters, and breast muscle myopathies of broiler chickens. *Poultry Science.* **101**(1), p101519.

Esatbeyoglu, T., Wagner, A.E., Schini-Kerth, V.B. and Rimbach, G. 2015. Betanin--a food colorant with biological activity. *Molecular Nutrition Food Research.* **59**(1), pp.36-47.

Esteban, M., Garcia, A., Ramos, P. and Márquez, M. 2007. Evaluation of fruit–vegetable and fish wastes as alternative feedstuffs in pig diets. *Waste Management.* **27**(2), pp.193-200.

Etxeberria, U., Arias, N., Boqué, N., Macarulla, M., Portillo, M., Martínez, J. and Milagro, F. 2015. Reshaping faecal gut microbiota composition by the intake of trans-resveratrol and quercetin in high-fat sucrose diet-fed rats. *Nutritional Biochemistry.* **26**(6), pp.651-660.

Everaert, N., Van Cruchten, S., Weström, B., Bailey, M., Van Ginneken, C., Thymann, T. and Pieper, R. 2017. A review on early gut maturation and colonization in pigs, including biological and dietary factors affecting gut homeostasis. *Animal Feed Science Technology.* **233**, pp.89-103.

Everard, A., Belzer, C., Geurts, L., Ouwerkerk, J.P., Druart, C., Bindels, L.B., Guiot, Y., Derrien, M., Muccioli, G.G. and Delzenne, N.M. 2013. Cross-talk between Akkermansia muciniphila and intestinal epithelium controls diet-induced obesity. *Proceedings of the National Academy of Sciences* **110**(22), pp.9066-9071.

Fadel, J., DePeters, E. and Arosemena, A. 2000. Composition and digestibility of beet pulp with and without molasses and dried using three methods. *Animal Feed Science Technology.* **85**(1-2), pp.121-129.

Fagerhol, M.K. 2000. Calprotectin, a faecal marker of organic gastrointestinal abnormality. *The Lancet.* **356**(9244), pp.1783-1784.

Fairbrother, J.M., Nadeau, É. and Gyles, C.L. 2005. Escherichia coli in postweaning diarrhea in pigs: an update on bacterial types, pathogenesis, and prevention strategies. *Animal Health Research Reviews.* **6**(1), pp.17-39.

Fan, Y. and Pedersen, O. 2021. Gut microbiota in human metabolic health and disease. *Nature Reviews Microbiology.* **19**(1), pp.55-71.

Fang, F., Junejo, S.A., Wang, K., Yang, X., Yuan, Y., Zhang, B.J.I.J.o.F.S. and Technology. 2022. Fibre matrices for enhanced gut health: a mini review. *International Journal of Food Science Technology.*

Fang, L., Li, M., Zhao, L., Han, S., Li, Y., Xiong, B. and Jiang, L.J.J.o.a.p. 2020. Dietary grape seed procyanidins suppressed weaning stress by improving antioxidant enzyme activity and mRNA expression in weanling piglets. *Journal of Animal Physiology Animal Nutrition.* **104**(4), pp.1178-1185.

FAO. 2011. *Global food losses and food waste - extent, causes and prevention.* The Swedish Institute for Food and Biotechnology.

FAO. 2014. FAO; Animal Production Health Division: Meat & Meat Products. *Food Agricultural Organization of the United Nations, Rome.*

FAO: Statistics data 2014. 2017. [Online database].

Farhadi, A., Banan, A., Fields, J. and Keshavarzian, A. 2003. Intestinal barrier: An interface between health and disease. *Journal of Gastroenterology and Hepatology.* **18**(5), pp.479-497.

Feder, M.E. and Hofmann, G.E. 1999. Heat-shock proteins, molecular chaperones, and the stress response: evolutionary and ecological physiology. *Annual Review of Physiology.* **61**(1), pp.243-282.

Fernando, G.S.N., Sergeeva, N.N., Frutos, M.J., Marshall, L.J. and Boesch, C. 2022. Novel approach for purification of major betalains using flash chromatography and comparison of radical scavenging and antioxidant activities. *Food Chemistry.* **385**, p132632.

Ferreira-Halder, C.V., de Sousa Faria, A.V. and Andrade, S.S. 2017. Action and function of Faecalibacterium prausnitzii in health and disease. *Best Practice Research Clinical Gastroenterology.* **31**(6), pp.643-648.

Fertner, M., Boklund, A., Dupont, N., Enøe, C., Stege, H. and Toft, N. 2015. Weaner production with low antimicrobial usage: a descriptive study. *Acta Veterinaria Scandinavica* **57**(1), pp.1-8.

Filippitzi, M.-E., Callens, B., Pardon, B., Persoons, D. and Dewulf, J. 2014. Antimicrobial use in pigs, broilers and veal calves in Belgium. *Vlaams diergeneeskundig tijdschrift* **83**(5).

Finkel, T. 2003. Oxidant signals and oxidative stress. *Current Opinion in Cell Biology.* **15**(2), pp.247-254.

Finlayson-Trick, E.C., Getz, L.J., Slaine, P.D., Thornbury, M., Lamoureux, E., Cook, J., Langille, M.G., Murray, L.E., McCormick, C. and Rohde, J.R. 2017. Taxonomic differences of gut microbiomes drive cellulolytic enzymatic potential within hind-gut fermenting mammals. *PloS One.* **12**(12), pe0189404.

Flis, M., Sobotka, W. and Antoszkiewicz, Z. 2017. Fiber substrates in the nutrition of weaned piglets – a review. *Annals of Animal Science.* **17**(3), pp.627-644.

Flohé, L. 2020. Looking back at the early stages of redox biology. *Antioxidants.* **9**(12), p1254.

Forman, H.J. and Zhang, H. 2021. Targeting oxidative stress in disease: Promise and limitations of antioxidant therapy. *Nature Reviews Drug Discovery.* **20**(9), pp.689-709.

Forsberg, K.J., Reyes, A., Wang, B., Selleck, E.M., Sommer, M.O. and Dantas, G. 2012. The shared antibiotic resistome of soil bacteria and human pathogens. *Science.* **337**(6098), pp.1107-1111.

Fouhse, J., Zijlstra, R. and Willing, B. 2016. The role of gut microbiota in the health and disease of pigs. *Animal Frontiers.* **6**(3), pp.30-36.

Francis, F. 1999. *Anthocyanins and betalains*. Colorants. Eagan Press: St. Paul, MN. pp.55-66.

Frank, T., Stintzing, F.C., Carle, R., Bitsch, I., Quaas, D., Straß, G., Bitsch, R. and Netzel, M. 2005. Urinary pharmacokinetics of betalains following consumption of red beet juice in healthy humans. *Pharmacological Research.* **52**(4), pp.290-297.

Franklin, C.C., Backos, D.S., Mohar, I., White, C.C., Forman, H.J. and Kavanagh, T.J. 2009. Structure, function, and post-translational regulation of the catalytic and modifier subunits of glutamate cysteine ligase. *Molecular aspects of Medicine.* **30**(1-2), pp.86-98.

Fricke, R., Bastert, O., Gotter, V., Brons, N., Kamp, J. and Selbitz, H.-J. 2015. Implementation of a vaccine against Shigatoxin 2e in a piglet producing farm with problems of Oedema disease: case study. *Porcine Health Management.* **1**(1), pp.1-5.

Fukunaga, S., Kuwaki, K., Mitsuyama, K., Takedatsu, H., Yoshioka, S., Yamasaki, H., Yamauchi, R., Mori, A., Kakuma, T. and Tsuruta, O. 2018. Detection of calprotectin in inflammatory bowel disease: Fecal and serum levels and immunohistochemical localization. *International Journal of Molecular Medicine.* **41**(1), pp.107-118.

Funaoka, H., Kanda, T. and Fujii, H.J.R.b.T.J.j.o.c.p. 2010. Intestinal fatty acid-binding protein (I-FABP) as a new biomarker for intestinal diseases. *Rinsho byori. The Japanese Journal of Clinical Pathology.* **58**(2), pp.162-168.

Funderburke, D.W. and Seerley, R.W. 1990. The effects of postweaning stressors on pig weight change, blood, liver and digestive tract characteristics. *Animal Science.* **68**(1), pp.155-162.

Furusawa, Y., Obata, Y., Fukuda, S., Endo, T.A., Nakato, G., Takahashi, D., Nakanishi, Y., Uetake, C., Kato, K. and Kato, T. 2013. Commensal microbe-derived butyrate induces the differentiation of colonic regulatory T cells. *Nature.***504**(7480), pp.446-450.

Gaillard, C., Brossard, L. and Dourmad, J.-Y. 2020. Improvement of feed and nutrient efficiency in pig production through precision feeding. *Animal Feed Science and Technology.* **268**, p114611.

Gajdács, M. and Albericio, F. 2019. *Antibiotic resistance: from the bench to patients*. MDPI Antibiotics. 8. p.129.

Galanakis, C.M. 2012. Recovery of high added-value components from food wastes: Conventional, emerging technologies and commercialized applications. *Trends in Food Science Technology.* **26**(2), pp.68-87.

Gammoh, N.Z. and Rink, L. 2019. Zinc and the immune system. *Nutrition Immunity.* pp.127-158.

Gan, Z., Wei, W., Li, Y., Wu, J., Zhao, Y., Zhang, L., Wang, T. and Zhong, X. 2019. Curcumin and resveratrol regulate intestinal bacteria and alleviate intestinal inflammation in weaned piglets. *Molecules.* **24**(7), p1220.

Gandía-Herrero, F., Escribano, J. and García-Carmona, F. 2016. Biological activities of plant pigments betalains. *Critical Reviews in Food Science Nutrition.* **56**(6), pp.937-945.

Ganesan, P., Phaiphan, A., Murugan, Y. and Baharin, B. 2013. Comparative study of bioactive compounds in curry and coriander leaves: An update. *Chemical Pharmaceutical Research.* **5**(11), pp.590-594.

Gao, J., Liu, Z., Wang, C., Ma, L., Chen, Y. and Li, T. 2022. Effects of Dietary Protein Level on the Microbial Composition and Metabolomic Profile in Postweaning Piglets. *Oxidative Medicine Cellular Longevity.* **2022**.

Gao, J., Yin, J., Xu, K., Li, T.J. and Yin, Y.L. 2019. What Is the Impact of Diet on Nutritional Diarrhea Associated with Gut Microbiota in Weaning Piglets: A System Review. *Biomed Research International.* **2019**.

Gao, X., Zhang, H.J., Guo, X.F., Li, K.L., Li, S. and Li, D. 2019. Effect of Betaine on Reducing Body Fat-A Systematic Review and Meta-Analysis of Randomized Controlled Trials. *Nutrients.* **11**(10), p2480.

Geng, S., Cheng, S., Li, Y., Wen, Z., Ma, X., Jiang, X., Wang, Y. and Han, X. 2018. Faecal microbiota transplantation reduces susceptibility to epithelial injury and modulates tryptophan metabolism of the microbial community in a piglet model. *Journal of Crohn's Colitis.* **12**(11), pp.1359-1374.

Georgiev, V.G., Weber, J., Kneschke, E.M., Denev, P.N., Bley, T. and Pavlov, A.I. 2010. Antioxidant activity and phenolic content of betalain extracts from intact plants and hairy root cultures of the red beetroot Beta vulgaris cv. Detroit dark red. *Plant Foods for Human Nutrition.* **65**(2), pp.105-111.

Gertenbach, W.D. and Dugmore. 2004. Crop residues for animal feeding. *South African Journal of Animal Science.* **5**, pp.49-51.

Gessner, D., Ringseis, R. and Eder, K. 2017. Potential of plant polyphenols to combat oxidative stress and inflammatory processes in farm animals. *Animal Physiology Animal Nutrition.* **101**(4), pp.605-628.

Ghani, M.A., Barril, C., Bedgood Jr, D.R. and Prenzler, P.D. 2017. Measurement of antioxidant activity with the thiobarbituric acid reactive substances assay. *Food Chemistry.* **230**, pp.195-207.

Gibson, G.R., Hutkins, R., Sanders, M.E., Prescott, S.L., Reimer, R.A., Salminen, S.J., Scott, K., Stanton, C., Swanson, K.S. and Cani, P.D. 2017. Expert consensus document: The International Scientific Association for Probiotics and Prebiotics (ISAPP) consensus statement on the definition and scope of prebiotics. *Nature Reviews Gastroenterology Hepatology.* **14**(8), pp.491-502.

Gilani, S., Howarth, G.S., Kitessa, S.M., Forder, R.E., Tran, C.D. and Hughes, R.J. 2016. New biomarkers for intestinal permeability induced by lipopolysaccharide in chickens. *Animal Production Science* **56**(12), pp.1984-1997.

Gill, S.K., Rossi, M., Bajka, B. and Whelan, K. 2021. Dietary fibre in gastrointestinal health and disease. *Nature Reviews Gastroenterology Hepatology.* **18**(2), pp.101-116.

Glass-Kaastra, S.K., Pearl, D.L., Reid-Smith, R.J., McEwen, B., McEwen, S.A., Amezcua, R. and Friendship, R.M. 2013. Describing antimicrobial use and reported treatment efficacy in Ontario swine using the Ontario swine veterinary-based surveillance program. *BMC Veterinary Research.* **9**(1), pp.1-7.

Gledhill, D. 2008. *The names of plants.* Cambridge University Press.

Gliszczyńska-Świgło, A., Szymusiak, H. and Malinowska, P. 2006. Betanin, the main pigment of red beet: Molecular origin of its exceptionally high free radical-scavenging activity. *Food Additives Contaminants.* **23**(11), pp.1079-1087.

Godinez, I., Raffatellu, M., Chu, H., Paixao, T.A., Haneda, T., Santos, R.L., Bevins, C.L., Tsolis, R.M. and Bäumler, A.J. 2009. Interleukin-23 orchestrates mucosal responses to Salmonella enterica serotype Typhimurium in the intestine. *Infection and Immunity* **77**(1), pp.387-398.

Gorinstein, S., Martín-Belloso, O., Park, Y.S., Haruenkit, R., Lojek, A., Ĉíž, M., Caspi, A., Libman, I. and Trakhtenberg, S. 2001. Comparison of some biochemical characteristics of different citrus fruits. *Food Chemistry.* **74**(3), pp.309-315.

Gracia, A. and Gómez, M.I. 2020. Food sustainability and waste reduction in Spain: consumer preferences for local, suboptimal, and/or unwashed fresh food products. *Sustainability.* **12**(10), p4148.

Gresse, R., Chaucheyras-Durand, F., Fleury, M.A., Van de Wiele, T., Forano, E. and Blanquet-Diot, S. 2017. Gut microbiota dysbiosis in postweaning piglets: understanding the keys to health. *Trends in Microbiology.* **25**(10), pp.851-873.

Gresse, R., Chaucheyras Durand, F., Duniere, L., Blanquet-Diot, S. and Forano, E. 2019. Microbiota Composition and Functional Profiling Throughout the Gastrointestinal Tract of Commercial Weaning Piglets. *Microorganisms.* **7**(9), p343.

Grilli, E., Tugnoli, B., Foerster, C. and Piva, A. 2016. Butyrate modulates inflammatory cytokines and tight junctions components along the gut of weaned pigs. *Journal of Animal Science.* **94**(suppl_3), pp.433-436.

Grilli, E., Tugnoli, B., Passey, J.L., Stahl, C.H., Piva, A. and Moeser, A.J. 2015. Impact of dietary organic acids and botanicals on intestinal integrity and inflammation in weaned pigs. *BMC Veterinary Research.* **11**(1), p96.

Grim, J.M., Simonik, E.A., Semones, M.C., Kuhn, D.E. and Crockett, E.L. 2013. The glutathione-dependent system of antioxidant defense is not modulated by temperature acclimation in muscle tissues from striped bass, Morone saxatilis. *Comparative Biochemistry Physiology Part A: Molecular Integrative Physiology.* **164**(2), pp.383-390.

Gu, C., Howell, K., Padayachee, A., Comino, T., Chhan, R., Zhang, P., Ng, K., Cottrell, J.J. and Dunshea, F.R. 2019. Effect of a polyphenol-rich plant matrix on colonic digestion and plasma antioxidant capacity in a porcine model. *Journal of Functional Foods.* **57**, pp.211-221.

Gu, Z., Eils, R. and Schlesner, M. 2016. Complex heatmaps reveal patterns and correlations in multidimensional genomic data. *Bioinformatics.* **32**(18), pp.2847-2849.

Guerra-Ordaz, A.A., Gonzalez-Ortiz, G., La Ragione, R.M., Woodward, M.J., Collins, J.W., Perez, J.F. and Martin-Orue, S.M. 2014. Lactulose and Lactobacillus plantarum, a potential complementary synbiotic to control postweaning colibacillosis in piglets. *Applied Environmental Microbiology.* **80**(16), pp.4879-4886.

Guevarra, R.B., Hong, S.H., Cho, J.H., Kim, B.-R., Shin, J., Lee, J.H., Kang, B.N., Kim, Y.H., Wattanaphansak, S. and Isaacson, R.E. 2018. The dynamics of the piglet gut microbiome during the weaning transition in association with health and nutrition. *Animal Science Biotechnology.* **9**(1), pp.1-9.

Guevarra, R.B., Lee, J.H., Lee, S.H., Seok, M.J., Kim, D.W., Kang, B.N., Johnson, T.J., Isaacson, R.E. and Kim, H.B. 2019. Piglet gut microbial shifts early in life: causes and effects. *Animal Science and Biotechnology.* **10**(1), p1.

Guil-Guerrero, J., Ramos, L., Moreno, C., Zúniga-Paredes, J., Carlosama-Yépez, M. and Ruales, P. 2016. Plant-food by-products to improve farm-animal health. *Animal Feed Science Technology.* **220**, pp.121-135.

Gutierrez, N., Serão, N., Kerr, B., Zijlstra, R. and Patience, J. 2014. Relationships among dietary fiber components and the digestibility of energy, dietary fiber, and amino acids and energy content of nine corn coproducts fed to growing pigs. *Journal of Animal Science.* **92**(10), pp.4505-4517.

Gyles, C. and Fairbrother, J. 2010. *Escherichia coli in Pathogenesis of Bacterial infections in Animals*. Wiley-Blackwell, Ames, Iowa.

Haase, H. and Rink, L. 2009. The immune system and the impact of zinc during aging. *Immunity & Ageing.* **6**(1), p9.

Hadipour, E., Taleghani, A., Tayarani-Najaran, N. and Tayarani-Najaran, Z. 2020. Biological effects of red beetroot and betalains: A review. *Phytotherapy Research.* **34**(8), pp.1847-1867.

Haines, R., Beard, R., Chen, L., Eitnier, R. and Wu, M. 2016. Interleukin-1β mediates β-catenin-driven downregulation of claudin-3 and barrier dysfunction in caco2 cells. *Digestive Diseases Sciences.* **61**(8), pp.2252-2261.

Håkenåsen, I.M., Øverland, M., Ånestad, R., Åkesson, C.P., Sundaram, A.Y., Press, C.M. and Mydland, L.T. 2020. Gene expression and gastrointestinal function is altered in piglet small intestine by weaning and inclusion of Cyberlindnera jadinii yeast as a protein source. *Functional Foods.* **73**, p104118.

Hammer, K.A., Carson, C.F. and Riley, T.V. 1999. Antimicrobial activity of essential oils and other plant extracts. *Applied Microbiology.* **86**(6), pp.985-990.

Hampson, D. 1986. Alterations in piglet small intestinal structure at weaning. *Research in Veterinary Science.* **40**(1), pp.32-40.

Han, J., Ma, D., Zhang, M., Yang, X. and Tan, D. 2015. Natural antioxidant betanin protects rats from paraquat-induced acute lung injury interstitial pneumonia. *BioMed Research International.* **2015**.

Han, R., Álvarez, A.J.H., Maycock, J., Murray, B.S. and Boesch, C. 2021. Comparison of alcalase-and pepsin-treated oilseed protein hydrolysates–Experimental validation of predicted antioxidant, antihypertensive and antidiabetic properties. *Current Research in Food Science.* **4**, pp.141-149.

Han, Y., Zhao, Q., Tang, C., Li, Y., Zhang, K., Li, F. and Zhang, J. 2020. Butyrate mitigates weanling piglets from lipopolysaccharide-induced colitis by regulating microbiota and energy metabolism of the gut–liver axis. *Frontiers in Microbiology.* **11**, p588666.

Hao, R., Li, Q., Zhao, J., Li, H., Wang, W. and Gao, J. 2015. Effects of grape seed procyanidins on growth performance, immune function and antioxidant capacity in weaned piglets. *Livestock Science.* **178**, pp.237-242.

Hashemi, S.R. and Davoodi, H. 2011. Herbal plants and their derivatives as growth and health promoters in animal nutrition. *Veterinary Research Communications.* **35**(3), pp.169-180.

Hayes, J.D. and McLellan, L.I. 1999. Glutathione and glutathione-dependent enzymes represent a co-ordinately regulated defence against oxidative stress. *Free Radical Research* **31**(4), pp.273-300.

Hedemann, M., Eskildsen, M., Laerke, H., Pedersen, C., Lindberg, J.E., Laurinen, P. and Knudsen, K.B. 2006. Intestinal morphology and enzymatic activity in newly weaned pigs fed contrasting fiber concentrations and fiber properties. *Animal Science.* **84**(6), pp.1375-1386.

Heinritz, S.N., Weiss, E., Eklund, M., Aumiller, T., Louis, S., Rings, A., Messner, S., Camarinha-Silva, A., Seifert, J., Bischoff, S.C. and Mosenthin, R. 2016. Intestinal Microbiota and Microbial Metabolites Are Changed in a Pig Model Fed a High-Fat/Low-Fiber or a Low-Fat/High-Fiber Diet. *Plos One.* **11**(4).

Henning, S.M., Yang, J., Shao, P., Lee, R.-P., Huang, J., Ly, A., Hsu, M., Lu, Q.-Y., Thames, G. and Heber, D. 2017. Health benefit of vegetable/fruit juice-based diet: Role of microbiome. *Scientific reports.* **7**(1), p2167.

Heo, J.M., Opapeju, F.O., Pluske, J.R., Kim, J.C., Hampson, D.J. and Nyachoti, C.M. 2013. Gastrointestinal health and function in weaned pigs: a review of feeding strategies to control post-weaning diarrhoea without using in-feed antimicrobial compounds. *Animal Physiology Animal Nutrition.* **97**(2), pp.207-237.

Højberg, O., Canibe, N., Poulsen, H.D., Hedemann, M.S. and Jensen, B.B. 2005. Influence of dietary zinc oxide and copper sulfate on the gastrointestinal ecosystem in newly weaned piglets. *Applied Environmental Microbiology.* **71**(5), pp.2267-2277.

Holman, D.B. and Chénier, M.R. 2015. Antimicrobial use in swine production and its effect on the swine gut microbiota and antimicrobial resistance. *Canadian Journal of Microbiology.* **61**(11), pp.785-798.

Holmes, E., Li, J.V., Athanasiou, T., Ashrafian, H. and Nicholson, J.K. 2011. Understanding the role of gut microbiome–host metabolic signal disruption in health and disease. *Trends in Microbiology.* **19**(7), pp.349-359.

Holscher, H.D. 2017. Dietary fiber and prebiotics and the gastrointestinal microbiota. *Gut microbes.* **8**(2), pp.172-184.

Hölzel, C.S., Müller, C., Harms, K.S., Mikolajewski, S., Schäfer, S., Schwaiger, K. and Bauer, J. 2012. Heavy metals in liquid pig manure in light of bacterial antimicrobial resistance. *Environmental Research.* **113**, pp.21-27.

Honda, K. and Littman, D.R. 2016. The microbiota in adaptive immune homeostasis and disease. *Nature.* **535**(7610), pp.75-84.

Hong, T.T.T., Linh, N.Q., Ogle, B. and Lindberg, J.E. 2006. Survey on the prevalence of diarrhoea in pre-weaning piglets and on feeding systems as contributing risk factors in smallholdings in Central Vietnam. *Tropical Animal Health Production.* **38**(5), pp.397-405.

Hoover, D.M., Boulegue, C., Yang, D., Oppenheim, J.J., Tucker, K., Lu, W. and Lubkowski, J. 2002. The structure of human macrophage inflammatory protein-3α/CCL20: linking antimicrobial and cc chemokine receptor-6-binding activities with human β-defensins. *Journal of Biological Chemistry* **277**(40), pp.37647-37654.

Hu, C., Xiao, K., Song, J. and Luan, Z. 2013. Effects of zinc oxide supported on zeolite on growth performance, intestinal microflora and permeability, and cytokines expression of weaned pigs. *Animal Feed Science and Technology.* **181**(1-4), pp.65-71.

Hu, J., Nie, Y., Chen, J., Zhang, Y., Wang, Z., Fan, Q. and Yan, X. 2016. Gradual changes of gut microbiota in weaned miniature piglets. *Frontiers in Microbiology.* **7**, p1727.

Hu, Y., Yang, X., Li, J., Lv, N., Liu, F., Wu, J., Lin, I.Y., Wu, N., Weimer, B.C., Gao, G.F., Liu, Y. and Zhu, B. 2016. The Bacterial Mobile Resistome Transfer Network Connecting the Animal and Human Microbiomes. *Applied Environmental Microbiology.* **82**(22), pp.6672-6681.

Huang, C., Song, P., Fan, P., Hou, C., Thacker, P. and Ma, X. 2015. Dietary sodium butyrate decreases postweaning diarrhea by modulating intestinal permeability and changing the bacterial communities in weaned piglets. *Journal of Nutrition.* **145**(12), pp.2774-2780.

Hung, Y.T., Hu, Q., Faris, R.J., Guo, J., Urriola, P.E., Shurson, G.C., Chen, C. and Saqui-Salces, M. 2020. Analysis of gastrointestinal responses revealed both shared and specific targets of zinc oxide and carbadox in weaned pigs. *Antibiotics.* **9**(8), p463.

Huting, A.M.S., Middelkoop, A., Guan, X. and Molist, F. 2021. Using Nutritional Strategies to Shape the Gastro-Intestinal Tracts of Suckling and Weaned Piglets. *Animals.* **11**.

Ikhtaire, S., Shajib, M.S., Reinisch, W. and Khan, W.I. 2016. Fecal calprotectin: its scope and utility in the management of inflammatory bowel disease. *Journal of gastroenterology.* **51**, pp.434-446.

Inagaki, T., Moschetta, A., Lee, Y.K., Peng, L., Zhao, G., Downes, M., Ruth, T.Y., Shelton, J.M., Richardson, J.A. and Repa, J.J. 2006. Regulation of antibacterial defense in the small intestine by the nuclear bile acid receptor. *Proceedings of the National Academy of Science.* **103**(10), pp.3920-3925.

Beetroot Powder Market: Food & Beverages End User Segment : . 2017. [Online database].

Isaacson, R. and Kim, H.B. 2012. The intestinal microbiome of the pig. *Animal Health Research Reviews.* **13**(1), pp.100-109.

Ivanov, I.I. and Honda, K. 2012. Intestinal commensal microbes as immune modulators. *Cell Host Microbe.* **12**(4), pp.496-508.

Iwai, S., Weinmaier, T., Schmidt, B.L., Albertson, D.G., Poloso, N.J., Dabbagh, K. and DeSantis, T.Z. 2016. Piphillin: Improved Prediction of Metagenomic Content by Direct Inference from Human Microbiomes. *PLoS One.* **11**(11), pe0166104.

Jakobsen, N., Goecke, N.B. and Pedersen, K.S. 2022. Evaluation of the diagnostic performance of a commercially available point-of-care test for post weaning diarrhoea in pigs-a pilot study. *Porcine Health Management.* **8**(1), pp.1-6.

Jensen, J., Kyvsgaard, N.C., Battisti, A. and Baptiste, K.E. 2018. Environmental and public health related risk of veterinary zinc in pig production-using Denmark as an example. *Environment International.* **114**, pp.181-190.

Jensen, V.F., Emborg, H.D. and Aarestrup, F.M. 2012. Indications and patterns of therapeutic use of antimicrobial agents in the Danish pig production from 2002 to 2008. *Veterinary Pharmacology Therapeutics.* **35**(1), pp.33-46.

Jha, R. and Berrocoso, J.D. 2015. Review: Dietary fiber utilization and its effects on physiological functions and gut health of swine. *Animal.* **9**(9), pp.1441-1452.

Jha, R. and Berrocoso, J.F. 2016. Dietary fiber and protein fermentation in the intestine of swine and their interactive effects on gut health and on the environment: A review. *Animal Feed Science.* **212**, pp.18-26.

Jian, C., Luukkonen, P., Yki-Järvinen, H., Salonen, A. and Korpela, K. 2020. Quantitative PCR provides a simple and accessible method for quantitative microbiota profiling. *PloS one.* **15**(1), pe0227285.

Jocken, J.W., González Hernández, M.A., Hoebers, N.T., Van der Beek, C.M., Essers, Y.P., Blaak, E.E. and Canfora, E.E. 2018. Short-chain fatty acids differentially affect intracellular lipolysis in a human white adipocyte model. *Frontiers in Endocrinology.* **8**, p372.

Johanns, V.C., Ghazisaeedi, F., Epping, L., Semmler, T., Lübke-Becker, A., Pfeifer, Y., Bethe, A., Eichhorn, I., Merle, R., Walther, B. and Wieler, L.H. 2019. Effects of a Four-Week High-Dosage Zinc Oxide Supplemented Diet on Commensal Escherichia coli of Weaned Pigs. *Frontiers in Microbiology.* **10**, pp.2734-2734.

Johansson, M.E., Ambort, D., Pelaseyed, T., Schütte, A., Gustafsson, J.K., Ermund, A., Subramani, D.B., Holmén-Larsson, J.M., Thomsson, K.A. and Bergström, J.H. 2011. Composition and functional role of the mucus layers in the intestine. *Cellular and Molecular Life Sciences.* **68**(22), pp.3635-3641.

Johansson, M.E. and Hansson, G.C. 2016. Immunological aspects of intestinal mucus and mucins. *Nature Reviews Immunology.* **16**(10), pp.639-649.

Jondreville, C., Revy, P. and Dourmad, J.-Y. 2003. Dietary means to better control the environmental impact of copper and zinc by pigs from weaning to slaughter. *Livestock Production Science.* **84**(2), pp.147-156.

Jugl-Chizzola, M., Ungerhofer, E., Gabler, C., Hagmüller, W., Chizzola, R., Zitterl-Eglseer, K. and Franz, C. 2006. Testing of the palatability of Thymus vulgaris L. and Origanum vulgare L. as flavouring feed additive for weaner pigs on the basis of a choice experiment. *Berliner Und Munchener Tierarztliche Wochenschrift.* **19**(5-6), pp.238-243.

Jukic, A., Bakiri, L., Wagner, E.F., Tilg, H. and Adolph, T.E. 2021. Calprotectin: from biomarker to biological function. *Gut.* **70**(10), pp.1978-1988.

Kaakoush, N.O. 2015. Insights into the Role of Erysipelotrichaceae in the Human Host. *Frontiers in Cellular and Infection Microbiology.* **5**, p84.

Kaevska, M., Lorencova, A., Videnska, P., Sedlar, K., Provaznik, I. and Trckova, M.J.V.m. 2016. Effect of sodium humate and zinc oxide used in prophylaxis of post-weaning diarrhoea on faecal microbiota composition in weaned piglets. **61**(6), pp.328-336.

Kaminsky, L.W., Al-Sadi, R. and Ma, T.Y. 2021. Il-1β and the intestinal epithelial tight junction barrier. *Frontiers in Immunology.* **12**, p767456.

Kanner, J., Harel, S. and Granit, R. 2001. Betalains a new class of dietary cationized antioxidants. *Journal of Agricultural Food chemistry.* **49**(11), pp.5178-5185.

Kao, D., Roach, B., Silva, M., Beck, P., Rioux, K., Kaplan, G.G., Chang, H.J., Coward, S., Goodman, K.J., Xu, H.P., Madsen, K., Mason, A., Wong, G.K.S., Jovel, J., Patterson, J. and Louie, T. 2017. Effect of Oral Capsule-vs Colonoscopy-Delivered Fecal Microbiota Transplantation on Recurrent Clostridium difficile Infection A Randomized Clinical Trial. *Journal of the American Medical Association.* **318**(20), pp.1985-1993.

Kapadia, G.J. and Rao, G.S. 2013. Anticancer effects of red beet pigments. *Red beet Biotechnology.* Springer, pp.125-154.

Kathiravan, T., Nadanasabapathi, S. and Kumar, R.J.I.F.R.J. 2014. Standardization of process condition in batch thermal pasteurization and its effect on antioxidant, pigment and microbial inactivation of Ready to Drink (RTD) beetroot (Beta vulgaris L.) juice. *International Food Research Journal.* **21**(4).

Kathirvelan, C., Banupriya, S. and Purushothaman, M. 2015. Azolla-an alternate and sustainable feed for livestock. *International Journal of Science, Environment Technology.* **4**(4), pp.1153-1157.

Katongole, C., Bareeba, F., Sabiiti, E. and Ledin, I. 2008. Nutritional characterization of some tropical urban market crop wastes. *Animal Feed Science and Technology.* **142**(3-4), pp.275-291.

Katongole, C.B., Sabiiti, E., Bareeba, F. and Ledin, I. 2011. Utilization of market crop wastes as animal feed in urban and peri-urban livestock production in Uganda. *Journal of Sustainable Agriculture.* **35**(3), pp.329-342.

Khan, M.I. 2016. Plant betalains: Safety, antioxidant activity, clinical efficacy, and bioavailability. *Comprehensive Reviews in Food Science, Food Safety.* **15**(2), pp.316-330.

Kick, A., Tompkins, M., Flowers, W., Whisnant, C. and Almond, G. 2012. Effects of stress associated with weaning on the adaptive immune system in pigs. *Animal Science.* **90**(2), pp.649-656.

Kil, D.Y. and Stein, H. 2010. Board invited review: management and feeding strategies to ameliorate the impact of removing antibiotic growth promoters from diets fed to weanling pigs. *Canadian Journal of Animal Science.* **90**(4), pp.447-460.

Kim, C.H., Park, J. and Kim, M. 2014. Gut microbiota-derived short-chain fatty acids, T cells, and inflammation. *Immune network.* **14**(6), pp.277-288.

Kim, D.P., Saegerman, C., Douny, C., Dinh, T.V., Xuan, B.H., Vu, B.D., Hong, N.P. and Scippo, M.-L. 2013. First survey on the use of antibiotics in pig and poultry production in the Red River Delta region of Vietnam. *Food Public Health.* **3**(5), pp.247-256.

Kim, H.B., Borewicz, K., White, B.A., Singer, R.S., Sreevatsan, S., Tu, Z.J. and Isaacson, R.E. 2011. Longitudinal investigation of the age-related bacterial diversity in the feces of commercial pigs. *Veternary Microbiology.* **153**(1-2), pp.124-133.

Kim, H.B., Borewicz, K., White, B.A., Singer, R.S., Sreevatsan, S., Tu, Z.J. and Isaacson, R.E. 2012. Microbial shifts in the swine distal gut in response to the treatment with antimicrobial growth promoter, tylosin. *Proceedings of the National Academy of Sciences USA.* **109**(38), pp.15485-15490.

Kim, H.B. and Isaacson, R.E. 2015. The pig gut microbial diversity: Understanding the pig gut microbial ecology through the next generation high throughput sequencing. *Veterinary Microbiology.* **177**(3-4), pp.242-251.

Kim, J.C., Hansen, C.F., Mullan, B.P. and Pluske, J.R. 2012. Nutrition and pathology of weaner pigs: Nutritional strategies to support barrier function in the gastrointestinal tract. *Animal Feed Science and Technology.* **173**(1-2), pp.3-16.

Kim, K. 2019. Nutritional Intervention for the Intestinal Development and Health of Weaned Pigs. *Frontiers in Veterinary Science.* **6**.

Kim, S., Lee, Y., Kim, Y., Seo, Y., Lee, H., Ha, J., Lee, J., Choi, Y., Oh, H. and Yoon, Y. 2020. Akkermansia muciniphila prevents fatty liver disease, decreases serum triglycerides, and maintains gut homeostasis. *Applied Environmental Microbiology.* **86**(7), pp.e03004-03019.

Kim, Y.S. and Ho, S.B. 2010. Intestinal goblet cells and mucins in health and disease: recent insights and progress. *Current Gastroenterology Reports.* **12**(5), pp.319-330.

Kim, Y.S., Young, M.R., Bobe, G., Colburn, N.H. and Milner, J.A. 2009. Bioactive Food Components, Inflammatory Targets, and Cancer PreventionDiet, Inflammation, and Cancer Prevention. *Cancer Prevention Research.* **2**(3), pp.200-208.

Klewicka, E. and Czyzowska, A. 2011. Biological stability of lactofermented beetroot juice during refrigerated storage. *Polish Journal of Food Nutrition Sciences.* **61**(4).

Klewicka, E., Zduńczyk, Z. and Juśkiewicz, J. 2009. Effect of lactobacillus fermented beetroot juice on composition and activity of cecal microflora of rats. *European Food Research Technology.* **229**, pp.153-157.

Klinger, A.C.K., Silva, L., de Toledo, G.S.P., Chimainski, C., Camera, A., Rodrigues, M. and Rocha, L. 2017. Carrot tops as a partial substitute for alfalfa hay on diets for growing rabbits. *Archivos de zootecnia.* **66**(256), pp.563-566.

Klurfeld, D.M., Davis, C.D., Karp, R.W., Allen-Vercoe, E., Chang, E.B., Chassaing, B., Fahey, G.C., Hamaker, B.R., Holscher, H.D. and Lampe, J.W. 2018. *Considerations for best practices in studies of fiber or other dietary components and the intestinal microbiome*. American Physiological Society Bethesda, MD.

Knudsen, K.E.B. 1997. Carbohydrate and lignin contents of plant materials used in animal feeding. *Animal Feed Science Technology.* **67**(4), pp.319-338.

Knudsen, K.E.B. 2014. Fiber and nonstarch polysaccharide content and variation in common crops used in broiler diets. *Poultry Science.* **93**(9), pp.2380-2393.

Koch, B.J., Hungate, B.A. and Price, L.B. 2017. Food-animal production and the spread of antibiotic resistance: the role of ecology. *Frontiers in Ecology the Environment.* **15**(6), pp.309-318.

Kohen, R. and Nyska, A. 2002. Invited review: Oxidation of biological systems: oxidative stress phenomena, antioxidants, redox reactions, and methods for their quantification. *Toxicologic Pathology.* **30**(6), pp.620-650.

Komatsu, T., Sugie, K., Inukai, N., Eguchi, O., Oyamada, T., Sawada, H., Yamanaka, N. and Shibahara, T. 2020. Chronic pancreatitis in farmed pigs fed excessive zinc oxide. *Veterinary Diagnostic Investigation.* **32**, pp.689 - 694.

Kozich, J.J., Westcott, S.L., Baxter, N.T., Highlander, S.K. and Schloss, P.D. 2013. Development of a dual-index sequencing strategy and curation pipeline for analyzing amplicon sequence data on the MiSeq Illumina sequencing platform. *Applied Environmental Microbiology.* **79**(17), pp.5112-5120.

Krajka-Kuźniak, V., Paluszczak, J., Szaefer, H. and Baer-Dubowska, W. 2013. Betanin, a beetroot component, induces nuclear factor erythroid-2-related factor 2-mediated expression of detoxifying/antioxidant enzymes in human liver cell lines. *British Journal of Nutrition.* **110**(12), pp.2138-2149.

Krajka-Kuźniak, V., Szaefer, H., Ignatowicz, E., Adamska, T. and Baer-Dubowska, W. 2012. Beetroot juice protects against N-nitrosodiethylamine-induced liver injury in rats. *Food Chemical Toxicology.* **50**(6), pp.2027-2033.

Krishnan, S., Ding, Y., Saedi, N., Choi, M., Sridharan, G.V., Sherr, D.H., Yarmush, M.L., Alaniz, R.C., Jayaraman, A. and Lee, K. 2018. Gut microbiota-derived tryptophan metabolites modulate inflammatory response in hepatocytes and macrophages. *Cell Reports.* **23**(4), pp.1099-1111.

Kujala, T., Loponen, J. and Pihlaja, K. 2001. Betalains and phenolics in red beetroot (Beta vulgaris) peel extracts: extraction and characterisation. *Z Naturforsch C Journal of Biosciences.* **56**(5-6), pp.343-348.

Kujala, T.S., Vienola, M.S., Klika, K.D., Loponen, J.M. and Pihlaja, K. 2002. Betalain and phenolic compositions of four beetroot (Beta vulgaris) cultivars. *European Food Research Technology.* **214**(6), pp.505-510.

Kumar, H., Bhardwaj, K., Sharma, R., Nepovimova, E., Kuča, K., Dhanjal, D.S., Verma, R., Bhardwaj, P., Sharma, S. and Kumar, D. 2020. Fruit and vegetable peels: Utilization of high value horticultural waste in novel industrial applications. *Molecules.* **25**(12), p2812.

Kumar, S. and Brooks, M.S.-L. 2018a. Use of red beet (Beta vulgaris L.) for antimicrobial applications—a critical review. *Food Bioprocess Technology.* **11**(1), pp.17-42.

Kumar, S. and Brooks, M.S.-L. 2018b. Use of red beet (Beta vulgaris L.) for antimicrobial applications—a critical review. *Food Bioprocess Technology.* **11**, pp.17-42.

Kumar, S. and Pandey, A.K. 2013. Chemistry and biological activities of flavonoids: an overview. *Scientific World Journal.* **2013**, p162750.

Kurundkar, A.R., Killingsworth, C.R., McIlwain, R.B., Timpa, J.G., Hartman, Y.E., He, D., Karnatak, R.K., Neel, M.L., Clancy, J.P. and Anantharamaiah, G. 2010. Extracorporeal membrane oxygenation causes loss of intestinal epithelial barrier in the newborn piglet. *Pediatric Research.* **68**(2), pp.128-133.

Lackeyram, D., Yang, C., Archbold, T., Swanson, K.C. and Fan, M.Z. 2010. Early weaning reduces small intestinal alkaline phosphatase expression in pigs. *Journal of Nutrition.* **140**(3), pp.461-468.

Laitat, M., Antoine, N., Cabaraux, J.F., Cassart, D., Mainil, J., Moula, N., Nicks, B., Wavreille, J. and Philippe, F.X. 2015. Influence of sugar beet pulp on feeding behavior, growth performance, carcass quality and gut health of fattening pigs. *Biotechnologie Agronomie Societe Et Environnement.* **19**(1), pp.20-31.

Lallès, J.-P. 2010. Intestinal alkaline phosphatase: multiple biological roles in maintenance of intestinal homeostasis and modulation by diet. *Nutrition Reviews.* **68**(6), pp.323-332.

Lallès, J.-P., Bosi, P., Smidt, H. and Stokes, C.R. 2007. Weaning—a challenge to gut physiologists. *Livestock Science.* **108**(1-3), pp.82-93.

Lallès, J.-P., Boudry, G., Favier, C., Le Floc'h, N., Luron, I., Montagne, L., Oswald, I.P., Pié, S., Piel, C. and Sève, B. 2004. Gut function and dysfunction in young pigs: physiology. *Animal Research.* **53**(4), pp.301-316.

Lallès, J.-P. and Fagerhol, M.K. 2005. Faecal calprotectin: a non invasive marker of inflammation in pigs. *ISAH.* **1**, pp.405-408.

Lamendella, R., Santo Domingo, J.W., Ghosh, S., Martinson, J. and Oerther, D.B. 2011. Comparative fecal metagenomics unveils unique functional capacity of the swine gut. *BMC Microbiology.* **11**, pp.1-17.

Lan, R., Tran, H. and Kim, I. 2017. Effects of probiotic supplementation in different nutrient density diets on growth performance, nutrient digestibility, blood profiles, fecal microflora and noxious gas emission in weaning pig. *Journal of the Science of Food Agriculture.* **97**(4), pp.1335-1341.

Landete, J.M. 2013. Dietary intake of natural antioxidants: vitamins and polyphenols. *Critical Reviews in Food Science and Nutrition.* **53**(7), pp.706-721.

Langille, M.G., Zaneveld, J., Caporaso, J.G., McDonald, D., Knights, D., Reyes, J.A., Clemente, J.C., Burkepile, D.E., Thurber, R.L.V. and Knight, R. 2013. Predictive functional profiling of microbial communities using 16S rRNA marker gene sequences. *Nature Biotechnology.* **31**(9), pp.814-821.

Lazarus, B., Paterson, D.L., Mollinger, J.L. and Rogers, B.A. 2015. Do human extraintestinal Escherichia coli infections resistant to expanded-spectrum cephalosporins originate from food-producing animals? A systematic review. *Clinical Infectious Diseases.* **60**(3), pp.439-452.

Lecce, J.G., Armstrong, W.D., Crawford, P.C. and Ducharme, G.A. 1979. Nutrition and management of early weaned piglets liquid vs dry feeding. *Journal of Animal Science.* **48**(5), pp.107-114.

Lechner, J.F. and Stoner, G.D. 2019. Red beetroot and betalains as cancer chemopreventative agents. *Molecules.* **24**(8), p1602.

Lechner, J.F., Wang, L.-S., Rocha, C.M., Larue, B., Henry, C., McIntyre, C.M., Riedl, K.M., Schwartz, S.J. and Stoner, G.D. 2010. Drinking water with red beetroot food color antagonizes esophageal carcinogenesis in N-nitrosomethylbenzylamine-treated rats. *Journal of Medicinal Food.* **13**(3), pp.733-739.

Lee, C.H., Wettasinghe, M., Bolling, B.W., Ji, L.-L. and Parkin, K.L. 2005. Betalains, phase II enzyme-inducing components from red beetroot (Beta vulgaris L.) extracts. *Nutrition cancer.* **53**(1), pp.91-103.

Lee, J.H., Son, C.W., Kim, M.Y., Kim, M.H., Kim, H.R., Kwak, E.S., Kim, S. and Kim, M.R. 2009. Red beet (Beta vulgaris L.) leaf supplementation improves antioxidant status in C57BL/6J mice fed high fat high cholesterol diet. *Nutrition Research Practice.* **3**(2), pp.114-121.

Lee, S.R. 2018. Critical role of zinc as either an antioxidant or a prooxidant in cellular systems. *Oxidative Medicine Cellular Longevity.* 2018**;** 8915684.

Lee, W.J. and Hase, K. 2014. Gut microbiota–generated metabolites in animal health and disease. *Nature Chemical Biology.* **10**(6), pp.416-424.

Leijdekkers, A.G., Aguirre, M., Venema, K., Bosch, G., Gruppen, H. and Schols, H.A. 2014. In vitro fermentability of sugar beet pulp derived oligosaccharides using human and pig fecal inocula. *Journal of Agricultural Food Chemistry.* **62**(5), pp.1079-1087.

Lekagul, A., Tangcharoensathien, V. and Yeung, S. 2019. Patterns of antibiotic use in global pig production: a systematic review. *Veterinary Animal Science.* **7**, p100058.

Leser, T.D., Lindecrona, R.H., Jensen, T.K., Jensen, B.B. and Moller, K. 2000. Changes in bacterial community structure in the colon of pigs fed different experimental diets and after infection with Brachyspira hyodysenteriae. *Applied Environmental Microbiology.* **66**(8), pp.3290-3296.

Leser, T.D. and Molbak, L. 2009. Better living through microbial action: the benefits of the mammalian gastrointestinal microbiota on the host. *Environmental Microbiology.* **11**(9), pp.2194-2206.

Lewis, K. 2001. Riddle of biofilm resistance. *Antimicrobial Agents Chemotherapy* **45**(4), pp.999-1007.

Li, B., Van Kessel, A., Caine, W., Huang, S. and Kirkwood, R. 2001. Small intestinal morphology and bacterial populations in ileal digesta and feces of newly weaned pigs receiving a high dietary level of zinc oxide. *Canadian Journal of Animal Science.* **81**(4), pp.511-516.

Li, F., Jiang, C., Krausz, K.W., Li, Y., Albert, I., Hao, H., Fabre, K.M., Mitchell, J.B., Patterson, A.D. and Gonzalez, F.J. 2013. Microbiome remodelling leads to inhibition of intestinal farnesoid X receptor signalling and decreased obesity. *Nature Communications.* **4**(1), pp.1-10.

Li, H., Li, H., Xie, P., Li, Z., Yin, Y., Blachier, F. and Kong, X. 2019. Dietary supplementation with fermented Mao-tai lees beneficially affects gut microbiota structure and function in pigs. *Amb Express.* **9**(1), pp.1-14.

Li, H.H., Li, Y.P., Zhu, Q., Qiao, J.Y. and Wang, W.J. 2018. Dietary supplementation with Clostridium butyricum helps to improve the intestinal barrier function of weaned piglets challenged with enterotoxigenic Escherichia coli K88. *Journal of Applied Microbiology.* **125**(4), pp.964-975.

Li, K., Xiao, Y., Chen, J., Chen, J., He, X. and Yang, H. 2017. Microbial composition in different gut locations of weaning piglets receiving antibiotics. *Asian-Australasian Journal of Animal Sciences.* **30**(1), pp.78-84.

Li, Q., Peng, X., Burrough, E.R., Sahin, O., Gould, S.A., Gabler, N.K., Loving, C.L., Dorman, K.S. and Patience, J.F. 2020. Dietary soluble and insoluble fiber with or without enzymes altered the intestinal microbiota in weaned pigs challenged with enterotoxigenic E. coli F18. *Frontiers in Microbiology.* **11**, p1110.

Li, Q., Yan, H., Li, H., Gao, J. and Hao, R. 2020. Effects of dietary supplementation with grape seed procyanidins on nutrient utilisation and gut function in weaned piglets. *Animal.* **14**(3), pp.491-498.

Li, S., Ru, Y., Liu, M., Xu, B., Péron, A. and Shi, X. 2012. The effect of essential oils on performance, immunity and gut microbial population in weaner pigs. *Livestock Science.* **145**(1-3), pp.119-123.

Li, T. and Chiang, J.Y. 2015. Bile acids as metabolic regulators. *Current Opinion in Gastroenterology.* **31**(2), p159.

Li, X., Dong, B., Li, D. and Yin, J. 2010. Mechanisms involved in the growth promotion of weaned piglets by high-level zinc oxide. *Animal Science and Biotechnology.* **1**(1), pp.59-67.

Li, Y., Guo, Y., Wen, Z., Jiang, X., Ma, X. and Han, X. 2018. Weaning stress perturbs gut microbiome and its metabolic profile in piglets. *Scientific Reports.* **8**(1), pp.1-12.

Liao, S.F. and Nyachoti, M. 2017. Using probiotics to improve swine gut health and nutrient utilization. *Animal Nutrition.* **3**(4), pp.331-343.

Lichtenthäler, R. and Marx, F. 2005. Total oxidant scavenging capacities of common European fruit and vegetable juices. *Journal of Agricultural Food Chemistry.* **53**(1), pp.103-110.

Lillehoj, H., Liu, Y., Calsamiglia, S., Fernandez-Miyakawa, M.E., Chi, F., Cravens, R.L., Oh, S. and Gay, C.G. 2018. Phytochemicals as antibiotic alternatives to promote growth and enhance host health. *Veterinary Research.* **49**(1), pp.1-18.

Lim, T. and Lim, T. 2016. Beta vulgaris. *Edible Medicinal Non-Medicinal Plants:.* Modified Stems, Roots, Bulbs, **10**, pp.26-68.

Lin, S., Yang, X., Long, Y., Zhong, H., Wang, P., Yuan, P., Zhang, X., Che, L., Feng, B. and Li, J. 2020. Dietary supplementation with Lactobacillus plantarum modified gut microbiota, bile acid profile and glucose homoeostasis in weaning piglets. *British Journal of Nutrition.* **124**(8), pp.797-808.

Lin, S., Yang, X., Yuan, P., Yang, J., Wang, P., Zhong, H., Zhang, X., Che, L., Feng, B. and Li, J. 2019. Undernutrition shapes the gut microbiota and bile acid profile in association with altered gut-liver FXR signaling in weaning pigs. *Journal of Agricultural Food Chemistry.* **67**(13), pp.3691-3701.

Lindstrom, M.J. and Bates, D.M. 1988. Newton-Raphson and Em Algorithms for Linear Mixed-Effects Models for Repeated-Measures Data. *American Statistical Association.* **83**(404), pp.1014-1022.

Lippolis, J. 2008. Immunological signaling networks: integrating the body's immune response. *Animal Science* **86**(suppl_14), pp.E53-E63.

Liu, B., Wang, W., Zhu, X., Sun, X., Xiao, J., Li, D., Cui, Y., Wang, C. and Shi, Y. 2018. Response of Gut Microbiota to Dietary Fiber and Metabolic Interaction With SCFAs in Piglets. *Frontiers in Microbiology.* **9**, p2344.

Liu, H., Hou, C., Li, N., Zhang, X., Zhang, G., Yang, F., Zeng, X., Liu, Z. and Qiao, S. 2019. Microbial and metabolic alterations in gut microbiota of sows during pregnancy and lactation. *The FASEB Journal.* **33**(3), pp.4490-4501.

Liu, H., Zhang, J., Zhang, S., Yang, F., Thacker, P.A., Zhang, G., Qiao, S. and Ma, X. 2014. Oral administration of Lactobacillus fermentum I5007 favors intestinal development and alters the intestinal microbiota in formula-fed piglets. *Agricultural Food Chemistry.* **62**(4), pp.860-866.

Liu, Y., Espinosa, C.D., Abelilla, J.J., Casas, G.A., Lagos, L.V., Lee, S.A., Kwon, W.B., Mathai, J.K., Navarro, D.M.D.L., Jaworski, N.W. and Stein, H.H. 2018. Non-antibiotic feed additives in diets for pigs: A review. *Animal Nutrition.* **4**(2), pp.113-125.

Liu, Y., Song, M., Che, T., Almeida, J., Lee, J., Bravo, D., Maddox, C. and Pettigrew, J. 2013. Dietary plant extracts alleviate diarrhea and alter immune responses of weaned pigs experimentally infected with a pathogenic Escherichia coli. *Animal Science.* **91**(11), pp.5294-5306.

Liu, Y., Song, M., Che, T., Bravo, D. and Pettigrew, J. 2012. Anti-inflammatory effects of several plant extracts on porcine alveolar macrophages in vitro. *Animal Science.* **90**(8), pp.2774-2783.

Liu, Y., Yu, X., Zhao, J., Zhang, H., Zhai, Q. and Chen, W. 2020. The role of MUC2 mucin in intestinal homeostasis and the impact of dietary components on MUC2 expression. *International Journal of Biological Macromolecules.* **164**, pp.884-891.

Livak, K.J. and Schmittgen, T.D. 2001. Analysis of relative gene expression data using real-time quantitative PCR and the 2− ΔΔCT method. *Methods.* **25**(4), pp.402-408.

Looft, T., Johnson, T.A., Allen, H.K., Bayles, D.O., Alt, D.P., Stedtfeld, R.D., Sul, W.J., Stedtfeld, T.M., Chai, B., Cole, J.R., Hashsham, S.A., Tiedje, J.M. and Stanton, T.B. 2012. In-feed antibiotic effects on the swine intestinal microbiome. *Proceedings of the National Academy of Sciences.* **109**(5), pp.1691-1696.

Lorizola, I.M., Furlan, C.P., Portovedo, M., Milanski, M., Botelho, P.B., Bezerra, R.M., Sumere, B.R., Rostagno, M.A. and Capitani, C.D. 2018. Beet stalks and leaves (Beta vulgaris L.) protect against high-fat diet-induced oxidative damage in the liver in mice. *Nutrients.* **10**(7), p872.

Louis, P., Duncan, S.H., Sheridan, P.O., Walker, A.W. and Flint, H.J. 2022. Microbial lactate utilisation and the stability of the gut microbiome. *Gut Microbiome.* **3**, pe3.

Louis, P. and Flint, H.J. 2017. Formation of propionate and butyrate by the human colonic microbiota. *Environmental microbiology.* **19**(1), pp.29-41.

Love, M.I., Huber, W. and Anders, S. 2014. Moderated estimation of fold change and dispersion for RNA-seq data with DESeq2. *Genome Biology.* **15**(12), pp.1-21.

Low, D.Y., Pluschke, A.M., Flanagan, B., Sonni, F., Grant, L.J., Williams, B.A. and Gidley, M.J. 2021. Isolated pectin (apple) and fruit pulp (mango) impact gastric emptying, passage rate and short chain fatty acid (SCFA) production differently along the pig gastrointestinal tract. *Food Hydrocolloids.* **118**, p106723.

Lu, S.C. 2009. Regulation of glutathione synthesis. *Molecular Aspects of Medicine.* **30**(1-2), pp.42-59.

Lu, X., Wang, Y. and Zhang, Z. 2009. Radioprotective activity of betalains from red beets in mice exposed to gamma irradiation. *European Journal of Pharmacology.* **615**(1-3), pp.223-227.

Luppi, A., Gibellini, M., Gin, T., Vangroenweghe, F., Vandenbroucke, V., Bauerfeind, R., Bonilauri, P., Labarque, G. and Hidalgo, Á. 2016. Prevalence of virulence factors in enterotoxigenic Escherichia coli isolated from pigs with post-weaning diarrhoea in Europe. *Porcine Health Management.* **2**(1), pp.1-6.

Ma, T.Y., Iwamoto, G.K., Hoa, N.T., Akotia, V., Pedram, A., Boivin, M.A. and Said, H.M. 2004. TNF-α-induced increase in intestinal epithelial tight junction permeability requires NF-κB activation. *American Journal of Physiology-Gastrointestinal Liver Physiology.* **286**(3), pp.G367-G376.

Ma, X., Fan, P.X., Li, L.S., Qiao, S.Y., Zhang, G.L. and Li, D.F. 2012. Butyrate promotes the recovering of intestinal wound healing through its positive effect on the tight junctions. *Journal of Animal Science.* **90**(suppl_4), pp.266-268.

Ma, Y., Liu, X. and Wang, J. 2022. Small molecules in the big picture of gut microbiome-host cross-talk. *EBioMedicine.* **81**, p104085.

Macfarlane, G.T. and Macfarlane, S. 2012. Bacteria, colonic fermentation, and gastrointestinal health. *Journal of AOAC International.* **95**(1), pp.50-60.

MacIntyre, C.R. and Bui, C.M. 2017. Pandemics, public health emergencies and antimicrobial resistance-putting the threat in an epidemiologic and risk analysis context. *Archives of Public Health.* **75**(1), pp.1-6.

Madadi, E., Mazloum-Ravasan, S., Yu, J.S., Ha, J.W., Hamishehkar, H. and Kim, K.H. 2020. Therapeutic application of betalains: A review. *Plants.* **9**(9), p1219.

Malan, M., Müller, F., Raitt, L., Aalbers, J., Cyster, L. and Brendonck, L. 2015. Farmyard manures: the major agronomic sources of heavy metals in the Philippi Horticultural Area in the Western Cape Province of South Africa. *Environmental Monitoring Assessment.* **187**(11), pp.1-7.

Malik, Y.S., Verma, A.K., Kumar, N., Touil, N., Karthik, K., Tiwari, R., Bora, D.P., Dhama, K., Ghosh, S. and Hemida, M.G. 2019. Advances in diagnostic approaches for viral etiologies of diarrhea: from the lab to the field. *Frontiers in Microbiology.* **10**, p1957.

Manach, C., Williamson, G., Morand, C., Scalbert, A. and Rémésy, C. 2005. Bioavailability and bioefficacy of polyphenols in humans. I. Review of 97 bioavailability studies. *American Journal of Clinical Nutrition* **81**(1), pp.230S-242S.

Mani, V., Weber, T.E., Baumgard, L.H. and Gabler, N.K. 2012. Growth and development symposium: endotoxin, inflammation, and intestinal function in livestock. *Animal Science.* **90**(5), pp.1452-1465.

Manzanilla, E., Perez, J., Martin, M., Kamel, C., Baucells, F. and Gasa, J. 2004. Effect of plant extracts and formic acid on the intestinal equilibrium of early-weaned pigs. *Animal Science.* **82**(11), pp.3210-3218.

Marchesi, J.R., Adams, D.H., Fava, F., Hermes, G.D., Hirschfield, G.M., Hold, G., Quraishi, M.N., Kinross, J., Smidt, H. and Tuohy, K.M. 2016. The gut microbiota and host health: a new clinical frontier. *Gut.* **65**(2), pp.330-339.

Maron, D.F., Smith, T.J. and Nachman, K.E. 2013. Restrictions on antimicrobial use in food animal production: an international regulatory and economic survey. *Global Health.* **9**, p48.

Marshall, B.M. and Levy, S.B. 2011. Food animals and antimicrobials: impacts on human health. *Clinical Microbiology Reviews* **24**(4), pp.718-733.

Martin, L., Pieper, R., Schunter, N., Vahjen, W. and Zentek, J. 2013. Performance, organ zinc concentration, jejunal brush border membrane enzyme activities and mRNA expression in piglets fed with different levels of dietary zinc. *Archives of Animal Nutrition.* **67**, pp.248 - 261.

Martínez-Rodríguez, P., Guerrero-Rubio, M.A., Henarejos-Escudero, P., García-Carmona, F. and Gandía-Herrero, F. 2022. Health-promoting potential of betalains in vivo and their relevance as functional ingredients: A review. *Trends in Food Science Technology.* **22,** pp.66-82.

Martínez, I., Perdicaro, D.J., Brown, A.W., Hammons, S., Carden, T.J., Carr, T.P., Eskridge, K.M. and Walter, J. 2013. Diet-induced alterations of host cholesterol metabolism are likely to affect the gut microbiota composition in hamsters. *Applied Environmental Microbiology.* **79**(2), pp.516-524.

Martínez, I., Wallace, G., Zhang, C., Legge, R., Benson, A.K., Carr, T.P., Moriyama, E.N. and Walter, J. 2009. Diet-induced metabolic improvements in a hamster model of hypercholesterolemia are strongly linked to alterations of the gut microbiota. *Applied Environmental Microbiology.* **75**(12), pp.4175-4184.

Martínez, R., Torres, P., Meneses, M.A., Figueroa, J.G., Pérez-Álvarez, J.A. and Viuda-Martos, M. 2012. Chemical, technological and in vitro antioxidant properties of mango, guava, pineapple and passion fruit dietary fibre concentrate. *Food Chemistry.* **135**(3), pp.1520-1526.

Masih, D., Singh, N. and Singh, A. 2019. Red beetroot: A source of natural colourant and antioxidants: A review. *Pharmacognosy Phytochemistry.* **8**(4), pp.162-166.

Matheson, S.M., Edwards, S.A. and Kyriazakis, I. 2022. Farm characteristics affecting antibiotic consumption in pig farms in England. *Porcine Health Management.* **8**(1), p7.

Mayeur, C., Gratadoux, J.-J., Bridonneau, C., Chegdani, F., Larroque, B., Kapel, N., Corcos, O., Thomas, M. and Joly, F. 2013. Faecal D/L lactate ratio is a metabolic signature of microbiota imbalance in patients with short bowel syndrome. *PLoS One.* **8**(1), pe54335.

McCrackin, M., Helke, K.L., Galloway, A.M., Poole, A.Z., Salgado, C.D. and Marriott, B.P. 2016. Effect of antimicrobial use in agricultural animals on drug-resistant foodborne campylobacteriosis in humans: a systematic literature review. *Critical Reviews in Food Science Nutrition.* **56**(13), pp.2115-2132.

McDermott, K., Lee, M.R., McDowall, K.J. and Greathead, H.M. 2020. Cross inoculation of rumen fluid to improve dry matter disappearance and its effect on bacterial composition using an in vitro batch culture model. *Frontiers in Microbiology.* **11**, p2293.

McMurdie, P.J. and Holmes, S. 2013. phyloseq: an R package for reproducible interactive analysis and graphics of microbiome census data. *PLoS One.* **8**(4), pe61217.

Meléndez, D.M., Marti, S., Faucitano, L., Haley, D.B., Schwinghamer, T.D. and Schwartzkopf-Genswein, K.S. 2021. Correlation between L-Lactate Concentrations in Beef Cattle, Obtained Using a Hand-Held Lactate Analyzer and a Lactate Assay Colorimetric Kit. *Animals.* **11**(4), p926.

Meng, Q., Luo, Z., Cao, C., Sun, S., Ma, Q., Li, Z., Shi, B. and Shan, A. 2020. Weaning alters intestinal gene expression involved in nutrient metabolism by shaping gut microbiota in pigs. *Frontiers in Microbiology.* **11**, p694.

Menon, V.P. and Sudheer, A.R. 2007. Antioxidant and anti-inflammatory properties of curcumin. *Advances in Experimental and Medical Biology.* **595**, pp.105-125.

Merle, R., Hajek, P., Käsbohrer, A., Hegger-Gravenhorst, C., Mollenhauer, Y., Robanus, M., Ungemach, F.-R. and Kreienbrock, L. 2012. Monitoring of antibiotic consumption in

livestock: a German feasibility study. *Preventive Veterinary Medicine.* **104**(1-2), pp.34-43.

Metzler-Zebeli, B.U., Schmitz-Esser, S., Klevenhusen, F., Podstatzky-Lichtenstein, L., Wagner, M. and Zebeli, Q. 2013. Grain-rich diets differently alter ruminal and colonic abundance of microbial populations and lipopolysaccharide in goats. *Anaerobe.* **20**, pp.65-73.

Mikołajczyk-Bator, K. and Czapski, J. 2018. Changes in the content of betalain pigments and their antioxidative capacity during storage. *Nauka Przyroda Technologie.* **12**(1), p# 10.

Miller, H., Toplis, P. and Slade, R. 2009. Can outdoor rearing and increased weaning age compensate for the removal of in-feed antibiotic growth promoters and zinc oxide? *Livestock Science.* **125**(2-3), pp.121-131.

Miller III, E.R., Erlinger, T.P., Sacks, F.M., Svetkey, L.P., Charleston, J., Lin, P.-H. and Appel, L.J. 2005. A dietary pattern that lowers oxidative stress increases antibodies to oxidized LDL: results from a randomized controlled feeding study. *Atherosclerosis.* **183**(1), pp.175-182.

Miller, J., Brzezinska-Slebodzinska, E. and Madsen, F. 1993. Oxidative stress, antioxidants, and animal function. *Dairy Science.* **76**(9), pp.2812-2823.

Millet, S., Aluwé, M., De Boever, J., De Witte, B., Douidah, L., Van den Broeke, A., Leen, F., De Cuyper, C., Ampe, B. and De Campeneere, S. 2018. The effect of crude protein reduction on performance and nitrogen metabolism in piglets (four to nine weeks of age) fed two dietary lysine levels. *Journal of Animal Science.* **96**(9), pp.3824-3836.

Mirmiran, P., Houshialsadat, Z., Gaeini, Z., Bahadoran, Z. and Azizi, F. 2020. Functional properties of beetroot (Beta vulgaris) in management of cardio-metabolic diseases. *Nutrition and Metabolism.* **17**(1), pp.1-15.

Moeser, A.J., Pohl, C.S. and Rajput, M. 2017. Weaning stress and gastrointestinal barrier development: Implications for lifelong gut health in pigs. *Animal Nutrition.* **3**(4), pp.313-321.

Moeser, A.J., Ryan, K.A. and Nighot, P.K. 2007. Gastrointestinal dysfunction induced by early weaning is attenuated by delayed weaning and mast cell blockade in pigs. *American Journal of Physiology-Gastrointestinal Liver Physiology.* **293**(2), pp.G413-G421.

Molina, F., López-Acedo, E., Tabla, R., Roa, I., Gómez, A. and Rebollo, J.E. 2015. Improved detection of Escherichia coli and coliform bacteria by multiplex PCR. *BMC Biotechnology.* **15**(1), pp.1-9.

Molinero, N., Ruiz, L., Sánchez, B., Margolles, A. and Delgado, S. 2019. Intestinal bacteria interplay with bile and cholesterol metabolism: implications on host physiology. *Frontiers in Physiology.* p185.

Montagne, L., Boudry, G., Favier, C., Le Huërou-Luron, I., Lalles, J.-P. and Seve, B. 2007. Main intestinal markers associated with the changes in gut architecture and function in piglets after weaning. *British Journal of Nutrition.* **97**(1), pp.45-57.

Montagne, L., Le Floc'H, N., Arturo-Schaan, M., Foret, R., Urdaci, M. and Le Gall, M. 2012. Comparative effects of level of dietary fiber and sanitary conditions on the growth and health of weanling pigs. *Journal of Animal Science.* **90**(8), pp.2556-2569.

Montagne, L., Pluske, J. and Hampson, D. 2003. A review of interactions between dietary fibre and the intestinal mucosa, and their consequences on digestive health in young non-ruminant animals. *Animal Feed Science Technology.* **108**(1-4), pp.95-117.

More, G.K. and Makola, R.T. 2020. In-vitro analysis of free radical scavenging activities and suppression of LPS-induced ROS production in macrophage cells by Solanum sisymbriifolium extracts. *Scientific Reports.* **10**(1), pp.1-9.

Moreno, D.A., García-Viguera, C., Gil, J.I. and Gil-Izquierdo, A. 2008. Betalains in the era of global agri-food science, technology and nutritional health. *Phytochemistry Reviews.* **7**(2), pp.261-280.

Morrison, D.J. and Preston, T.J.G.m. 2016. Formation of short chain fatty acids by the gut microbiota and their impact on human metabolism. *Gut Microbes.* **7**(3), pp.189-200.

Mueller, K., Blum, N.M., Kluge, H. and Mueller, A.S. 2012. Influence of broccoli extract and various essential oils on performance and expression of xenobiotic-and antioxidant enzymes in broiler chickens. *British Journal of Nutrition.* **108**(4), pp.588-602.

Mukhopadhya, A., O'Doherty, J.V. and Sweeney, T. 2019. A combination of yeast beta-glucan and milk hydrolysate is a suitable alternative to zinc oxide in the race to alleviate post-weaning diarrhoea in piglets. *Scientific reports.* **9**(1), p616.

Munita, J.M. and Arias, C.A. 2016. Mechanisms of antibiotic resistance. *Microbiology Spectrum.* **4**(2), p4.2. 15.

Muniz, L.R., Knosp, C. and Yeretssian, G. 2012. Intestinal antimicrobial peptides during homeostasis, infection, and disease. *Frontiers in Immunology.* **3**, p310.

MyAssays. *"Four Parameter Logistic Curve" online data analysis tool, MyAssays Ltd accessed 10th August, 2021. http://www.myassays.com/four-parameter-logistic-curve.assay*

Nabuurs, M., Hoogendoorn, A., Van Der Molen, E. and Van Osta, A. 1993. Villus height and crypt depth in weaned and unweaned pigs, reared under various circumstances in the Netherlands. *Research in Veterinary Science.* **55**(1), pp.78-84.

Nair, S., Singh, S.V. and Krishan, A. 1991. Flow cytometric monitoring of glutathione content and anthracycline retention in tumor cells. *Cytometry: The Journal of the International Society for Analytical Cytology.* **12**(4), pp.336-342.

Nakatani, M., Inoue, R., Tomonaga, S., Fukuta, K. and Tsukahara, T. 2018. Production, absorption, and blood flow dynamics of short-chain fatty acids produced by fermentation in piglet hindgut during the suckling–weaning period. *Nutrients.* **10**(9), p1220.

Namkung, H., Gong, J., Yu, H. and De Lange, C. 2006. Effect of pharmacological intakes of zinc and copper on growth performance, circulating cytokines and gut microbiota of

newly weaned piglets challenged with coliform lipopolysaccharides. *Canadian Journal of Animal Science.* **86**(4), pp.511-522.

Namkung, H., Yu, H., Gong, J. and Leeson, S. 2011. Antimicrobial activity of butyrate glycerides toward Salmonella Typhimurium and Clostridium perfringens. *Poultry Science.* **90**(10), pp.2217-2222.

Nemzer, B., Pietrzkowski, Z., Spórna, A., Stalica, P., Thresher, W., Michałowski, T. and Wybraniec, S. 2011. Betalainic and nutritional profiles of pigment-enriched red beet root (Beta vulgaris L.) dried extracts. *Food Chemistry.* **127**(1), pp.42-53.

Netzel, M., Stintzing, F., Quaas, D., Strass, G., Carle, R., Bitsch, R., Bitsch, I. and Frank, T. 2005. Renal excretion of antioxidative constituents from red beet in humans. *Food Research International.* **38**(8-9), pp.1051-1058.

Ngoc, T., Hong, T., Len, N. and Lindberg, J. 2012. Effect of Fibre Level and Fibre Source on Gut Morphology and Micro-environment in Local (Mong Cai) and Exotic (Landrace 휡 Yorkshire) Pigs. *Asian-Australasian Journal of Animal Sciences.* **25**(12), pp.1726-1733.

Ngoc, T., Len, N. and Lindberg, J. 2013. Impact of fibre intake and fibre source on digestibility, gut development, retention time and growth performance of indigenous and exotic pigs. *Animal.* **7**(5), pp.736-745.

Nie, Y.-f., Hu, J. and Yan, X.-h. 2015. Cross-talk between bile acids and intestinal microbiota in host metabolism and health. *Journal of Zhejiang University-Science B.* **16**(6), pp.436-446.

Niewold, T. 2015. Intestinal health biomarkers in vivo. *Intestinal Health, Key to Maximise Growth Performance in Livestock.* Wageningen Academic Publishers Wageningen, The Netherlands, pp.219-228.

Ninfali, P. and Angelino, D. 2013. Nutritional and functional potential of Beta vulgaris cicla and rubra. *Fitoterapia.* **89**, pp.188-199.

Ninfali, P., Antonini, E., Frati, A. and Scarpa, E.S. 2017. C-glycosyl flavonoids from Beta vulgaris cicla and betalains from Beta vulgaris rubra: antioxidant, anticancer and antiinflammatory activities—A review. *Phytotherapy Research.* **31**(6), pp.871-884.

Nishitsuji, K., Xiao, J., Nagatomo, R., Umemoto, H., Morimoto, Y., Akatsu, H., Inoue, K. and Tsuneyama, K. 2017. Analysis of the gut microbiome and plasma short-chain fatty acid profiles in a spontaneous mouse model of metabolic syndrome. *Scientific Reports.* **7**(1), p15876.

Niu, Q., Li, P., Hao, S., Zhang, Y., Kim, S.W., Li, H., Ma, X., Gao, S., He, L., Wu, W., Huang, X., Hua, J., Zhou, B. and Huang, R. 2015. Dynamic distribution of the gut microbiota and the relationship with apparent crude fiber digestibility and growth stages in pigs. *Scientific Reports.* **5**(1), p9938.

Niyonsaba, F., Nagaoka, I., Ogawa, H. and Okumura, K. 2009. Multifunctional antimicrobial proteins and peptides: natural activators of immune systems. *Current Pharmaceutical Design.* **15**(21), pp.2393-2413.

Nowland, T.L. and Kirkwood, R.N. 2020. Faecal microbiota transplantation: is it the future for pig production? *Microbiology Australia.* **41**(2), pp.91-94.

Nowland, T.L., Plush, K.J., Barton, M. and Kirkwood, R.N. 2019. Development and function of the intestinal microbiome and potential implications for pig production. *Animals.* **9**(3), p76.

Nwafor, I.C., Shale, K. and Achilonu, M.C. 2017. Chemical composition and nutritive benefits of chicory (Cichorium intybus) as an ideal complementary and/or alternative livestock feed supplement. *Scientific World Journal.* **2017**.

OECD/FAO. 2019. *OECD-FAO Agricultural Outlook 2019–2028.* Food Agriculture Organization of the United Nations Paris, France.

Oh, H.J., Park, Y.J., Cho, J.H., Song, M.H., Gu, B.H., Yun, W., Lee, J.H., An, J.S., Kim, Y.J. and Lee, J.-S. 2021. Changes in diarrhea score, nutrient digestibility, zinc utilization, intestinal immune profiles, and fecal microbiome in weaned piglets by different forms of zinc. *Animals.* **11**(5), p1356.

Oksanen, J., Blanchet, F.G., Kindt, R., Legendre, P., Minchin, P.R., O'hara, R., Simpson, G.L., Solymos, P., Stevens, M.H.H. and Wagner, H. 2013. Package 'vegan'. *Community Ecology Package, version.* **2**(9), pp.1-295.

Olech, M. 2022. Current State of Molecular and Serological Methods for Detection of Porcine Epidemic Diarrhea Virus. *Pathogens.* **11**(10), p1074.

Opal, S.M. and DePalo, V.A. 2000. Anti-inflammatory cytokines. *Chest.* **117**(4), pp.1162-1172.

Orayaga, K.T., Oluremi, O., Tuleun, C.D. and Carew, S.N. 2015. The feed value of composite mango (Mangifera indica) fruit reject meal in the finisher broiler chickens nutrition. *African Journal Food Science Technology.* **6**(6), pp.177-184.

Ormsbee, M.J., Lox, J. and Arciero, P.J. 2013. Beetroot juice and exercise performance. *Nutrition Dietary Supplements.* **5**, pp.27-35.

Oruç, E.Ö. 2010. Oxidative stress, steroid hormone concentrations and acetylcholinesterase activity in Oreochromis niloticus exposed to chlorpyrifos. *Pesticide Biochemistry and Physiology.* **96**(3), pp.160-166.

Ottman, N., Smidt, H., De Vos, W.M. and Belzer, C. 2012. The function of our microbiota: who is out there and what do they do? *Frontiers in Cellular Infection Microbiology.* p104.

Ozdal, T., Sela, D.A., Xiao, J., Boyacioglu, D., Chen, F. and Capanoglu, E. 2016. The reciprocal interactions between polyphenols and gut microbiota and effects on bioaccessibility. *Nutrients.* **8**(2), p78.

Pajarillo, E.A.B., Chae, J.P., Balolong, M.P., Kim, H.B. and Kang, D.K. 2014. Assessment of fecal bacterial diversity among healthy piglets during the weaning transition. *General and Applied Microbiology.* **60**(4), pp.140-146.

Palm, N.W., De Zoete, M.R., Cullen, T.W., Barry, N.A., Stefanowski, J., Hao, L., Degnan, P.H., Hu, J., Peter, I. and Zhang, W. 2014. Immunoglobulin A coating identifies colitogenic bacteria in inflammatory bowel disease. *Cell.* **158**(5), pp.1000-1010.

Panda, S.K., Mishra, S.S., Kayitesi, E. and Ray, R.C. 2016. Microbial-processing of fruit and vegetable wastes for production of vital enzymes and organic acids: Biotechnology and scopes. *Environmental Research.* **146**, pp.161-172.

PAnghAl, A., VirKAr, K., KumAr, V., Dhull, S.B., Gat, Y. and Chhikara, N. 2017. Development of probiotic beetroot drink. *Current Research in Nutrition Food Science* **5**(3).

Papatsiros, V., Tzika, E., Papaioannou, D., Kyriakis, S., Tassis, P. and Kyriakis, C. 2009. Effect of Origanum vulgaris and Allium sativum extracts for the control of proliferative enteropathy in weaning pigs. *Polish Journal of Veterinary Sciences.* **12**(3), p407.

Park, H.S., Min, B. and Oh, S.-H. 2017. Research trends in outdoor pig production—A review. *Asian-Australasian Journal of Animal Sciences.* **30**(9), p1207.

Park, M., Choi, J. and Lee, H.J. 2020. Flavonoid-rich orange juice intake and altered gut microbiome in young adults with depressive symptom: a randomized controlled study. *Nutrients.* **12**(6), p1815.

Park, S., Kim, B., Kim, Y., Kim, S., Jang, K., Kim, Y., Park, J., Song, M. and Oh, S. 2016. Nutrition and feed approach according to pig physiology. *Korean Journal of Agricultural Science.* **43**(5), pp.750-760.

Parks, D.H., Tyson, G.W., Hugenholtz, P. and Beiko, R.G. 2014. STAMP: statistical analysis of taxonomic and functional profiles. *Bioinformatics.* **30**(21), pp.3123-3124.

Pascoal, L.A.F., Thomaz, M.C., Watanabe, P.H., Ruiz, U.D.S., Amorim, A.B., Daniel, E. and da Silva, S.Z. 2015. Purified cellulose, soybean hulls and citrus pulp as a source of fiber for weaned piglets. *Scientia Agricola.* **72**(5), pp.400-410.

Pastore, A., Piemonte, F., Locatelli, M., Lo Russo, A., Gaeta, L., Tozzi, G. and Federici, G. 2001. TECHNICAL BRIEFS-Determination of Blood Total, Reduced, and Oxidized Glutathione in Pediatric Subjects. *Clinical Chemistry-International Journal of Laboratory Medicine Molecular Diagnostic.* **47**(8), pp.1467-1468.

Patterson, E., Cryan, J.F., Fitzgerald, G.F., Ross, R.P., Dinan, T.G. and Stanton, C.J.P.o.t.N.S. 2014. Gut microbiota, the pharmabiotics they produce and host health. *Proceedings of the Nutrition Society.* **73**(4), pp.477-489.

Pavlov, A., Georgiev, V. and Ilieva, M. 2005. Betalain biosynthesis by red beet (Beta vulgaris L.) hairy root culture. *Process Biochemistry.* **40**(5), pp.1531-1533.

Peng, X., Zhou, Q., Wu, C., Zhao, J., Tan, Q., He, Y., Hu, L., Fang, Z., Lin, Y. and Xu, S. 2022. Effects of dietary supplementation with essential oils and protease on growth performance, antioxidation, inflammation and intestinal function of weaned pigs. *Animal Nutrition.* **9**, pp.39-48.

Pereira, A.C., Bandeira, V., Fonseca, C. and Cunha, M.V. 2020. Egyptian Mongoose (Herpestes ichneumon) Gut Microbiota: Taxonomical and Functional Differences across Sex and Age Classes. *Microorganisms.* **8**(3).

Pérez-Jiménez, J. and Saura-Calixto, F. 2018. Fruit peels as sources of non-extractable polyphenols or macromolecular antioxidants: Analysis and nutritional implications. *Food Research International.* **111**, pp.148-152.

Perez-Lopez, A., Behnsen, J., Nuccio, S.-P. and Raffatellu, M. 2016. Mucosal immunity to pathogenic intestinal bacteria. *Nature Reviews Immunology.* **16**(3), pp.135-148.

Pettigrew, J. 2006. Reduced use of antibiotic growth promoters in diets fed to weanling pigs: dietary tools, part 1. *Animal Biotechnology.* **17**(2), pp.207-215.

Pi, J., Zhang, Q., Fu, J., Woods, C.G., Hou, Y., Corkey, B.E., Collins, S. and Andersen, M.E. 2010. ROS signaling, oxidative stress and Nrf2 in pancreatic beta-cell function. *Toxicology Applied Pharmacology.* **244**(1), pp.77-83.

Pi, Y., Gao, K., Peng, Y., Mu, C. and Zhu, W. 2018. Antibiotic-induced alterations of the gut microbiota and microbial fermentation in protein parallel the changes in host nitrogen metabolism of growing pigs. *Animal.* **13**, pp.1-11.

Picard, C., Fioramonti, J., Francois, A., Robinson, T., Neant, F. and Matuchansky, C. 2005. bifidobacteria as probiotic agents–physiological effects and clinical benefits. *Alimentary Pharmacology and Therapeutics.* **22**(6), pp.495-512.

Pickard, J.M., Zeng, M.Y., Caruso, R. and Núñez, G. 2017. Gut microbiota: role in pathogen colonization, immune responses, and inflammatory disease. *Immunological Reviews.* **279**(1), pp.70-89.

Pié, S., Lalles, J.P., Blazy, F., Laffitte, J., Sève, B. and Oswald, I.P. 2004. Weaning is associated with an upregulation of expression of inflammatory cytokines in the intestine of piglets. *Journal of Nutrition.* **134**(3), pp.641-647.

Pieper, R., Vahjen, W., Neumann, K., Van Kessel, A. and Zentek, J. 2012. Dose-dependent effects of dietary zinc oxide on bacterial communities and metabolic profiles in the ileum of weaned pigs. *Animal Physiology and Animal Nutrition.* **96**(5), pp.825-833.

Pietrzkowski, Z., Nemzer, B., Spórna, A., Stalica, P., Tresher, W., Keller, R., Jimenez, R., Michałowski, T. and Wybraniec, S. 2010. Influence of betalain-rich extract on reduction of discomfort associated with osteoarthritis. *New Medicine.* **1**, pp.12-17.

Pinedo-Gil, J., Martín-Diana, A.B., Bertotto, D., Sanz-Calvo, M.Á., Jover-Cerdá, M. and Tomás-Vidal, A. 2018. Effects of dietary inclusions of red beet and betaine on the acute stress response and muscle lipid peroxidation in rainbow trout. *Fish Physiology Biochemistry.* **44**(3), pp.939-948.

Pinedo-Gil, J., Tomás-Vidal, A., Jover-Cerdá, M., Tomás-Almenar, C., Sanz-Calvo, M.Á. and Martín-Diana, A.B. 2017. Red beet and betaine as ingredients in diets of rainbow trout (Oncorhynchus mykiss): effects on growth performance, nutrient retention and flesh quality. *Archives in Animal Nutrition.* **71**(6), pp.486-505.

Ploegmarkers, M. 2022. A health gut is key: Zero Zinc summit. *All About Feed.* **30**(6-2022).

Plöger, S., Stumpff, F., Penner, G.B., Schulzke, J.D., Gäbel, G., Martens, H., Shen, Z., Günzel, D. and Aschenbach, J.R. 2012. Microbial butyrate and its role for barrier function in the

gastrointestinal tract. *Annals of the New York Academy of Sciences.* **1258**(1), pp.52-59.

Pluske, J. 2016. Invited review: aspects of gastrointestinal tract growth and maturation in the pre-and postweaning period of pigs. *Animal Science.* **94**(suppl_3), pp.399-411.

Pluske, J.R., Miller, D., Sterndale, S. and Turpin, D. 2019. Associations between gastrointestinal-tract function and the stress response after weaning in pigs. *Animal Production Science.* **59**(11), pp.2015-2022.

Pluske, J.R. 2013. Feed-and feed additives-related aspects of gut health and development in weanling pigs. *Animal Science and Biotechnology.* **4**(1), pp.1-7.

Pluske, J.R., Hampson, D.J. and Williams, I.H. 1997. Factors influencing the structure and function of the small intestine in the weaned pig: a review. *Livestock Production Science.* **51**(1-3), pp.215-236.

Pluske, J.R., Pethick, D.W., Hopwood, D.E. and Hampson, D.J. 2002. Nutritional influences on some major enteric bacterial diseases of pig. *Nutrition Research Reviews.* **15**(2), pp.333-371.

Pluske, J.R., Turpin, D.L. and Kim, J.C. 2018. Gastrointestinal tract (gut) health in the young pig. *Animal Nutrition.* **4**(2), pp.187-196.

Poonsuk, K. and Zimmerman, J. 2018. Historical and contemporary aspects of maternal immunity in swine. *Animal Health Research Reviews.* **19**(1), pp.31-45.

Pop, M., Walker, A.W., Paulson, J., Lindsay, B., Antonio, M., Hossain, M.A., Oundo, J., Tamboura, B., Mai, V. and Astrovskaya, I. 2014. Diarrhea in young children from low-income countries leads to large-scale alterations in intestinal microbiota composition. *Genome Biology.* **15**(6), pp.1-12.

Prandi, B., Baldassarre, S., Babbar, N., Bancalari, E., Vandezande, P., Hermans, D., Bruggeman, G., Gatti, M., Elst, K. and Sforza, S. 2018. Pectin oligosaccharides from sugar beet pulp: Molecular characterization and potential prebiotic activity. *Functional Foods.* **9**(3), pp.1557-1569.

Punda, I., Prikhodko, D., Duvall and Merlin. 2009. Agribusiness Handbook: Sugar beet white sugar. *United Nations Food Agriculture Organization* **7**, pp.27-28.

Rahal, A., Kumar, A., Singh, V., Yadav, B., Tiwari, R., Chakraborty, S. and Dhama, K. 2014. Oxidative stress, prooxidants, and antioxidants: the interplay. *BioMedical Research International.* **2014**.

Rahimi, P., Abedimanesh, S., Mesbah-Namin, S.A. and Ostadrahimi, A. 2019. Betalains, the nature-inspired pigments, in health and diseases. *Critical Reviews in Food Science and Nutrition.* **59**(18), pp.2949-2978.

Raila, J., Schweigert, F., Stanitznig, A., Lambacher, B., Franz, S., Baldermann, S. and Wittek, T. 2017. No detectable carotenoid concentrations in serum of llamas and alpacas. *Animal Physiology and Animal Nutrition.* **101**(4), pp.629-634.

Ravichandran, K., Saw, N.M.M.T., Mohdaly, A.A., Gabr, A.M., Kastell, A., Riedel, H., Cai, Z., Knorr, D. and Smetanska, I. 2013. Impact of processing of red beet on betalain content and antioxidant activity. *Food Research International.* **50**(2), pp.670-675.

Re, R., Pellegrini, N., Proteggente, A., Pannala, A., Yang, M. and Rice-Evans, C. 1999. Antioxidant activity applying an improved ABTS radical cation decolorization assay. *Free Radical Biology Medicine.* **26**(9-10), pp.1231-1237.

Rebecca, L.J., Sharmila, S., Das, M.P. and Seshiah, C. 2014. Extraction and purification of carotenoids from vegetables. *Journal of Chemical Pharmaceutical Research.* **6**(4), pp.594-598.

Rebezov, M., Tokhtarov, Z., Tretyak, L., Kenijz, N., Gayvas, A., Konovalov, S., Rybchenko, T., Ermolaev, V. and Belyakov, A. 2020. Role of beetroot as a dietary supplement in food products. *Plant Cell Biotechnology and Molecular Biology.* pp.8-16.

Rebucci, R., Staurenghi, V., Marchetti, L., Giromini, C. and Bontempo, V. 2022. Effects of nature identical essential oils (carvacrol, thymol and cinnamaldehyde) on growth performance of piglets and non-invasive markers of antioxidant status and calprotectin release. *Livestock Science.* p104959.

Reddy, M.K., Alexander-Lindo, R.L. and Nair, M.G.J.J.o.a. 2005. Relative inhibition of lipid peroxidation, cyclooxygenase enzymes, and human tumor cell proliferation by natural food colors. *Journal of Agricultural Food Chemistry.* **53**(23), pp.9268-9273.

Regassa, A. and Nyachoti, C.M. 2018. Application of resistant starch in swine and poultry diets with particular reference to gut health and function. *Animal Nutrition.* **4**(3), pp.305-310.

Ren, J., Yan, X.M., Ai, H.S., Zhang, Z.Y., Huang, X., Ouyang, J., Yang, M., Yang, H.G., Han, P.F., Zeng, W.H., Chen, Y.J., Guo, Y.M., Xiao, S.J., Ding, N.S. and Huang, L.S. 2012. Susceptibility towards Enterotoxigenic Escherichia coli F4ac Diarrhea Is Governed by the MUC13 Gene in Pigs. *Plos One.* **7**(9).

Ren, M., Zhang, S.H., Zeng, X.F., Liu, H. and Qiao, S.Y. 2015. Branched-chain Amino Acids are Beneficial to Maintain Growth Performance and Intestinal Immune-related Function in Weaned Piglets Fed Protein Restricted Diet. *Asian-Australian Journal of Animal Sciences.* **28**(12), pp.1742-1750.

Renaudeau, D., Brochain, J., Giorgi, M., Bocage, B., Hery, M., Crantor, E., Marie-Magdeleine, C. and Archimède, H. 2014. Banana meal for feeding pigs: digestive utilization, growth performance and feeding behavior. *Animal.* **8**(4), pp.565-571.

Rescigno, M. 2014. Intestinal microbiota and its effects on the immune system. *Cellular Microbiology.* **16**(7), pp.1004-1013.

Reuter, S., Gupta, S.C., Chaturvedi, M.M. and Aggarwal, B.B. 2010. Oxidative stress, inflammation, and cancer: how are they linked? *Free Radical Biology Medicine.* **49**(11), pp.1603-1616.

Revelle, W. 2016. How to: Use the psych package for factor analysis and data reduction. *Evanston, IL: Northwestern University, Department of Psychology.*

Rhouma, M., Fairbrother, J.M., Beaudry, F. and Letellier, A. 2017. Post weaning diarrhea in pigs: risk factors and non-colistin-based control strategies. *Acta Veterinaria Scandinavica.* **59**, pp.1-19.

Ridlon, J.M., Kang, D.J., Hylemon, P.B. and Bajaj, J.S. 2014. Bile acids and the gut microbiome. *Current Opinion in Gastroenterology.* **30**(3), p332.

Riis Poulsen, A.-S., Luise, D., Curtasu, M.V., Sugiharto, S., Canibe, N., Trevisi, P. and Lauridsen, C. 2018. Effects of alpha-(1, 2)-fucosyltransferase genotype variants on plasma metabolome, immune responses and gastrointestinal bacterial enumeration of pigs pre-and post-weaning. *PLoS One.* **13**(8), pe0202970.

Rizzetto, L., Fava, F., Tuohy, K.M. and Selmi, C. 2018. Connecting the immune system, systemic chronic inflammation and the gut microbiome: the role of sex. *Autoimmunity.* **92**, pp.12-34.

Rohilla, P. and Bujarbaruah, K. 2000. Effect of banana leaves feeding on growth of rabbits. *Indian Veterinary Journal.* **77**(10), pp.902-903.

Ross, J., Hale, B., Gabler, N., Rhoads, R., Keating, A. and Baumgard, L. 2015. Physiological consequences of heat stress in pigs. *Animal Production Science.* **55**(12), pp.1381-1390.

Rossi, B., Toschi, A., Piva, A. and Grilli, E. 2020. Single components of botanicals and nature-identical compounds as a non-antibiotic strategy to ameliorate health status and improve performance in poultry and pigs. *Nutrition Research Reviews.* **33**(2), pp.218-234.

Rostagno, M.H. 2022. Managing Intestinal Health in Farm Animals: A Critical View. *Gut Microbiota, Immunity, Health in Production Animals.* pp.1-12.

Rousset, F., Peyrol, S., Garcia, E., Vezzio, N., Andujar, M., Grimaud, J.A. and Banchereau, J. 1995. Long-term cultured CD40-activated B lymphocytes differentiate into plasma cells in response to IL-10 but not IL-4. *International Immunology.* **7**(8), pp.1243-1253.

Ruberto, G., Renda, A., Daquino, C., Amico, V., Spatafora, C., Tringali, C. and De Tommasi, N. 2007. Polyphenol constituents and antioxidant activity of grape pomace extracts from five Sicilian red grape cultivars. *Food Chemistry.* **100**(1), pp.203-210.

Rudra, S.G., Nishad, J., Jakhar, N. and Kaur, C. 2015. Food industry waste: mine of nutraceuticals. *International Journal of Science Environment and Technology.* **4**(1), pp.205-229.

Sagara, Y. and Schubert, D. 1998. The activation of metabotropic glutamate receptors protects nerve cells from oxidative stress. *Neuroscience.* **18**(17), pp.6662-6671.

Sales, J. 2013. Effects of pharmacological concentrations of dietary zinc oxide on growth of post-weaning pigs: a meta-analysis. *Biological Trace Element Research.* **152**(3), pp.343-349.

Santana, M.B.d., Melo, A.D.B., Cruz, D.R., Garbossa, C.A.P., Andrade, C.d., Cantarelli, V.d.S. and Costa, L.B. 2015. Alternatives to antibiotic growth promoters for weanling pigs. *Ciência Rural.* **45**, pp.1093-1098.

Sarcia, R. and Hebraud, P. 2005. Crackling of a coagulating suspension. *Phys Rev E Stat Nonlin Soft Matter Physics.* **72**(Pt 1), p011402.

Sargeant, H.R., McDowall, K.J., Miller, H.M. and Shaw, M.A. 2010. Dietary zinc oxide affects the expression of genes associated with inflammation: transcriptome analysis in piglets challenged with ETEC K88. *Veterinary Immunology and Immunopathology.* **137**(1-2), pp.120-129.

Satessa, G.D., Tamez-Hidalgo, P., Hui, Y., Cieplak, T., Krych, L., Kjærulff, S., Brunsgaard, G., Nielsen, D.S. and Nielsen, M.O. 2020. Impact of dietary supplementation of lactic acid bacteria fermented rapeseed with or without macroalgae on performance and health of piglets following omission of medicinal zinc from weaner diets. *Animals.* **10**(1), p137.

Sawicki, T., Bączek, N. and Wiczkowski, W. 2016. Betalain profile, content and antioxidant capacity of red beetroot dependent on the genotype and root part. *Functional Foods.* **27**, pp.249-261.

Sawicki, T., Topolska, J., Bączek, N., Szawara-Nowak, D., Juśkiewicz, J. and Wiczkowski, W. 2020. Characterization of the profile and concentration of betacyanin in the gastric content, blood and urine of rats after an intragastric administration of fermented red beet juice. *Food Chemistry.* **313**, p126169.

Schloss, P.D., Westcott, S.L., Ryabin, T., Hall, J.R., Hartmann, M., Hollister, E.B., Lesniewski, R.A., Oakley, B.B., Parks, D.H., Robinson, C.J., Sahl, J.W., Stres, B., Thallinger, G.G., Van Horn, D.J. and Weber, C.F. 2009. Introducing mothur: open-source, platform-independent, community-supported software for describing and comparing microbial communities. *Applied Environmental Microbiology.* **75**(23), pp.7537-7541.

Schokker, D., Fledderus, J., Jansen, R., Vastenhouw, S.A., de Bree, F.M., Smits, M.A. and Jansman, A.A. 2018. Supplementation of fructooligosaccharides to suckling piglets affects intestinal microbiota colonization and immune development. *Journal of Animal Science.* **96**(6), pp.2139-2153.

Schokker, D., Zhang, J., Vastenhouw, S.A., Heilig, H.G., Smidt, H., Rebel, J.M. and Smits, M.A. 2015. Long-lasting effects of early-life antibiotic treatment and routine animal handling on gut microbiota composition and immune system in pigs. *PLoS One.* **10**(2), pe0116523.

Schroyen, M., Stinckens, A., Verhelst, R., Geens, M., Cox, E., Niewold, T. and Buys, N. 2012. Susceptibility of piglets to enterotoxigenic Escherichia coli is not related to the expression of MUC13 and MUC20. *Animal Genetics.* **43**(3), pp.324-327.

Schwarz, N.A. and McKinley-Barnard, S.K. 2020. Acute Oral ingestion of a multi-ingredient Preworkout supplement increases exercise performance and alters Postexercise hormone responses: a randomized crossover, double-blinded, placebo-controlled trial. *Dietary Supplements.* **17**(2), pp.211-226.

Scott, A.M., Beller, E., Glasziou, P., Clark, J., Ranakusuma, R.W., Byambasuren, O., Bakhit, M., Page, S.W., Trott, D. and Del Mar, C. 2018. Is antimicrobial administration to food animals a direct threat to human health? A rapid systematic review. *International Journal of Antimicrobial Agents.* **52**(3), pp.316-323.

Seal, B.S., Lillehoj, H.S., Donovan, D.M. and Gay, C.G. 2013. Alternatives to antibiotics: a symposium on the challenges and solutions for animal production. *Animal Health Research Reviews.* **14**(1), pp.78-87.

Seiler, C. and Berendonk, T.U. 2012. Heavy metal driven co-selection of antibiotic resistance in soil and water bodies impacted by agriculture and aquaculture. *Frontiers in Microbiology.* **3**, p399.

Selim, N., Youssef, S., Abdel-Salam, A. and Nada, S.A. 2013. Evaluations of some natural antioxidant sources in broiler diets: 1-effect on growth, physiological and immunological performance of broiler chicks. *International Journal of Poultry Science* **12**, pp.561-571.

Shang, Q., Liu, S., Liu, H., Mahfuz, S. and Piao, X. 2021. Impact of sugar beet pulp and wheat bran on serum biochemical profile, inflammatory responses and gut microbiota in sows during late gestation and lactation. *Animal Sciencen Biotechnology.* **12**(1), pp.1-14.

Shang, Q., Ma, X., Liu, H., Liu, S. and Piao, X. 2020. Effect of fibre sources on performance, serum parameters, intestinal morphology, digestive enzyme activities and microbiota in weaned pigs. *Archives of Animal Nutrition.* **74**(2), pp.121-137.

Shannon, M.C. and Hill, G.M. 2019. Trace mineral supplementation for the intestinal health of young monogastric animals. *Frontiers in Veterinary Science.* **6**, p73.

Shen, J., Chen, Y., Wang, Z., Zhou, A., He, M., Mao, L., Zou, H., Peng, Q., Xue, B. and Wang, L. 2014. Coated zinc oxide improves intestinal immunity function and regulates microbiota composition in weaned piglets. *British Journal of Nutrition.* **111**(12), pp.2123-2134.

Shi, J.-T., Chen, N., Xu, J., Goyal, H., Wu, Z.-Q., Zhang, J.-X. and Xu, H.-G. 2023. Diagnostic Accuracy of Fecal Calprotectin for Predicting Relapse in Inflammatory Bowel Disease: A Meta-Analysis. *Journal of Clinical Medicine.* **12**(3), p1206.

Shyamala, B.N. and Jamuna, P. 2010. Nutritional Content and Antioxidant Properties of Pulp Waste from Daucus carota and Beta vulgaris. *Mal NJ Nutr* **16**(3), pp.397-408.

Sies, H., Berndt, C. and Jones, D.P. 2017. Oxidative stress. *Annual Review of Biochemistry.* **86**, pp.715-748.

Singh, B. and Hathan, B.S. 2014. Chemical composition, functional properties and processing of beetroot—a review. *Interanationa Journal of Scientific and Enginnering Research.* **5**(1), pp.679-684.

Singh, P., Teal, T.K., Marsh, T.L., Tiedje, J.M., Mosci, R., Jernigan, K., Zell, A., Newton, D.W., Salimnia, H. and Lephart, P. 2015. Intestinal microbial communities associated with acute enteric infections and disease recovery. *Microbiome.* **3**(1), pp.1-12.

Sjölund-Karlsson, M., Joyce, K., Blickenstaff, K., Ball, T., Haro, J., Medalla, F.M., Fedorka-Cray, P., Zhao, S., Crump, J.A. and Whichard, J.M. 2011. Antimicrobial susceptibility to azithromycin among Salmonella enterica isolates from the United States. *Antimicrobial Agents Chemotherapy.* **55**(9), pp.3985-3989.

Sjölund, M., Postma, M., Collineau, L., Lösken, S., Backhans, A., Belloc, C., Emanuelson, U., Beilage, E.G., Stärk, K. and Dewulf, J. 2016. Quantitative and qualitative antimicrobial usage patterns in farrow-to-finish pig herds in Belgium, France, Germany and Sweden. *Preventive Veterinary Medicine.* **130**, pp.41-50.

Slade, R., Kyriazakis, I., Carroll, S., Reynolds, F., Wellock, I., Broom, L. and Miller, H. 2011. Effect of rearing environment and dietary zinc oxide on the response of group-housed weaned pigs to enterotoxigenic Escherichia coli O149 challenge. *Animal.* **5**(8), pp.1170-1178.

Slavov, A., Karagyozov, V., Denev, P., Kratchanova, M. and Kratchanov, C. 2013. Antioxidant activity of red beet juices obtained after microwave and thermal pretreatments. *Czech Journal of Food Sciences.* **31**(2), pp.139-147.

Slifierz, M.J., Park, J., Friendship, R.M. and Weese, J.S. 2014. Zinc-resistance gene CzrC identified in methicillin-resistant Staphylococcus hyicus isolated from pigs with exudative epidermitis. *Canadian Veterinary Journal* **55 5**, pp.489-490.

Sloup, V., Jankovská, I., Nechybová, S., Peřinková, P. and Langrová, I. 2017. Zinc in the animal organism: a review. *Scientia Agriculturae Bohemica.* **48**(1), pp.13-21.

Smith, P.M., Howitt, M.R., Panikov, N., Michaud, M., Gallini, C.A., Bohlooly-y, M., Glickman, J.N. and Garrett, W.S. 2013. The microbial metabolites, short-chain fatty acids, regulate colonic Treg cell homeostasis. *Science.* **341**(6145), pp.569-573.

Sokmen, A., Gulluce, M., Akpulat, H.A., Daferera, D., Tepe, B., Polissiou, M., Sokmen, M. and Sahin, F. 2004. The in vitro antimicrobial and antioxidant activities of the essential oils and methanol extracts of endemic Thymus spathulifolius. *Food Control.* **15**(8), pp.627-634.

Soler, C., Goossens, T., Bermejo, A., Migura-García, L., Cusco, A., Francino, O. and Fraile, L.J.P.o. 2018. Digestive microbiota is different in pigs receiving antimicrobials or a feed additive during the nursery period. **13**(5), pe0197353.

Soler, L., Miller, I., Nöbauer, K., Carpentier, S. and Niewold, T. 2015. Identification of the major regenerative III protein (RegIII) in the porcine intestinal mucosa as RegIIIγ, not RegIIIα. *Veterinary Immunology Immunopathology.* **167**(1-2), pp.51-56.

Song, M., Zhang, F., Chen, L., Yang, Q., Su, H., Yang, X., He, H., Ling, M., Zheng, J. and Duan, C. 2021. Dietary chenodeoxycholic acid improves growth performance and intestinal health by altering serum metabolic profiles and gut bacteria in weaned piglets. *Animal Nutrition.* **7**(2), pp.365-375.

Soong, Y.Y. and Barlow, P.J. 2004. Antioxidant activity and phenolic content of selected fruit seeds. *Food Chemistry.* **88**(3), pp.411-417.

Spees, A.M., Lopez, C.A., Kingsbury, D.D., Winter, S.E. and Bäumler, A.J. 2013. Colonization resistance: battle of the bugs or ménage à trois with the host? *PLoS Pathogens.* **9**(11), pe1003730.

Šplíchal, I., Fagerhol, M.K., Trebichavský, I., Šplíchalová, A. and Schulze, J. 2005. The effect of intestinal colonization of germ-free pigs with Escherichia coli on calprotectin levels in plasma, intestinal and bronchoalveolar lavages. *Immunobiology.* **209**(9), pp.681-687.

Stärk, K.D. 2000. Epidemiological investigation of the influence of environmental risk factors on respiratory diseases in swine—a literature review. *Veterinary Journal.* **159**(1), pp.37-56.

Starke, I.C., Pieper, R., Neumann, K., Zentek, J. and Vahjen, W. 2014. The impact of high dietary zinc oxide on the development of the intestinal microbiota in weaned piglets. *FEMS Microbiology Ecology.* **87**(2), pp.416-427.

Stensland, I., Kim, J.C., Bowring, B., Collins, A.M., Mansfield, J.P. and Pluske, J.R. 2015. A comparison of diets supplemented with a feed additive containing organic acids, cinnamaldehyde and a permeabilizing complex, or zinc oxide, on post-weaning diarrhoea, selected bacterial populations, blood measures and performance in weaned pigs experimentally infected with enterotoxigenic E. coli. *Animals.* **5**(4), pp.1147-1168.

Strack, D., Vogt, T. and Schliemann, W. 2003. Recent advances in betalain research. *Phytochemistry.* **62**(3), pp.247-269.

Su, Y., Li, X., Li, D. and Sun, J. 2021. Fecal microbiota transplantation shows marked shifts in the multi-omic profiles of porcine post-weaning diarrhea. *Frontiers in Microbiology.* **12**, p619460.

Su, Y., Yao, W., Perez-Gutierrez, O.N., Smidt, H. and Zhu, W.-Y.J.A. 2008. 16S ribosomal RNA-based methods to monitor changes in the hindgut bacterial community of piglets after oral administration of Lactobacillus sobrius S1. *Anaerobe.* **14**(2), pp.78-86.

Suganyadevi, P., Saravanakumar, M., Aravinthan, K., Arunkumar, A., Krishna, R.K. and Karthikeyani, S. 2010. Extraction of betacyanin from red beet root (Beta vulgaris L.) and to evaluate its antioxidant potential. *Pharmacy Research.* **3**(11), pp.2693-2696.

Sugiharto, S. and Ranjitkar, S.J.A.n. 2019. Recent advances in fermented feeds towards improved broiler chicken performance, gastrointestinal tract microecology and immune responses: A review. *Animal Nutrition.* **5**(1), pp.1-10.

Sun, C., Wang, X., Hui, Y., Fukui, H., Wang, B. and Miwa, H. 2021. The Potential Role of REG Family Proteins in Inflammatory and Inflammation-Associated Diseases of the Gastrointestinal Tract. *International Journal of Molecular Science.* **22**(13), p7196.

Sun, Y., Teng, T., Bai, G., Qiu, S., Shi, B., Ju, D. and Zhao, X. 2020. Protein-restricted diet balanced for lysine, methionine, threonine, and tryptophan for nursery pigs elicits subsequent compensatory growth and has long term effects on protein metabolism and organ development. *Animal Feed ScienceTechnology.* **270**, p114712.

Sutton, T.A., McDermott, K., Bedford, M. R. and Miller, H. M. 2020. Real-time lactate concentration analysis in pig faeces as a potential gut health indicator. *In: Proceedings of the British Society of Animal Sciences Annual Conference: The Challenge of Change; 2020:.* p122.

Suzuki, M.T., Taylor, L.T. and DeLong, E.F. 2000. Quantitative analysis of small-subunit rRNA genes in mixed microbial populations via 5′-nuclease assays. *Applied Environmental Microbiology.* **66**(11), pp.4605-4614.

Suzuki, T. 2020. Regulation of the intestinal barrier by nutrients: The role of tight junctions. *Animal Science Journal.* **91**(1), pe13357.

Suzuki, T. and Hara, H. 2010. Dietary fat and bile juice, but not obesity, are responsible for the increase in small intestinal permeability induced through the suppression of tight junction protein expression in LETO and OLETF rats. *Nutrition Metabolism.* **7**(1), pp.1-17.

Swarna, J., Lokeswari, T., Smita, M. and Ravindhran, R. 2013. Characterisation and determination of in vitro antioxidant potential of betalains from Talinum triangulare (Jacq.) Willd. *Food Chemistry.* **141**(4), pp.4382-4390.

Sweeney, T. and O'doherty, J. 2016. Marine macroalgal extracts to maintain gut homeostasis in the weaning piglet. *Domestic Animal Endocrinology.* **56**, pp.S84-S89.

Szeto, Y.T., Tomlinson, B. and Benzie, I.F. 2002. Total antioxidant and ascorbic acid content of fresh fruits and vegetables: implications for dietary planning and food preservation. *British Journal of nutrition.* **87**(1), pp.55-59.

Szopińska, A.A. and Gawęda, M.J.J.o.H.R. 2013. Comparison of yield and quality of red beet roots cultivated using conventional, integrated and organic method. *Journal of Horticultural Research.* **21**(1), pp.107-114.

Taira, J., Tsuchida, E., Katoh, M.C., Uehara, M. and Ogi, T. 2015. Antioxidant capacity of betacyanins as radical scavengers for peroxyl radical and nitric oxide. *Food Chemistry.* **166**, pp.531-536.

Tan, D., Wang, Y., Bai, B., Yang, X. and Han, J. 2015. Betanin attenuates oxidative stress and inflammatory reaction in kidney of paraquat-treated rat. *Food Chemical Toxicology.* **78**, pp.141-146.

Tan, J., McKenzie, C., Potamitis, M., Thorburn, A.N., Mackay, C.R. and Macia, L. 2014. The role of short-chain fatty acids in health and disease. *Advances in Immunology.* **121**, pp.91-119.

Tan, M.L. and Hamid, S.B.S. 2021. Beetroot as a potential functional food for cancer chemoprevention, a narrative review. *Cancer Prevention.* **26**(1), p1.

Tang, W., Chen, D., Yu, B., He, J., Huang, Z., Zheng, P., Mao, X., Luo, Y., Luo, J. and Wang, Q. 2020. Capsulized faecal microbiota transplantation ameliorates post-weaning diarrhoea by modulating the gut microbiota in piglets. *Veterinary Research.* **51**, pp.1-14.

Taylor, A.E., Bedford, M.R. and Miller, H.M. 2018. The effects of xylanase on grower pig performance, concentrations of volatile fatty acids and peptide YY in portal and peripheral blood. *Animal.* **12**(12), pp.2499-2504.

Tesoriere, L., Allegra, M., Butera, D. and Livrea, M.A. 2004a. Absorption, excretion, and distribution of dietary antioxidant betalains in LDLs: potential health effects of betalains in humans. *American Journal of Clinical Nutrition.* **80**(4), pp.941-945.

Tesoriere, L., Butera, D., Pintaudi, A.M., Allegra, M. and Livrea, M.A. 2004b. Supplementation with cactus pear (Opuntia ficus-indica) fruit decreases oxidative stress in healthy humans: a comparative study with vitamin C. *American Journal of Clinical Nutrition.* **80**(2), pp.391-395.

Tesoriere, L., Gentile, C., Angileri, F., Attanzio, A., Tutone, M., Allegra, M. and Livrea, M. 2013. Trans-epithelial transport of the betalain pigments indicaxanthin and betanin across Caco-2 cell monolayers and influence of food matrix. *European Journal of Nutrition.* **52**(3), pp.1077-1087.

Thacker. 2013. Alternatives to antibiotics as growth promoters for use in swine production: a review *Animal Science and Biotechnology.* **4**(35), pp.2-12.

Thai, J.D. and Gregory, K.E. 2020. Bioactive factors in human breast milk attenuate intestinal inflammation during early life. *Nutrients.* **12**(2), p581.

Thompson, C.L., Wang, B. and Holmes, A.J. 2008. The immediate environment during postnatal development has long-term impact on gut community structure in pigs. *ISME Journal.* **2**(7), pp.739-748.

Tian, G., Wu, X.Y., Chen, D.W., Yu, B. and He, J. 2017. Adaptation of gut microbiome to different dietary nonstarch polysaccharide fractions in a porcine model. *Molecular Nutrition & Food Research.* **61**(10).

Tian, L.M., Scholte, J., Borewicz, K., van den Bogert, B., Smidt, H., Scheurink, A.J.W., Gruppen, H. and Schols, H.A. 2016. Effects of pectin supplementation on the fermentation patterns of different structural carbohydrates in rats. *Molecular Nutrition & Food Research.* **60**(10), pp.2256-2266.

Tian, Y., Gui, W., Koo, I., Smith, P.B., Allman, E.L., Nichols, R.G., Rimal, B., Cai, J., Liu, Q. and Patterson, A.D. 2020. The microbiome modulating activity of bile acids. *Gut microbes.* **11**(4), pp.979-996.

Tietze, F. 1969. Enzymic method for quantitative determination of nanogram amounts of total and oxidized glutathione: applications to mammalian blood and other tissues. *Analytical Biochemistry.* **27**(3), pp.502-522.

Timmerman, H., Veldman, A., Van den Elsen, E., Rombouts, F. and Beynen, A. 2006. Mortality and growth performance of broilers given drinking water supplemented with chicken-specific probiotics. *Poultry Science.* **85**(8), pp.1383-1388.

Ting, Y., Jiang, Y., Ho, C.-T. and Huang, Q. 2014. Common delivery systems for enhancing in vivo bioavailability and biological efficacy of nutraceuticals. *Journal of Functional Foods.* **7**, pp.112-128.

Tkachenko, H., Kurhaluk, N., Andriichuk, A., Gasiuk, E. and Beschasniu, S. 2014. Oxidative stress biomarkers in liver of sea trout (Salmo trutta m. trutta L.) affected by ulcerative dermal necrosis syndrome. *Turkish Journal of Fisheries Aquatic Sciences.* **14**(2), pp.391-402.

Toutain, P.-L., Bousquet-mélou, A.J.J.o.v.p. and therapeutics. 2004. Bioavailability and its assessment. *Veterinary Pharmacology Therapeutics.* **27**(6), pp.455-466.

Trckova, M., Lorencova, A., Hazova, K. and Zajacova, Z.S. 2015. Prophylaxis of post-weaning diarrhoea in piglets by zinc oxide and sodium humate. *Veterinarni Medicina.* **60**(7).

Trefflich, I., Dietrich, S., Braune, A., Abraham, K. and Weikert, C. 2021. Short-and branched-chain fatty acids as fecal markers for microbiota activity in vegans and omnivores. *Nutrients.* **13**(6), p1808.

Tretter, L., Patocs, A. and Chinopoulos, C. 2016. Succinate, an intermediate in metabolism, signal transduction, ROS, hypoxia, and tumorigenesis. *Biochimica et Biophysica Acta -Bioenergetics.* **1857**(8), pp.1086-1101.

Trinchieri, G. 2003. Interleukin-12 and the regulation of innate resistance and adaptive immunity. *Nature Reviews Immunology.* **3**(2), pp.133-146.

Tsukahara, T., Azuma, Y. and Ushida, K. 2001. The effect of a mixture of live lactic acid bacteria on intestinal gas production in pigs. *Microbial Ecology in Health and Disease.* **13**(2), pp.105-110.

Tuddenham, S. and Sears, C.L.J.C.o.i.i.d. 2015. The intestinal microbiome and health. *Current Opinion in Infectious Diseases.* **28**(5), p464.

Tuohy, K.M., Conterno, L., Gasperotti, M. and Viola, R. 2012. Up-regulating the human intestinal microbiome using whole plant foods, polyphenols, and/or fiber. *Journal of Agricultural and Food Chemistry.* **60**(36), pp.8776-8782.

Turnbaugh, P.J., Ley, R.E., Hamady, M., Fraser-Liggett, C.M., Knight, R. and Gordon, J.I. 2007. The human microbiome project. *Nature.* **449**(7164), pp.804-810.

Uerlings, J., Arevalo Sureda, E., Schroyen, M., Kroeske, K., Tanghe, S., De Vos, M., Bruggeman, G., Wavreille, J., Bindelle, J. and Purcaro, G.J.F.i.N. 2021. Impact of citrus pulp or inulin on intestinal microbiota and metabolites, barrier, and immune function of weaned piglets. *Frontiers in Nutrition.* p934.

Ulluwishewa, D., Anderson, R.C., McNabb, W.C., Moughan, P.J., Wells, J.M. and Roy, N.C. 2011. Regulation of tight junction permeability by intestinal bacteria and dietary components. *Journal of Nutrition.* **141**(5), pp.769-776.

Ural, M.Ş. 2013. Chlorpyrifos-induced changes in oxidant/antioxidant status and haematological parameters of Cyprinus carpio carpio: ameliorative effect of lycopene. *Chemosphere.* **90**(7), pp.2059-2064.

USEPA. 2012. *Institutional Controls: A Guide to Planning, Implementing, Maintaining, and Enforcing Institutional Controls at Contaminated Sites.*

Vahjen, W., Pieper, R. and Zentek, J. 2011. Increased dietary zinc oxide changes the bacterial core and enterobacterial composition in the ileum of piglets. *Animal Science.* **89**(8), pp.2430-2439.

Vahjen, W., Pietruszyńska, D., Starke, I.C. and Zentek, J. 2015. High dietary zinc supplementation increases the occurrence of tetracycline and sulfonamide resistance genes in the intestine of weaned pigs. *Gut Pathogens.* **7**.

Vaishnava, S., Yamamoto, M., Severson, K.M., Ruhn, K.A., Yu, X., Koren, O., Ley, R., Wakeland, E.K. and Hooper, L.V. 2011. The antibacterial lectin RegIIIγ promotes the spatial segregation of microbiota and host in the intestine. *Science.* **334**(6053), pp.255-258.

Valeriano, V.D., Balolong, M.P. and Kang, D.K. 2017. Probiotic roles of Lactobacillus sp. in swine: insights from gut microbiota. *Journal of Applied Microbiology.* **122**(3), pp.554-567.

Vallance, A., Ma, C., Huang, T., Velcich, A., Xia, L., Zarepour, B.M., Bhullar, K. and Montero, M. 2013. The Mucin Muc2 Limits Pathogen Burdens. *Infection and Immunity.* **81**(10), p3672.

van Beers-Schreurs, H.M., Nabuurs, M.J., Vellenga, L., Valk, H.J.K.-v.d., Wensing, T. and Breukink, H.J. 1998. Weaning and the weanling diet influence the villous height and crypt depth in the small intestine of pigs and alter the concentrations of short-chain fatty acids in the large intestine and blood. *Journal of Nutrition.* **128**(6), pp.947-953.

Van Boeckel, T.P., Glennon, E.E., Chen, D., Gilbert, M., Robinson, T.P., Grenfell, B.T., Levin, S.A., Bonhoeffer, S. and Laxminarayan, R. 2017. Reducing antimicrobial use in food animals. *Science.* **357**(6358), pp.1350-1352.

van den Berg, R., Haenen, G.R., van den Berg, H. and Bast, A. 1999. Applicability of an improved Trolox equivalent antioxidant capacity (TEAC) assay for evaluation of antioxidant capacity measurements of mixtures. *Food Chemistry.* **66**(4), pp.511-517.

van der Beek, C.M., Dejong, C.H., Troost, F.J., Masclee, A.A. and Lenaerts, K. 2017. Role of short-chain fatty acids in colonic inflammation, carcinogenesis, and mucosal protection and healing. *Nutrition Reviews.* **75**(4), pp.286-305.

Van der Meulen, J., Koopmans, S., Dekker, R. and Hoogendoorn, A. 2010. Increasing weaning age of piglets from 4 to 7 weeks reduces stress, increases post-weaning feed intake but does not improve intestinal functionality. *Animal.* **4**(10), pp.1653-1661.

Vancamelbeke, M. and Vermeire, S. 2017. The intestinal barrier: a fundamental role in health and disease. *Expert Review of Gastroenterology and Hepatology.* **11**(9), pp.821-834.

Vasquez, R., Oh, J.K., Song, J.H. and Kang, D.K. 2022. Gut microbiome-produced metabolites in pigs: a review on their biological functions and the influence of probiotics. *Journal of Animal Science and Technology.* **64**(4), pp.671-695.

Vavricka, S.R., Galván, J.A., Dawson, H., Soltermann, A., Biedermann, L., Scharl, M., Schoepfer, A.M., Rogler, G., Prinz Vavricka, M.B. and Terracciano, L. 2018. Expression patterns of TNFα, MAdCAM1, and STAT3 in intestinal and skin manifestations of inflammatory bowel disease. *Journal of Crohn's Colitis.* **12**(3), pp.347-354.

Velisek, J., Stara, A., Li, Z.-H., Silovska, S. and Turek, J. 2011. Comparison of the effects of four anaesthetics on blood biochemical profiles and oxidative stress biomarkers in rainbow trout. *Aquaculture.* **310**(3-4), pp.369-375.

Ventura, M., O'flaherty, S., Claesson, M.J., Turroni, F., Klaenhammer, T.R., Van Sinderen, D. and O'toole, P.W. 2009. Genome-scale analyses of health-promoting bacteria: probiogenomics. *Nataure Reviews in Microbiology.* **7**(1), pp.61-71.

Ventura, M., van Sinderen, D., Fitzgerald, G.F. and Zink, R. 2004. Insights into the taxonomy, genetics and physiology of bifidobacteria. *Antonie Van Leeuwenhoek.* **86**(3), pp.205-223.

Verbrugghe, P., Van Aken, O., Hållenius, F. and Nilsson, A. 2021. Development of a real-time quantitative PCR method for detection and quantification of Prevotella copri. *BMC Microbiology.* **21**(1), pp.1-10.

Vergauwen, H., Degroote, J., Prims, S., Wang, W., Fransen, E., De Smet, S., Casteleyn, C., Van Cruchten, S., Michiels, J. and Van Ginneken, C. 2017. Artificial rearing influences the morphology, permeability and redox state of the gastrointestinal tract of low and normal birth weight piglets. *Animal Science and Biotechnology.* **8**(1), pp.1-14.

Verhelst, R., Schroyen, M., Buys, N. and Niewold, T. 2014. Dietary polyphenols reduce diarrhea in enterotoxigenic Escherichia coli (ETEC) infected post-weaning piglets. *Livestock Science.* **160**, pp.138-140.

Vicente, B., Valencia, D.G., Pérez-Serrano, M., Lázaro, R. and Mateos, G.G. 2008. The effects of feeding rice in substitution of corn and the degree of starch gelatinization of rice on the digestibility of dietary components and productive performance of young pigs1,2. *Animal Science.* **86**(1), pp.119-126.

Víctor, V.M., Guayerbas, N. and De la Fuente, M. 2002. Changes in the antioxidant content of mononuclear leukocytes from mice with endotoxin-induced oxidative stress. *Molecular Cellular Biochemistry.* **229**(1), pp.107-111.

Vidal, P.J., López-Nicolás, J.M., Gandía-Herrero, F. and García-Carmona, F. 2014. Inactivation of lipoxygenase and cyclooxygenase by natural betalains and semi-synthetic analogues. *Food Chemistry.* **154**, pp.246-254.

Vieira, M.M., Paik, J., Blaner, W.S., Soares, A.M., Mota, R.M., Guerrant, R.L. and Lima, A.A. 2008. Carotenoids, retinol, and intestinal barrier function in children from northeastern Brazil. *Pediatric Gastroenterology Nutrition.* **47**(5), p652.

Vighi, G., Marcucci, F., Sensi, L., Di Cara, G. and Frati, F. 2008. Allergy and the gastrointestinal system. *Clinical Experimental Immunology* **153**(Supplement_1), pp.3-6.

Vikram, A., Woolston, J. and Sulakvelidze, A. 2021. Phage biocontrol applications in food production and processing. *Current issues in Molecular Biology.* **40**(1), pp.267-302.

Viuda-Martos, M., Ruiz Navajas, Y., Sánchez Zapata, E., Fernández-López, J. and Pérez-Álvarez, J.A. 2010. Antioxidant activity of essential oils of five spice plants widely used in a Mediterranean diet. *Flavour Fragrance Journal.* **25**(1), pp.13-19.

Voreades, N., Kozil, A. and Weir, T.L. 2014. Diet and the development of the human intestinal microbiome. *Frontiers in Microbiology.* **5**, p494.

Vulić, J.J., Ćebović, T.N., Čanadanović-Brunet, J.M., Ćetković, G.S., Čanadanović, V.M., Djilas, S.M. and Šaponjac, V.T.T. 2014. In vivo and in vitro antioxidant effects of beetroot pomace extracts. *Functional Foods* **6**, pp.168-175.

Vulić, J.J., Ćebović, T.N., Čanadanović, V.M., Ćetković, G.S., Djilas, S.M., Čanadanović-Brunet, J.M., Velićanski, A.S., Cvetković, D.D. and Tumbas, V.T. 2013. Antiradical, antimicrobial and cytotoxic activities of commercial beetroot pomace. *Food and Function.* (5), pp.713-721.

Wadhwa, M. and Bakshi, M. 2013. Utilization of fruit and vegetable wastes as livestock feed and as substrates for generation of other value-added products. *Rap Publication.* **4**(2013), p67.

Wadhwa, M., Bakshi, M. and Makkar, H. 2016. Wastes to worth: value added products from fruit and vegetable wastes. *CABI Reviews.* (2015), pp.1-25.

Wahlström, A., Sayin, S.I., Marschall, H.-U. and Bäckhed, F. 2016. Intestinal crosstalk between bile acids and microbiota and its impact on host metabolism. *Cell metabolism.* **24**(1), pp.41-50.

Walker, A.W., Ince, J., Duncan, S.H., Webster, L.M., Holtrop, G., Ze, X., Brown, D., Stares, M.D., Scott, P. and Bergerat, A. 2011. Dominant and diet-responsive groups of bacteria within the human colonic microbiota. *ISME Journal.* **5**(2), pp.220-230.

Wan, M.L.Y., Co, V.A. and El-Nezami, H. 2021. Dietary polyphenol impact on gut health and microbiota. *Critical Reviews in Food Science and Nutrition.* **61**(4), pp.690-711.

Wang, C.-Q. and Yang, G.-Q. 2010. Betacyanins from Portulaca oleracea L. ameliorate cognition deficits and attenuate oxidative damage induced by D-galactose in the brains of senescent mice. *Phytomedicine.* **17**(7), pp.527-532.

Wang, C., Shen, Z., Cao, S., Zhang, Q., Peng, Y., Hong, Q., Feng, J. and Hu, C. 2019. Effects of tributyrin on growth performance, intestinal microflora and barrier function of weaned pigs. *Animal Feed Science Technology.* **258**, p114311.

Wang, C., Shi, C., Zhang, Y., Song, D., Lu, Z. and Wang, Y. 2018. Microbiota in fermented feed and swine gut. *Applied Microbiology and Biotechnology.* **102**(7), pp.2941-2948.

Wang, C.C., Wu, H., Lin, F.H., Gong, R., Xie, F., Peng, Y., Feng, J. and Hu, C.H. 2018. Sodium butyrate enhances intestinal integrity, inhibits mast cell activation, inflammatory mediator production and JNK signaling pathway in weaned pigs. *Innate Immunity.* **24**(1), pp.40-46.

Wang, J., Ghosh, S.S. and Ghosh, S. 2017. Curcumin improves intestinal barrier function: modulation of intracellular signaling, and organization of tight junctions. *American Journal of Physiology-Cell Physiology.* **312**(4), pp.C438-C445.

Wang, J., Ji, H., Wang, S., Liu, H., Zhang, W., Zhang, D. and Wang, Y. 2018. Probiotic Lactobacillus plantarum promotes intestinal barrier function by strengthening the epithelium and modulating gut microbiota. *Frontiers in Microbiology.* **9**, p1953.

Wang, L.F., Beltranena, E. and Zijlstra, R.T. 2016. Diet nutrient digestibility and growth performance of weaned pigs fed sugar beet pulp. *Animal Feed Science and Technology.* **211**, pp.145-152.

Wang, S., Yao, B., Gao, H., Zang, J., Tao, S., Zhang, S., Huang, S., He, B. and Wang, J. 2019. Combined supplementation of Lactobacillus fermentum and Pediococcus acidilactici promoted growth performance, alleviated inflammation, and modulated intestinal microbiota in weaned pigs. *BMC Veterinary Research.* **15**(1), p239.

Wang, S., Zhang, C., Yang, J., Wang, X., Wu, K., Zhang, B., Zhang, J., Yang, A., Rajput, S.A. and Qi, D. 2020. Sodium butyrate protects the intestinal barrier by modulating intestinal host defense peptide expression and gut microbiota after a challenge with deoxynivalenol in weaned piglets. *Agricultural Food Chemistry.* **68**(15), pp.4515-4527.

Wang, S.P., Rubio, L.A., Duncan, S.H., Donachie, G.E., Holtrop, G., Lo, G., Farquharson, F.M., Wagner, J., Parkhill, J. and Louis, P. 2020. Pivotal roles for pH, lactate, and lactate-utilizing bacteria in the stability of a human colonic microbial ecosystem. *Msystems.* **5**(5), pp.e00645-00620.

Wang, T., Yao, W., Li, J., Shao, Y., He, Q., Xia, J. and Huang, F. 2020. Dietary garcinol supplementation improves diarrhea and intestinal barrier function associated with its modulation of gut microbiota in weaned piglets. *J Anim Sci Biotechnol.* **11**(1), p12.

Wang, W., Van Noten, N., Degroote, J., Romeo, A., Vermeir, P. and Michiels, J. 2019. Effect of zinc oxide sources and dosages on gut microbiota and integrity of weaned piglets. *Journal of Animal Physiology Animal Nutrition (Berl).* **103**(1), pp.231-241.

Wang, Y., Fernando, G.S.N., Sergeeva, N.N., Vagkidis, N., Chechik, V., Do, T., Marshall, L.J. and Boesch, C. 2022. Uptake and Immunomodulatory Properties of Betanin, Vulgaxanthin I and Indicaxanthin towards Caco-2 Intestinal Cells. *Antioxidants.* **11**(8), p1627.

Watford, W.T., Moriguchi, M., Morinobu, A. and O'Shea, J.J. 2003. The biology of IL-12: coordinating innate and adaptive immune responses. *Cytokine Growth Factor Reviews.* **14**(5), pp.361-368.

Watford, W.T. and O'Shea, J.J. 2003. A case of mistaken identity. *Nature.* **421**(6924), pp.706-707.

Wei, H.-K., Xue, H.-X., Zhou, Z. and Peng, J. 2017. A carvacrol–thymol blend decreased intestinal oxidative stress and influenced selected microbes without changing the messenger RNA levels of tight junction proteins in jejunal mucosa of weaning piglets. *Animal.* **11**(2), pp.193-201.

Wei, X., Tsai, T., Knapp, J., Bottoms, K., Deng, F., Story, R., Maxwell, C. and Zhao, J. 2020. ZnO modulates swine gut microbiota and improves growth performance of nursery pigs when combined with peptide cocktail. *Microorganisms.* **8**(2), p146.

Weiss, S.L. and Deutschman, C.S. 2014. Elevated malondialdehyde levels in sepsis-something to'stress' about? *Critical Care.* **18**(2), pp.1-2.

Wells, J.M., Brummer, R.J., Derrien, M., MacDonald, T.T., Troost, F., Cani, P.D., Theodorou, V., Dekker, J., Méheust, A. and De Vos, W.M. 2017. Homeostasis of the gut barrier and potential biomarkers. *American Journal of Physiology-Gastrointestinal Liver Physiology.* **312**(3), pp.G171-G193.

WHO. 2001. *World Health Organization; Global strategy for containment of antimicrobial resistance.* World Health Organization.

WHO. 2013. *World Health Organization*

;Global action plan for the prevention and control of noncommunicable diseases 2013-2020. World Health Organization.

WHO. 2016. *World health statistics 2016: Monitoring health for the SDGs sustainable development goals.* World Health Organization.

Wickham, H. 2011. ggplot2. *Wiley Interdisciplinary Reviews: Computational Statistics* **3**(2), pp.180-185.

Wiczkowski, W., Romaszko, E., Szawara-Nowak, D. and Piskula, M.K. 2018. The impact of the matrix of red beet products and interindividual variability on betacyanins bioavailability in humans. *Food Research International.* **108**, pp.530-538.

Wiecek, M., Szymura, J., Maciejczyk, M., Kantorowicz, M. and Szygula, Z. 2018. Anaerobic exercise-induced activation of antioxidant enzymes in the blood of women and men. *Frontiers in Physiology.* p1006.

Wijtten, P.J., van der Meulen, J. and Verstegen, M.W. 2011. Intestinal barrier function and absorption in pigs after weaning: a review. *British Journal of Nutrition.* **105**(7), pp.967-981.

Williams, I. 2003. Growth of the weaned pig. *Weaning the Pig: Concepts and Consequences, 1st ed.; Pluske, JR, Le Dividich, J., Verstegen, MWA, Eds.* pp.17-37.

Williams, R.J., Spencer, J.P. and Rice-Evans, C. 2004. Flavonoids: antioxidants or signalling molecules? *Free Radical Biology and Medicine.* **36**(7), pp.838-849.

Windey, K., De Preter, V. and Verbeke, K. 2012. Relevance of protein fermentation to gut health. *Molecular Nutrition Food Research.* **56**(1), pp.184-196.

Winter, S.E., Winter, M.G., Xavier, M.N., Thiennimitr, P., Poon, V., Keestra, A.M., Laughlin, R.C., Gomez, G., Wu, J. and Lawhon, S.D. 2013. Host-derived nitrate boosts growth of E. coli in the inflamed gut. *Science.* **339**(6120), pp.708-711.

Wong, S.Y., Grant, I.R., Friedman, M., Elliott, C.T. and Situ, C. 2008. Antibacterial activities of naturally occurring compounds against Mycobacterium avium subsp. paratuberculosis. *Applied Environmental Microbiology.* **74**(19), pp.5986-5990.

Wootton-Beard, P.C., Brandt, K., Fell, D., Warner, S. and Ryan, L. 2014. Effects of a beetroot juice with high neobetanin content on the early-phase insulin response in healthy volunteers. *Nutritional Science.* **3**.

Wootton-Beard, P.C., Moran, A. and Ryan, L. 2011. Stability of the total antioxidant capacity and total polyphenol content of 23 commercially available vegetable juices before and after in vitro digestion measured by FRAP, DPPH, ABTS and Folin–Ciocalteu methods. *Food Research international.* **44**(1), pp.217-224.

Wu, J., Wang, J., Lin, Z., Liu, C., Zhang, Y., Zhang, S., Zhou, M., Zhao, J., Liu, H. and Ma, X. 2023. Clostridium butyricum alleviates weaned stress of piglets by improving intestinal immune function and gut microbiota. *Food Chemistry.* **405**, p135014.

Wybraniec, S., Stalica, P., Spórna-Kucab, A., Nemzer, B., Pietrzkowski, Z. and Michałowski, T. 2011. Antioxidant Activity of Betanidin: Electrochemical Study in Aqueous Media. *Journal of Agricultural and Food Chemistry.* **59**, pp.12163-12170.

Xiang, Q., Wu, X., Pan, Y., Wang, L., Cui, C., Guo, Y., Zhu, L., Peng, J. and Wei, H. 2020. Early-life intervention using fecal microbiota combined with probiotics promotes gut microbiota maturation, regulates immune system development, and alleviates weaning stress in piglets. *International journal of molecular sciences.* **21**(2), p503.

Xiao, D., Wang, Y., Liu, G., He, J., Qiu, W., Hu, X., Feng, Z., Ran, M., Nyachoti, C.M. and Kim, S.W. 2014. Effects of chitosan on intestinal inflammation in weaned pigs challenged by enterotoxigenic Escherichia coli. *PLoS One.* **9**(8), pe104192.

Xiao, L., Sun, Y. and Tsao, R. 2022. Paradigm Shift in Phytochemicals Research: Evolution from Antioxidant Capacity to Anti-Inflammatory Effect and to Roles in Gut Health and Metabolic Syndrome. *Journal of Agricultural Food Chemistry.* **70**(28), pp.8551-8568.

Xiong, X., Tan, B., Song, M., Ji, P., Kim, K., Yin, Y. and Liu, Y. 2019. Nutritional Intervention for the Intestinal Development and Health of Weaned Pigs. *Frontiers in Veterinary Science* **6**, p46.

Xu, B., Qin, W., Xu, Y., Yang, W., Chen, Y., Huang, J., Zhao, J. and Ma, L. 2021. Dietary quercetin supplementation attenuates diarrhea and intestinal damage by regulating gut microbiota in weanling piglets. *Oxidative Medicine Cellular Longevity.* **202,** p19.

Xu, J., Xu, C., Chen, X., Cai, X., Yang, S., Sheng, Y. and Wang, T. 2014. Regulation of an antioxidant blend on intestinal redox status and major microbiota in early weaned piglets. *Nutrition.* **30**(5), pp.584-589.

Xu, M., Jiang, Z., Wang, C., Li, N., Bo, L., Zha, Y., Bian, J., Zhang, Y. and Deng, X. 2019. Acetate attenuates inflammasome activation through GPR43-mediated Ca2+-dependent NLRP3 ubiquitination. *Experimental Molecular Medicine.* **51**(7), pp.1-13.

Yan, C.L., Kim, H.S., Hong, J.S., Lee, J.H., Han, Y.G., Jin, Y.H., Son, S.W., Ha, S.H. and Kim, Y.Y. 2017. Effect of Dietary sugar beet pulp supplementation on growth performance, nutrient digestibility, fecal Microflora, blood profiles and Diarrhea incidence in weaning pigs. *Animal Science and Technology.* **59**, p18.

Yan, L., Zhang, Z., Park, J. and Kim, I. 2012. Evaluation of Houttuynia cordata and Taraxacum officinale on growth performance, nutrient digestibility, blood characteristics, and fecal microbial shedding in diet for weaning pigs. *Asian-Australasian Journal of Animal Sciences.* **25**(10), p1439.

Yang, Q., Huang, X., Wang, P., Yan, Z., Sun, W., Zhao, S. and Gun, S. 2019. Longitudinal development of the gut microbiota in healthy and diarrheic piglets induced by age-related dietary changes. *Microbiologyopen.* **8**(12), pe923.

Yazdankhah, S., Rudi, K. and Bernhoft, A. 2014. Zinc and copper in animal feed—Development of resistance and co-resistance to antimicrobial agents in bacteria of animal origin. *Microbial Ecology in Health and Disease.* **25**,p7

Ye, D., Ma, I., Ma, T.Y.J.A.J.o.P.-G. and Physiology, L. 2006. Molecular mechanism of tumor necrosis factor-α modulation of intestinal epithelial tight junction barrier. *American Journal of Physiology-Gastrointestinal Liver Physiology* **290**(3), pp.G496-G504.

Ye, J., Coulouris, G., Zaretskaya, I., Cutcutache, I., Rozen, S. and Madden, T.L. 2012. Primer-BLAST: a tool to design target-specific primers for polymerase chain reaction. *BMC Bioinformatics.* **13**(1), pp.1-11.

Yin, J., Ren, W., Liu, G., Duan, J., Yang, G., Wu, L., Li, T. and Yin, Y. 2013. Birth oxidative stress and the development of an antioxidant system in newborn piglets. *Free Radical Research.* **47**(12), pp.1027-1035.

Yin, J., Wu, M., Xiao, H., Ren, W., Duan, J., Yang, G., Li, T. and Yin, Y. 2014. Development of an antioxidant system after early weaning in piglets. *Animal Science.* **92(**2), pp.612-619.

Yin, R., Kuo, H.C., Hudlikar, R., Sargsyan, D., Li, S., Wang, L., Wu, R. and Kong, A.N. 2019. Gut microbiota, dietary phytochemicals and benefits to human health. *Current Pharmacology Reports.* **5**(5), pp.332-344.

Yin, X., Ming, D., Bai, L., Wu, F., Liu, H., Chen, Y., Sun, L., Wan, Y., Thacker, P.A. and Wu, G. 2019. Effects of pyrroloquinoline quinone supplementation on growth performance and small intestine characteristics in weaned pigs. *Animal Science.* **97**(1), pp.246-256.

Yoshino, F., Yoshida, A., Okada, E., Okada, Y., Maehata, Y., Miyamoto, C., Kishimoto, S., Otsuka, T., Nishimura, T. and Lee, M.C.-i. 2012. Dental resin curing blue light induced oxidative stress with reactive oxygen species production. *Journal of Photochemistry Photobiology B: Biology.* **114**, pp.73-78.

Yu, T., Zhu, C., Chen, S., Gao, L., Lv, H., Feng, R., Zhu, Q., Xu, J., Chen, Z. and Jiang, Z. 2017. Dietary High Zinc Oxide Modulates the Microbiome of Ileum and Colon in Weaned Piglets. *Frontiers in Microbiology.* **8**, p825.

Yusuf, M., Khan, R.A., Khan, M. and Ahmed, B. 2012. Plausible antioxidant biomechanics and anticonvulsant pharmacological activity of brain-targeted β-carotene nanoparticles. *International Journal of Nanomedicine.* **7**, p4311.

Zarepour, M., Bhullar, K., Montero, M., Ma, C., Huang, T., Velcich, A., Xia, L. and Vallance, B.A. 2013. The mucin Muc2 limits pathogen burdens and epithelial barrier dysfunction during Salmonella enterica serovar Typhimurium colitis. *Infection and Immunity.* **81**(10), pp.3672-3683.

Zeb, A. and Zeb, A. 2021. Phenolic Antioxidants in Vegetables. *Phenolic Antioxidants in Foods: Chemistry, Biochemistry Analysis.* pp.131-148.

Zelnickova, P., Leva, L., Stepanova, H., Kovaru, F. and Faldyna, M. 2008. Age-dependent changes of proinflammatory cytokine production by porcine peripheral blood phagocytes. *Veterinary Immunology and Immunopathology.* **124**(3-4), pp.367-378.

Zeng, M.Y., Cisalpino, D., Varadarajan, S., Hellman, J., Warren, H.S., Cascalho, M., Inohara, N. and Núñez, G. 2016. Gut microbiota-induced immunoglobulin G controls systemic infection by symbiotic bacteria and pathogens. *Immunity.* **44**(3), pp.647-658.

Zhan, K., Zheng, H., Li, J., Wu, H., Qin, S., Luo, L. and Huang, S. 2020. Gut microbiota-bile acid crosstalk in diarrhea-irritable bowel syndrome. *BioMed Research International.* **2020**, p16

Zhang, B. and Guo, Y. 2009. Supplemental zinc reduced intestinal permeability by enhancing occludin and zonula occludens protein-1 (ZO-1) expression in weaning piglets. *British Journal of Nutrition.* **102**(5), pp.687-693.

Zhang, D., Ji, H., Liu, H., Wang, S., Wang, J. and Wang, Y. 2016. Changes in the diversity and composition of gut microbiota of weaned piglets after oral administration of Lactobacillus or an antibiotic. *Applied Microbiology and Biotechnology.* **100**(23), pp.10081-10093.

Zhang, G., Hu, G., Yang, Z. and Zhao, J. 2022. Effects of tetrabasic zinc chloride on growth performance, nutrient digestibility and fecal microbial community in weaned piglets. *Frontiers in Veterinary Science.* **9**:905242.

Zhang, H., Wielen, N.v.d., Hee, B.v.d., Wang, J., Hendriks, W. and Gilbert, M. 2020. Impact of fermentable protein, by feeding high protein diets, on microbial composition, microbial catabolic activity, gut health and beyond in pigs. *Microorganisms.* **8**(11), p1735.

Zhang, L., Wu, W., Lee, Y.K., Xie, J. and Zhang, H. 2018. Spatial Heterogeneity and Co-occurrence of Mucosal and Luminal Microbiome across Swine Intestinal Tract. *Frontiers in Microbiology.* **9**, p48.

Zhang, W., Li, D., Liu, L., Zang, J., Duan, Q., Yang, W. and Zhang, L. 2013. The effects of dietary fiber level on nutrient digestibility in growing pigs. *Animal Science Biotechnology.* **4**(1), pp.1-7.

Zhang, W. and Sack, D.A. 2012. Progress and hurdles in the development of vaccines against enterotoxigenic Escherichia coli in humans. *Expert Review of Vaccines.* **11**(6), pp.677-694.

Zhang, Y., Wang, Y., Chen, D.W., Yu, B., Zheng, P., Mao, X.B., Luo, Y.H., Li, Y. and He, J. 2018. Dietary chlorogenic acid supplementation affects gut morphology, antioxidant capacity and intestinal selected bacterial populations in weaned piglets. *Food & Function.* **9**(9), pp.4968-4978.

Zhao, J., Zhang, Z., Zhang, S., Page, G. and Jaworski, N. 2021. The role of lactose in weanling pig nutrition: a literature and meta-analysis review. *Animal Science and Biotechnology.* **12,** 10 (2021).

Zhao, P.Y. and Kim, I.S. 2015. Effect of direct-fed microbial on growth performance, nutrient digestibility, fecal noxious gas emission, fecal microbial flora and diarrhea score in weaning pigs. *Animal Feed Science and Technology.* **200**(2015), p86-92.

Zhao, Y., Su, J.Q., An, X.L., Huang, F.Y., Rensing, C., Brandt, K.K. and Zhu, Y.G. 2018. Feed additives shift gut microbiota and enrich antibiotic resistance in swine gut. *Science of Total Environment.* **621**, pp.1224-1232.

Zhong, X., Zhang, Z., Wang, S., Cao, L., Zhou, L., Sun, A., Zhong, Z. and Nabben, M. 2019. Microbial-driven butyrate regulates jejunal homeostasis in piglets during the weaning stage. *Frontiers in Microbiology.* **9**, p3335.

Zhou, M., Johnston, L.J., Wu, C. and Ma, X. 2021. Gut microbiota and its metabolites: Bridge of dietary nutrients and obesity-related diseases. *Critical Reviews in Food Science Nutrition.* pp.1-18.

Zhu, C., Lv, H., Chen, Z., Wang, L., Wu, X., Chen, Z., Zhang, W., Liang, R. and Jiang, Z. 2017. Dietary Zinc Oxide Modulates Antioxidant Capacity, Small Intestine Development, and Jejunal Gene Expression in Weaned Piglets. *Biology of Trace Element Research.* **175**(2), pp.331-338.

Zhu, L., Han, J., Li, L., Wang, Y., Li, Y. and Zhang, S. 2019. Claudin family participates in the pathogenesis of inflammatory bowel diseases and colitis-associated colorectal cancer. *Frontiers in Immunology.* **10**, p1441.

Zhu, L., Zhao, K., Chen, X. and Xu, J. 2012. Impact of weaning and an antioxidant blend on intestinal barrier function and antioxidant status in pigs. *Animal Science.* **90**(8), pp.2581-2589.

Zielińska-Przyjemska, M., Olejnik, A., Dobrowolska-Zachwieja, A. and Grajek, W. 2009. In vitro effects of beetroot juice and chips on oxidative metabolism and apoptosis in neutrophils from obese individuals. *Phytotherapy Research.* **23**(1), pp.49-55.

Zijlmans, M.A., Korpela, K., Riksen-Walraven, J.M., de Vos, W.M. and de Weerth, C. 2015. Maternal prenatal stress is associated with the infant intestinal microbiota. *Psychoneuroendocrinology.* **53**, pp.233-245.