The effect of pre-weaning rearing environment and post-weaning provision of zinc oxide on the gastrointestinal tract health and microbiome in pigs

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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 reference has been made to the work of others.

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Abstract

The use of pharmacological levels of zinc oxide in pig diets for 14 days post-weaning has been common practice for many farmers across the EU. High dietary zinc oxide frequently improves growth performance and reduces the incidence of diarrhoea immediately after weaning, thereby reducing mortality and in turn improving profitability for farmers. However, environmental concerns and reports of contribution to the spread of antibiotic resistance genes in pathogenic bacteria, have resulted in the upcoming ban of pharmacological levels of zinc oxide in weaner pig diets across the EU in 2022. Alternative strategies to improve pig performance and health after weaning are continuously being sought, with the potential for rearing piglets outdoors before weaning, showing such benefits. Therefore, the research presented in this thesis aimed to investigate whether pre-weaning rearing environment and post-weaning supplementation of zinc oxide improved pig performance from farrow to finish and whether there are components of the gastrointestinal tract microbiome that showed similarities between rearing environment and dietary treatment. It was found that pigs reared outdoors showed lifetime performance benefits; enabling the majority of pigs reared outdoors to be sent to slaughter sooner than those reared indoors, whilst zinc oxide supplementation improved performance of pigs during the first two weeks after weaning but did not provide lifetime advantages, in support of previous findings. Remarkably, similar shifts in the composition of some bacteria of the small intestine were detected when comparing indoor pigs without zinc oxide supplementation to all outdoor pigs as well as indoor pigs receiving zinc oxide supplementation. Both outdoor rearing and the supplementation of zinc oxide in the diets of indoor-reared pigs, improved performance during the period of treatment after weaning, with added lifetime performance improvements of outdoor-reared pigs. This suggests that outdoor rearing and zinc oxide supplementation might cause similar changes in the microbiota that could be associated with improved pig performance immediately after weaning. The significance of these findings are discussed with a focus on continuing the work to investigate associations between the microbiota and pig performance.

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List of Abbreviations

ADFI	**Average daily feed intake**
ADG	**Average daily gain**
AGPs	**Antibiotic growth promotors**
AHDB	**Agriculture and horticulture development board**
APP	**Acute phase protein**
APR	**Acute phase response**
cAMP	**Cyclic adenosine monophosphate**
cDNA	**Complimentary DNA**
CFU	**Colonyforming unit**
Cq	**Quantification cycle**
Ct	**Cycle threshold**
CV	**Coefficient variation**
DCP	**Dicalcium phosphate**
DEFRA	**Department for environment, food and rural affairs**
DNA	**Deoxyribonucleinc acid**
dNTP	**Deoxynucleoside triphosphate**
EDTA	**Ethylenediaminetetraacetic acid**
ELISA	**Enzyme linked immunosorbent assay**
EMM	**Estimated marginal means**
EPEC	**Enteropathogenic *Escherichia coli***
ETEC	**Enterotoxigenic *Escherichia coli***
FCE	**Feed conversion efficiency**
FCR	**Feed conversion ratio**
FOS	**Fructooligosaccharides**
GAPDH	**Glyceralehyde-3-phosphate-dehydrogenase**
GIT	**Gastrointestinal tract**
GLM	**General linear model**
HRP	**Horseraddish peroxidase conjugte**
HSD	**Honestly signficant difference**
IFN	**Interferon**
IL	**Interleukin**

IU	**International unit**
MH	**Mycoplasma hyopneumoniae**
MOS	**Mannanologosaccharide**
mRNA	**Messenger ribonucleic acid**
NGS	**Next generation sequencing**
NMDS	**Non-metric multidimensional scaling**
NO	**Nitric Oxide**
NRC	**National research council**
NRQ	**Normalised relative quantities**
OD	**Optical density**
OTU	**Operational taxonomic unit**
PBS	**Phosphate buffered saline**
PCR	**Polymerase chain reaction**
PERMANOVA	**Permutational analysis of variation**
pig-MAP	**Pig major acute phase protein**
PWD	**Post-weaning diarrhoea**
qPCR	**Quantitative polymerase chain reaction**
RNA	**Ribonucleic acid**
rRNA	**Ribosomal RNA**
RT	**Reverse transcriptase**
SCFA	**Scort-chain fatty acids**
SEM	**Standard error of the mean**
SID	**Standardised ileal digestibility**
SOP	**Standard operating procedure**
TAE	**Tris-acetate-EDTA**
TBZC	**Tetrabasic zinc chloride**
TNF-α	**Tumour necrosis factor**
TOS	**Trans-galactooligosaccharides**
UK	**United Kingdom**
UV	**Ultraviolet**
ZnO	**Zinc oxide**
ZO-1	**Zonula occludens protein-1**

Chapter 1
General Introduction

1.1 The UK pig industry

The growing human population, which is expected to exceed 10 billion by 2055 (UN, 2017), along with the increase in average per capita income, is increasing the demand for livestock products worldwide (Gunnarsson et al., 2020). This growing demand increases pressure within the pig industry to produce more pigs, that are leaner and heavier in weight, in less time. Worldwide, in 2019, nearly 1.4 billion pigs were produced and slaughtered for the meat industry, equating to over 110 million tonnes of pig meat; the second highest volume of production animal, behind chickens (Nations, 2019). In the same year, the UK produced 0.8% of these pigs, equating to over 10 million pigs sent to slaughter, or over 950,000 tonnes of pig meat, excluding cull sows and boars (DEFRA, 2021). Total pig herd size within the UK has steadily increased year-on-year since 2015, with 2019 showing the largest pig herd since 2003, at 5.1 million pigs in June and 4.7 million pigs in December across ~10,500 holdings (AHDB, 2020). The female breeding herd across the UK has also steadily increased since 2015, with 2020 recording over 400,000 females.

Economically important traits such as the potential for—and speed of—growth have been selected for in pigs over time and are an integral part of commercial pig production success in the UK (Fabian et al., 2003). The pig price within the UK fluctuates dramatically, causing variable pressure on farmers to ensure they can continue producing when outgoings are higher, or close to, their income. When pig prices fluctuate dramatically overtime, and little can be done to account for rising feed prices, optimising pig weight and numbers is a key area for continued improvement.

1.1.1 Outdoor pig breeding in the UK

The UK is unique, in that 40% of the breeding sow herd is managed outdoors, which brings many benefits and some disadvantages compared to more typical intensive, indoor systems (AHDB, 2021c). Although 40% of UK pigs are bred outdoors, these piglets are typically weaned into either hut-and-run outdoor systems, or more commonly, into fully enclosed buildings (Edwards, 2005), with only an estimated 2% of UK pigs living outdoors for their entire production life

(AHDB, 2019). In outdoor breeding systems, straw bedded arks within pasture paddocks provide the sow the opportunity to perform more natural nesting behaviours prior to farrowing and subsequently provide a more enriched environment for her piglets (Cox and Cooper, 2001). Hötzel et al. (2004) showed that outdoor bred piglets were also more active and had a more extensive behavioural repertoire compared to indoor-reared piglets; exhibiting more locomotion, exploration of the environment as well as standing and feeding behaviours. In turn, the increase in an outdoor pigs behavioural repertoire reportedly enables development of strategies to overcome environmental challenges and exposes them to a wider variety of microbes, which potentially enhances the development of their gastrointestinal tract (GIT) and their immune system. Outdoor breeding of pigs can also be associated with cost benefits to the farmer. Historically, breeding pigs outdoors has provided significant cost reductions due to reduced building costs, whilst still achieving similar gross margins to indoor-reared pigs (Gentry and McGlone, 2003).

Outdoor production can be beneficial for sow and piglet welfare but pre-weaning mortality of piglets has historically been different between the rearing environments. O'Reilly et al. (2006) identified a trend towards an increase in mortality of piglets reared in outdoor systems (14%) compared to those reared indoors (10%) when they performed a cross-sectional study of 67 farms across England and Wales in 2006. Since this date, UK average mortality percentages have become more balanced across environments with AHDB figures showing mortality of 12.8% and 12.2% of pigs reared outdoors and indoors, respectively as of September 2021 (AHDB, 2021b; AHDB, 2021a). Increased mortality from predation and disease from other animals is a risk considered to be associated with outdoor, but not indoor, pig breeding. Furthermore, general management of outdoor bred piglets can be harder given the increased difficulty and risk of accessing a litter frequently, due to the freedom of the sow. Furthermore, the inability to control temperatures for sows and piglets outdoors can lead to heat stress in sows during the summer months (Schild et al., 2016). Conversely, new-born piglets can suffer from hypothermia immediately after birth as a result of the cold temperatures in winter (Baxter et al., 2011). This can lead to lethargy and increases the chance of mortality due to sow crushing (Baxter et al., 2011). Any increases in mortality of piglets before weaning reduces the number of piglets

weaned, which is directly linked to profitability for farmers and can have a significant role in a farms overall viability (Vande Pol et al., 2021; Park et al., 2017).

1.1.2 Indoor pig breeding in the UK

Piglets reared on indoor units can often be perceived by the consumer as having poor welfare, with limited environmental stimuli (Lau et al., 2015). This is particularly relevant regarding the use of farrowing crates for indoor-housed sows; although, crates are a cost-effective way to ensure the safety of piglets and minimises mortality through sow crushing (Guy et al., 2012). This is why the use of farrowing crates indoors is the most predominant method of production currently in the UK. However, the restraint associated with the use of farrowing crates prevents natural mothering behaviours, including nest building, that is commonly seen in outdoor sows (Damm et al., 2000). As a result, Jarvis et al. (2001) identified an increase in markers of stress, such as plasma cortisol and heart rate, just before parturition in crated sows, compared to sows in straw bedded pens. Furthermore, Oliviero et al. (2008) found that sows who had restricted movement due to being crated, had a longer farrowing duration (considered to be above 300 minutes) which was associated with an increase in stillborn piglets (Oliviero et al., 2010; Van Dijk et al., 2005)

While an increase in stillborn piglets has previously been identified when sows are crated, the use of farrowing crates are primarily designed to reduce the number of piglets that die from sow crushing after birth, due to her restricting mobility. Crushing of piglets by the sow is the leading cause of pre-weaning mortality worldwide and is a significant welfare concern (Villanueva-García et al., 2020; KilBride et al., 2012).The majority of pre-weaning piglet mortality occurs within the first 48 hours after birth, when piglets are most vulnerable in terms of their thermal and nutritional needs. This vulnerability can result in reduced mobility, making them more at risk of crushing by the sow (Marchant et al., 2001). Reducing pre-weaning mortality means more piglets survive to weaning and potentially to slaughter (KilBride et al., 2012). The cost of these additional pigs to slaughter must be balanced against the cost of building maintenance and space required, to determine the true benefit to a farmer of either indoor or outdoor rearing.

Nonetheless, sows farrowing in an indoor environment, are easier to access for the stockperson, thus enabling better control and management of disease or illness in both sow and piglets, potentially further reducing pre-weaning mortality (Cox and Cooper, 2001). In addition, indoor environments offer increased control over ambient temperature and lighting for pigs. Unlike outdoor pig production, indoor environments can provide a more stable, ambient temperature within the sows thermal comfort zone for optimal milk production (~18ºC) all year (Black et al., 1993). In addition, targeted heat for piglets in the form of heat lamps or heat mats, can provide an area with increased temperature, for their thermal comfort zone of ~30ºC to also be achieved (Baxter et al., 2011).

1.1.3 The effect of rearing environment on piglet weight prior to weaning

Increased weight of piglets at birth, alongside reduced weight variation, are key factors that can influence overall performance traits in pigs. This is particularly the case as low birth weights can impair growth performance during the pre-weaning stage, and can have lifelong impacts to slaughter. It is established that an increase in litter size is negatively associated with birth weight of piglets (Milligan et al., 2002). On this basis, outdoor-reared piglets could be expected to weigh more at birth compared to those reared indoors, as a result of smaller litter sizes. Differences in litter sizes are often reported as a result of different genetics used across rearing environments, with AHDB reporting an average litter size of 14 piglets indoors and 12 piglets outdoors (AHDB, 2021b; AHDB, 2021a). Previous work conducted by Miller et al. (2009) at the University of Leeds identified no differences in birth weight of pigs reared in an indoor or outdoor environment.

Low birth weight pigs have also been associated with reduced milk intake during the pre-weaning stage, leading to reduced growth overall (Milligan et al., 2002). Although Miller et al. (2009) did not find significant differences in birth weight between indoor and outdoor environments, the same study did find that outdoor piglets weaned significantly heavier than those reared indoors. In many instances, although weaning weight of outdoor pigs is heavier, there are often fewer pigs within the litter, thus heavier weights may be as a result of increased intake of sow milk as there are fewer pigs to compete with (Miller et al., 2009; Johnson et al., 2001). Weaning weight is an important factor to consider in overall

pig performance as it can have a profound influence on post-weaning growth performance (Collins et al., 2017).

1.2 Weaning: A critical stage in pig production

The weaning process of pigs is arguably one of the most critical phases in the pig production cycle (Lau et al., 2015). Naturally, pigs would be weaned gradually between 14 and 17 weeks of age, yet in commercial settings, piglets are typically weaned abruptly between 3 and 4 weeks old (Cox and Cooper, 2001). The Welfare of Farmed Animals, England, Regulation 2007, states that piglets in the UK should not be weaned prior to 28 days of age, unless the welfare and health of the sow or piglets are otherwise compromised. However, the legislation allows piglets to be weaned up to seven days early if specialised housing is provided and thoroughly disinfected before reintroduction of new piglets. Although even later weaning may be more beneficial for the piglets, the post-weaning growth check of any weaned piglet has to be balanced with returning the sow to the production system to produce another litter, enabling more piglets to be produced per year.

In any circumstance, weaning is typically characterised by two major changes and challenges. Firstly, the piglet's food supply changes from their sow's milk—made up of 80% water, with the remaining dry matter comprising predominantly fat (40%) and protein (30%)—to a solid, often plant and animal protein-based diet, that is 88% dry, in the form of a pellet (Pluske et al., 2003a; Pluske et al., 2003b). Food presentation also changes from the sow's udder, typically to a plastic feed trough or creep feeder (Pluske et al., 2003b). A further challenge faced by pigs at weaning is the change in their physical environment, with their transportation away from the sow, into new rooms and pens (Lau et al., 2015). This change in environment is also frequently associated with mixing of litters; forcing new social hierarchies to form and thus potentially inducing aggressive behaviours post-weaning (Colson et al., 2006). Evidence of stress post-weaning is seen through the increase in cortisol and corticotrophin-releasing factor within the blood of pigs post-weaning (Moeser et al., 2007). Overall, the pig experiences significant physiological, environmental and social challenges when weaned, that can predispose them to disease and result in production losses. These losses arise as a result of intestinal, immunological and behavioural changes (Campbell et al., 2013).

1.2.1 Behavioural effects of weaning

Removal of the sow at weaning also results in the removal of her stimuli to feed; which conditions piglets to suckle at regular intervals, as well as encourages them to feed as a group (Pluske et al., 2003b). The removal of this stimuli and mixing of litters, can result in a prolonged period of time where some piglets do not consume any food. According to Bruininx et al. (2001), approximately 50% of newly weaned pigs eat within four hours after weaning, but 50 hours had to pass (post-weaning) before 95% of pigs had eaten. These differences in time to feed may be a direct result of the differing reactions to the stressors faced at weaning.

To reduce the time to feed post-weaning, creep feed is often provided to indoor-reared piglets before weaning, although results are varied with how much this helps feed intake after weaning and subsequent growth, which may at least in part be a result of varying intake pre-weaning (Bruininx et al., 2002). Outdoor-reared pigs are rarely provided creep feed, but have been reported to show more feeding behaviours in the first hour post-weaning, compared to indoor-reared piglets receiving creep (Cox and Cooper, 2001). This could be linked to eating sow feed and vegetation found in the outdoor environment during the pre-weaning stage (Hötzel et al., 2004). Ultimately, growth performance can be hindered by low feed intake, making it imperative to encourage pigs to feed as soon as possible after weaning. Reduced intake, alongside an immature digestive and immune system can result in GIT disturbances and reduced overall health (Jayaraman and Nyachoti, 2017).

1.3 Gastrointestinal tract health and the impact of weaning

The GIT is a hugely complex organ and is responsible for regulating physiological homeostasis to enable the host to withstand both non-infectious and infectious stressors, that are naturally present throughout their lifetime (Maslowski and Mackay, 2011; Kogut and Arsenault, 2016). The term GIT health, or ‘gut health’ is widely used, with a broad range of definitions. Celi et al. (2017) has defined the term gut heath in mature production animals as being a ‘steady state where the microbiome and the intestinal tract exist in symbiotic equilibrium and where the welfare and performance of the animal is not constrained by intestinal dysfunction’. The definition incorporates three principle components: the digestion and absorption of nutrients; GIT barrier functionality and mucosal

immune system; and GIT microbiome and host interactions. It is also imperative to include the interactions between these components when considering gut health (Celi et al., 2017). As previously discussed, weaning is one of the most challenging times within a production pig's lifetime and is often accompanied by reduced feed intake and growth after weaning (Bruininx et al., 2001). Feed intake after weaning has been directly related to physiological and histological changes in the small intestine that impact on nutrient digestion and absorption in the GIT (Pluske et al., 1996; Pluske et al., 1997).

1.3.1 Gastrointestinal tract morphology and subsequent impact on nutrient digestion and absorption

Digestion and absorption of feed is complete by the stomach, small intestine, large intestine, pancreas and liver. Digestive and absorptive abilities of a young pig's GIT is dependent on the physical capacity of the GIT, the secretions it can provide, the mechanisms to control these secretions as well as the digestive and absorptive capacity of the mucosa within the small intestine (Cranwell, 1995; Pluske et al., 2003a). The small intestine has a variety of functions, including the absorption of nutrients and electrolytes as well as barrier protection against harmful antigens and pathogens (Lallès et al., 2004). Dietary fibre is resistant to digestion by endogenous enzymes within the small intestine and is instead, fermented by the microbiota within the large intestine (Williams et al., 2001). Fermentation of fibre in the large intestine produces volatile fatty acids that can be absorbed to provide the animal with energy and also has an important role in regulating host metabolism and immune system (Zhao et al., 2020; Koh et al., 2016). Although all parts of the GIT are influenced by the weaning process, it is predominantly the small intestine that sees the greatest anatomical, physiological and immunological adaptations (Pluske, 2016; Lallès et al., 2004; Wijtten et al., 2011). The epithelial lining of the small intestine has finger-like projections known as villi, that help to increase its surface area for digestion and absorption, as well as tubular glands that open into the intestinal lumen, at the base of the villi, known as crypts (Heo et al., 2013). As it is not possible to explain the multitude of changes that occur within the small intestine within this review, focus will firstly be prioritised to the changes seen in villous height and crypt depth and the impact on nutrient digestion and absorption this has within the newly weaned pig.

1.3.1.1 Gastrointestinal tract morphology

It has long been documented that stress at weaning causes a reduction in villous height (villous atrophy) and an increase in crypt depth (crypt hyperplasia) in the small intestine, immediately after weaning (Pluske et al., 1997; Xu et al., 2000; Miller et al., 1986; Hampson, 1986). Villous atrophy is caused either by an increased rate of cell loss, associated with increased crypt-cell production, which leads to an increased crypt-depth (Pluske et al., 1997). Alternatively, villous atrophy can also be caused by a reduced rate of cell renewal, from reduced cell division in the crypts of the small intestine (Pluske et al., 1997). The extent to which villous atrophy occurs has been linked to the level of feed intake in pigs post-weaning, with the lack of luminal stimulation by nutrients shown to induce villous atrophy (Kelly et al., 1985; van Beers-Schreurs et al., 1998; Pluske et al., 1996; Goldstein et al., 1985). Composition of the diet after weaning has not had the same impact on villous atrophy as time of first feed (van Beers-Schreurs et al., 1998; Pluske et al., 1996), indicating time to first feed is of most importance in reducing the impact on GIT structure. Villous height has previously shown to reduce by around 25 to 35% of pre-weaning height, within the first 24 hours post-weaning (when weaned at 21 days of age (Campbell, 2013)). This decrease was shown to continue until around day five, when villi were half the initial height. The changes seen in villous height and crypt depth have been shown to be transient, in agreement with the effect of fasting, with Boudry et al. (2004) also seeing a recovery in villous atrophy two days after feeding had resumed. The transient changes in villous height and crypt depth lead to a temporary decrease in the digestive and absorptive capacity of the small intestine (Pluske et al., 1997; Xu et al., 2000).

1.3.1.2 Brush-border enzymes and digestion

For optimal digestion and absorption of the small intestine, an increase in villous height is desirable, although this is often not seen post-weaning (Pluske et al., 1997).The reduced villous height and increased crypt depth seen in piglets after weaning have been associated with reduced activity of brush-border enzymes (Jayaraman and Nyachoti, 2017; Pluske et al., 1997). Brush border enzymes are nutritionally important for the break-down of carbohydrates and peptides into monosaccharides and small peptides or amino acids (Sangild et al., 1995). These enzymes are released by epithelial cells, which make up 90 and 95% of the crypt

and villi, respectively and perform digestive actions within the small intestine (Heo et al., 2013). Reduced lactase activity after weaning has been widely reported (Pluske et al., 2003a; Lallès et al., 2004). However, the effect of weaning on other disaccharidases, such as maltase and sucrase is inconsistent, with reports of increases (Kelly et al., 1991) and decreases in their activities (Miller et al., 1986). Nonetheless, in studies where reductions in brush-border enzyme activity is reported, this is likely linked to villous atrophy and the loss of mature enterocytes, which are intestinal villous epithelial cells (Hedemann et al., 2003). Furthermore, reduction in brush-border enzyme activity is also transient, and 3 to 5 days after weaning, their levels gradually increase (Heo et al., 2013). As with GIT morphology, this increase 3 to 5 days after weaning is also likely to be a result of increased substrate availability from increased daily feed intake (Pluske et al., 1997; Heo et al., 2013).

1.3.1.3 Nutrient absorption

The GIT is responsible for maintenance of fluid and electrolyte balance as well as elimination of waste products (Celi et al., 2017). Secretion of fluids and electrolytes from crypt cells is essential for nutrient absorption and digestion (Heo et al., 2013). Active absorption occurs in transporters that specifically transport nutrients such as glucose or amino acids over the intestinal epithelium (Wijtten et al., 2011). This coincides with electrolyte transport over the epithelium, which further coincides with water movement through tight junction proteins (Wijtten et al., 2011). At weaning, there is a reduction in the net absorption of fluids and electrolytes within the small intestine of piglets (Nabuurs et al., 1994). Boudry et al. (2004) weaned pigs at 21 days of age and found transient increases in net ion transport in the jejunum and colon and in glucose absorption within the jejunum when piglets were fasted for two days after weaning, although these returned to pre-weaning values at five days after weaning. However, these results may not be replicated in pigs weaned at 28 days old. In conjunction, ileal glucose absorption decreased after weaning in the same study, with differences likely due to the proximal region depending more on luminal nutrient supply than the distal region. As with morphology and digestion, the short-term impact on active absorption in the proximal small intestine after weaning was likely to be as a result of low feed intake. In addition, a decrease in digestive efficiency and reduced absorption of macro and micronutrients can accompany an increase of defensive

responses as a result of a proliferation in pathogens within the GIT, see Section 1.3.3 (Celi et al., 2017). Malabsorption, which is most noticeable for micronutrients, can be attributed to a loss of integrity and function of the GIT barrier, increasing passage rate of digesta and reducing time available for nutrients to be absorbed and inducing an immune response (Celi et al., 2017).

1.3.2 Gastrointestinal barrier function and mucosal immunity

When considering overall gut health of pigs, GIT barrier functionality and the mucosal immune system are important factors because one of the main functions of the immune system is to identify and eliminate pathogens (Burkey et al., 2009). As a result of this, it is imperative to also consider the interaction between the immune system, GIT barrier functionality and the GIT microbiome (Celi et al., 2017). These interactions will be detailed below, but specific effects of the microbiome on overall gut health will be discussed in further detail in Section 1.3.3. The immune system of vertebrates is subdivided into the innate immune system and the adaptive immune system. The innate system is composed of anatomical, physiological, phagocytic and inflammatory barriers, it is these barriers that provide the first line of defence against infectious diseases (Burkey et al., 2009). The adaptive components of the immune system are characterised by their response to specific antigens and holds immunologic memory (Burkey et al., 2009). The GIT barrier is made up of multiple layers: a microbial barrier, a chemical barrier, physical barrier and an inner immunological barrier consisting of digestive secretions, and cell products such as cytokines and inflammatory mediators (Figure 1.1) (Gao et al., 2020; Celi et al., 2017). There is a mucus layer that covers enterocytes and prevents acidic damage and damage to proteases within the stomach and duodenum as well as providing resistance to pathogen colonisation by adhesion of commensal bacterial to the luminal surface (Kim et al., 2012). Overall, barrier function is dependent upon the interactions between the microbiota within the lumen, the epithelial and immune cells (Gao et al., 2020). The epithelium is where breakdown and uptake of nutrients via brush-border enzymes occurs as described in Section 1.3.1.3. This epithelial barrier also provides a separation barrier for the gut contents and the host (Celi et al., 2017), including separation for pathogens and toxins, thereby acting as the first line of defence (Moeser et al., 2017).

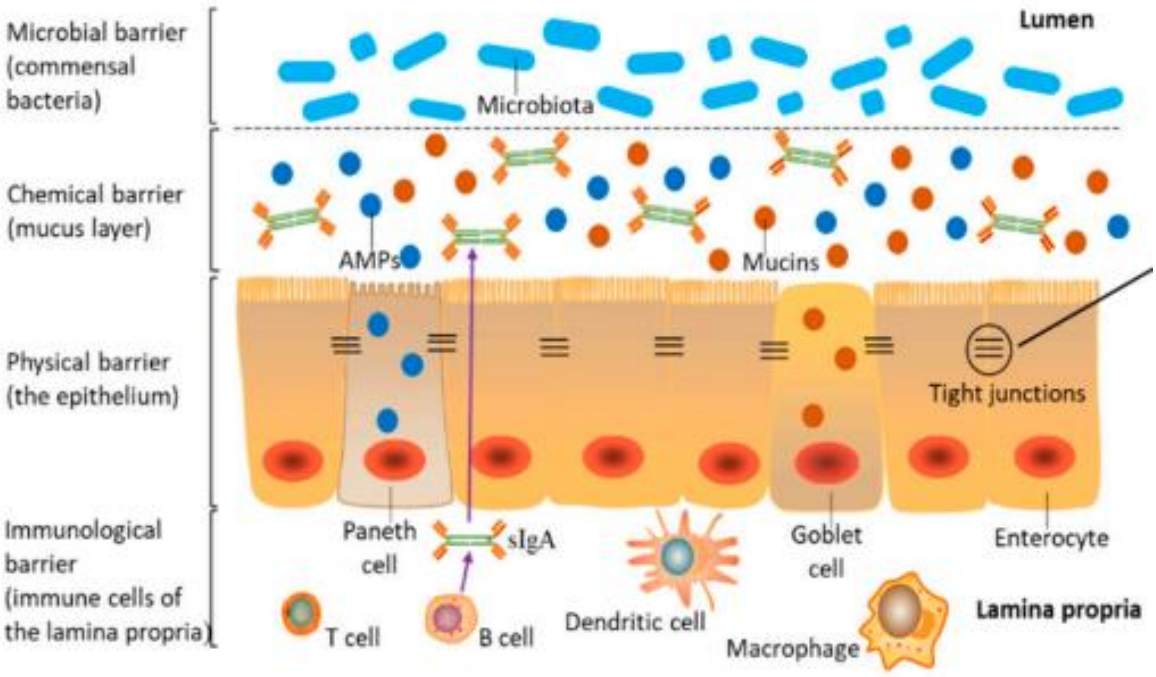

Figure 1.1 Intestinal structure of healthy pigs. Including four levels of protection from external stimuli: physical barrier, chemical barrier with mucins and antimicrobial peptides, immunological barrier with secreted immune mediators such as cytokines and secretory immunoglobulin A (sIgA) and a microbial barrier. Epithelial cells are connected by tight junctions. Source: Gao et al. (2020).

The gut mucosal immune system has an abundance of cells such as dendritic cells, lymphocytes, macrophages and mast cells, that regulate the communication between the GIT microbiome and the mucosal immune system, to recognise bacterial antigens and maintain a healthy GIT microbiome (Figure 1.1) (Celi et al., 2017). Intestinal epithelial cells act as immune sentinel cells and recognise pathogenic signal molecules and secreting interleukins (IL; e.g. IL-17A, IL-23) and growth factors that have important immunomodulatory properties (Moeser et al., 2017). There are a number of immunological biomarkers that can be used to assess gut health and this functionality, including secreting IL and growth factors (Bischoff, 2011). Given the continual exposure to luminal products, the GIT immune system is regulated by several molecular mechanisms to prevent excessive activation and inflammation (Pluske et al., 2018b). Excessive stimulation of the immune system as a result of large numbers of non-pathogenic organisms, can have negative effects on pig performance and is therefore crucial

to regulate (Pluske et al., 2018a; Murphy et al., 2012). Both epithelial barrier function and mucosal immunity can be compromised in the period immediately post-weaning.

1.3.2.1 The effect of weaning on gastrointestinal tract barrier function

There are four key factors associated with weaning that have been identified as having a major effect on GIT barrier function: weaning age, weaning stress, feed intake and diet composition (Wijtten et al., 2011). These factors can affect barrier function differently from the proximal to the distal small intestine, with the proximal and mid-small intestine being affected more than the distal end (Figure 1.2). Immediately after weaning, the epithelial barrier function in pigs is frequently compromised, leading to increased permeability which is associated with inflammation (Moeser et al., 2017). Paracellular pathways are made up of a network of proteins called tight junctions, which connect the epithelial cells (Figure 1.1). Tight junction proteins are selectively permeable for ions, small molecules and water (Pacha, 2000). Any reduction in tightness favours the translocation of pro-inflammatory compounds and microbes from the gut lumen (Farré et al., 2020). In turn, this can result in an immunological response, liberating metabolites and increasing the likelihood of an inflammatory response and infections (Pluske et al., 2018b; Berg, 1995). The combination of reactions of the host to infection, inflammation or trauma are collectively termed the acute phase response (Jain et al., 2011; Kim et al., 2012). The time in which pigs are weaned in commercial settings coincides with a decline of passive immunity from the sows milk, which adds additional challenges to the pigs immune system (Moeser et al., 2017). The breakdown in intestinal barrier function at weaning has been identified through a reduced transepithelial electrical resistance and an increase in permeability to paracellular probes, in experiments using Ussing chambers, which can measure properties of the epithelial membrane (Hu et al., 2013a; Moeser et al., 2007). The loss of paracellular barrier function is typically exclusively seen within the first week post-weaning and returns to pre-weaning levels two weeks post-weaning.

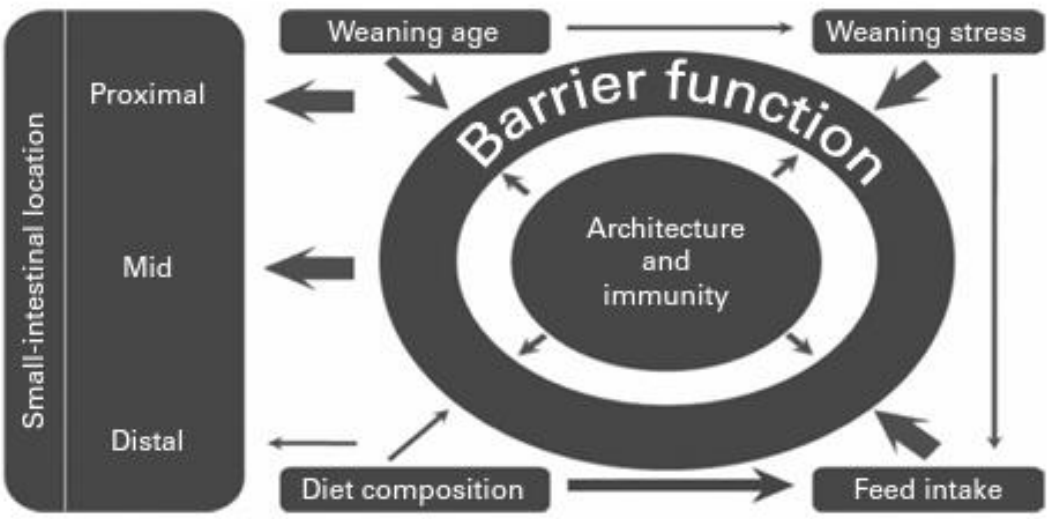

Figure 1.2 Relationship between the small intestinal barrier function, small intestine location and factors (weaning age, stress, diet composition and feed intake) that affect barrier function. The thickness of arrows indicate significance of relationship. Source: Wijtten et al. (2011).

Pigs are less susceptible to compromises in GIT barrier function when weaned at an older age, which is likely as a result of increased intestinal maturity (Wijtten et al., 2011). McLamb et al. (2013) found increased incidence of clinical disease and increased permeability when pigs were challenges with F18 enterotoxigenic *Escherichia coli* (ETEC), a known pathogen in pigs, and weaned at 15/16 days old compared to 22 days old. This would therefore be expected to improve again when pigs are weaned at 28 days old, as is legally required within the UK (see Section 1.2). The improvements seen are likely as a result of the small intestine being more mature when weaned later, enabling a more stable number of lymphocytes to produce antibodies in response to invading bacteria (Moeser et al., 2017). McLamb et al. (2013) also identified changes in the mucosal innate immune response, with suppressed IL-6, IL-8 and neutrophil responses in earlier weaned piglets facing an ETEC challenge.

1.3.2.2 The effect of weaning on the mucosal immune response

The GIT mucosal immune system faces continuous challenge from the GIT microbiota and is therefore abundant in cells capable of recognising bacterial antigens, such as dendritic cells, lymphocytes, macrophages and mast cells (Celi et al., 2017). Each of these cells play a critical role in communication between the GIT microbiome and the mucosal immune system. Intestinal epithelial cells signal the onset of innate and acquired immune responses to the host through

the production of cytokines and chemokines. Cytokines are peptide molecules that act as mediators in the regulation of the immune and inflammatory responses, they are typically derived from lymphocytes and macrophages (Pié et al., 2004). As a result of the increased permeability of the GIT barrier upon weaning, the up-regulation of pro-inflammatory cytokines within the intestine can cause intestinal inflammation (Hu et al., 2014). Some cytokines are primarily expressed by intestinal epithelial cells and have a role in epithelial cell growth, homeostasis and basal influx of immune cells into the mucosa, including IL-1α and IL-6 (Pié et al., 2004). Other cytokines, such as IL-1β and tumour necrosis factor-alpha (TNF-α), can be expressed by normal epithelial cells and are significantly upregulated in response to microbial infection (Pié et al., 2004).

The activation of the immune system has positive consequences in the destruction of invading pathogens. However, activation resulting in the release of IL-1β, IL-6, TNF-α and Interferons (IFN) can increase energy expenditure, induce fever and cause anorexia, further negatively impacting pig performance after weaning (Pluske et al., 2018a). Local expression of these ILs has been largely documented after bacterial or viral infection in pigs and humans during GIT diseases such as Crohn's disease (Lallès et al., 2004). The expression of pro-inflammatory cytokines can be used to determine post-weaning effects on the immune response. For example, Pié et al. (2004) utilised 28 day old piglets and measured mRNA expression of TNF-α over eight days after weaning and identified increased TNF-α one day after weaning in the proximal and mid intestine, followed by increases in the distal small intestine and proximal colon from day two until day eight. This indicated that weaning was associated with early up-regulation of pro-inflammatory cytokines that may contribute to subsequent reduced performance and onset of diarrhoea.

The use of biomarkers to determine the level of GIT inflammation, in readily available samples such as blood and faeces, has been previously documented in humans. Calprotectin is an antibacterial protein that is released at sites of inflammation by neutrophil granulocytes and activated macrophages (Brandtzaeg et al., 1995). Calprotectin has been shown to be highly sensitive and specific in the diagnosis of inflammatory bowel disease (Alibrahim et al., 2015) and could therefore be considered a successful marker for identifying inflammation in humans, without requiring invasive surgery. Non-invasive markers of

inflammation in farm animals are limited, Lallès and Fagerhol (2005) identified faecal calprotectin concentrations in adult sows were similar to that observed in adult humans, but inflammation was not present during their analysis. An acute phase response (APR) includes the host's response to inflammation and infection, and can be directly measured by the livers production of plasma proteins, known as acute phase proteins (APP) (Jain et al., 2011). Acute phase protein concentration in blood samples have been used as a quantitative biomarker for diagnosis, prognosis and monitoring of disease and inflammation in veterinary science (Eckersall and Bell, 2010) and may be an interesting way to identify overall herd health in pigs, without the use of invasive techniques in the future. Determining immune responses, through invasive or non-invasive markers could help identify overall health status on a farm and the subsequent impact of weaning and it would be beneficial to determine whether non-invasive makers are accurate in their results, mimicking results also obtained from more invasive samples directly from the GIT. Although this would be useful, it still would not determine exact causes of poor health and reduced immunity, such as changes in the GIT microbial communities and the presence of pathogens.

1.3.3 Gastrointestinal tract microbiome

The mammalian GIT is made up of an estimated 500-1000 different bacterial species that interact with one another, other microbes and their host species (Kim and Isaacson, 2015). Although bacteria (~10^{14}) are the most abundant microbes that make up the microbiome, archaea, yeasts, fungi and protozoa also make up the collective genomes of the microbiome (Leser and Mølbak, 2009). Bacteria are essential to assist with nutrient digestion, vitamin synthesis, pathogen displacement and immune system maturation (Looft et al., 2014a). To determine differences in bacterial diversity within and between environmental samples, the use of bacterial 16S rRNA next generation (or "high throughput") DNA sequencing is now commonly used (Takahashi et al., 2014; Kumar et al., 2019; Wang et al., 2019; De Rodas et al., 2018). Next generation sequencing (NGS) was developed in 2005, as a progression of first generation, or "Sanger sequencing", which was a dominant approach, considered the gold standard in DNA sequencing, since its development in the 1970s (Sanger et al., 1977). Sanger sequencing was the technology used to complete the 'Human Genome Project' in the early 2000s but was technically limited in the amount and extent of

DNA sequence that could be generated within a single run (Kumar et al., 2019) and was costly, preventing its widespread use (Besser et al., 2018). In comparison, NGS allows for both large and small regions of the genome to be sequenced and specific approaches have subsequently been developed (Kumar et al., 2019). One such approach is the targeted sequencing approach, whereby specific genes of interest can be sequenced (Kumar et al., 2019).

1.3.3.1 16S Sequencing to determine biodiversity

The 16S rRNA gene is present in all prokaryotes and is comprised of approximately 1542 base pairs, with nine hypervariable regions (V1 – V9), that correlate with bacterial species (McCabe et al., 1999; Barb et al., 2016). These regions can be specifically targeted using 16S sequencing. Across research using 16S sequencing, multiple hypervariable regions have been reported used with no single region, or combination of regions being dominant. Sequences generated using 16S sequencing can be clustered into similar sequences, known collectively as operational taxonomic units (OTUs) (Kim et al., 2011). Similarities are computed as the percentage of sites that match in a pairwise sequence alignment; the percentage is typically 97%, as derived from a previous empirical study (Nguyen et al., 2016; Konstantinidis and Tiedje, 2005). These OTUs can then be used to determine bacterial diversity within and between environments, or ecosystems. Biological diversity, or biodiversity is widely defined as “the variability among living organisms from all sources and the ecological complexes of which they are part; this includes diversity within species, between species and of an ecosystem” (Mace et al., 2012). One measure of diversity, the number of species within a given area, is called within-area diversity, or more commonly, alpha diversity (Council, 1999). An additional diversity measure commonly used to assess microbial diversity is beta diversity, which looks at differences in composition between-area diversity (Council, 1999; Koleff et al., 2003).

Alpha diversity is typically measured in terms of species richness (i.e. the number of species) as well as evenness, which compares the uniformity of the population size of each species present (Kim et al., 2017a). Typically, a combination of diversity measures are looked at to determine alpha diversity, including Shannon-Weaver, Simpson and Chao1 diversity indices (Kim et al., 2017a). The Shannon-Weaver diversity index, or Shannon diversity, is an estimator of species (or in

microbial data, OTU) richness and evenness, providing more weight to species richness using the following equation:

$$H = \sum \frac{n}{N} x \ \ln \frac{n}{N}$$

Whereby, $\frac{n}{N}$ is the proportion of the total number of individual OTUs that belong to the ith OTU (Swingland, 2001). Alongside Shannon diversity, Simpsons diversity index is commonly used in bacterial diversity measurement, Simpsons index (D) indicates species/OTU dominance and reflects the probability of two individuals that belong to the same species being randomly chosen, the index varies from 0 to 1 and increases as diversity increases (Simpson, 1949). The equation used to calculate Simpsons diversity index is:

$$D = \frac{1}{\sum_{i=1}^{s} \left(\frac{n}{N}\right)^2}$$

Whereby, s is the total number of species/OTUs within the community, and $\frac{n}{N}$ is the proportion of community represented by OTU i (Kim et al., 2017a). Finally, Chao1 index is an abundance-based estimator of richness based on the measurement of OTUs expected in samples, given all the bacterial OTUs identified in the samples (Kim et al., 2017a). This indices considers the rarity of species represented by one individual, or singletons and two individuals, or doubletons (Gatti et al., 2020). Chao1 is calculated using the following equation:

$$S_{Chao1} = S_{obs} + \frac{F_1(F_1 - 1)}{2(F_2 + 1)}$$

Whereby $F_1$and F_2 are the count of singletons and doubletons, respectively and S_{obs} is the number of observed species. In addition to alpha diversity, the difference in composition between-area diversity, or beta diversity, is also commonly measured to determine differences between environments (Council, 1999; Koleff et al., 2003).

1.3.3.2 Succession of the gastrointestinal tract microbiome from birth

In humans, bacterial colonisation of a sterile infants' GIT occurs within hours of birth and the microbiome then goes through a succession of changes as a result of environmental factors, such as diet (Penders et al., 2006; Leser and Mølbak,

2009). The dominant bacterial phyla within the GIT of infants are Bacteroidetes and Firmicutes (Palmer et al., 2007; Penders et al., 2006), which have also been seen in other species such as ruminants (Malmuthuge et al., 2015) and pigs (Kim and Isaacson, 2015; Crespo-Piazuelo et al., 2018).

As with humans, bacterial colonization of the piglet's intestine occurs immediately after birth, by commensal bacteria from the maternal vagina, faeces and skin as well as the immediate external environment (Fouhse et al., 2016; De Rodas et al., 2018; Wang et al., 2019). From here, a continuous succession of microbial populations have been identified through NGS 16S sequencing (Wang et al., 2019). Initial colonization of the pigs' GIT has been reported as being from the family *Enterobacteriaceae*, including *E. coli*, as well as *Clostrideaceae* such as *C. perfringens* (Inoue et al., 2005; Petri et al., 2010). However, Petri et al. (2010), who used 16S rRNA sequencing, found that levels of *Enterobacteriaceae* and *Clostrideaceae* decreased at around day three, when a secondary colonization occurred and resulted in predominantly *Lactobacillaceae* species present within the GIT. This may be as a result of transfer from the sows milk, causing initial colonisation of the piglets GIT (Mach et al., 2015). *Lactobacillaceae* are a family of lactic acid producing bacteria that have prebiotic properties in improving health and disease resistance in the GIT (Heo et al., 2013).

Succession can be influenced by a multitude of factors, for example bacterial distribution throughout the GIT is influenced by the spatial organisation of the intestine, including from the proximal to the distal regions, as well as across the lumen and mucosal layers of the intestines (Looft et al., 2014a; Kim and Isaacson, 2015). Therefore samples obtained from faeces, or the large intestine, cannot be assumed to represent the small intestine as well. Further influencing factors in mammals such as pigs include genotype, sex, age, diet, weaning, rearing environment, and birthing method, some of which will be further discussed below.

1.3.3.3 The effect of birth environment on the pigs gastrointestinal tract microbiome and immune response

The neonatal GIT undergoes a dramatic transition from a sterile state to an extremely densely colonised microbial community and is considered less stable, and more variable than an established microbial community of mature animals or humans (Bian et al., 2016). In humans, birth environment is one of the most

prominent elements that can influence neonatal intestinal microbial development (Chong et al., 2018; Nagpal et al., 2017).

Forty percent of pigs in the UK are reared in outdoor environments, where they are exposed to a wide range of bacteria and nutrients within soil, pasture and straw from birth. These factors are likely to impact on the GIT microbiome and development compared to indoor-reared piglets. Pluske et al. (2007) identified that indoor-reared piglets had a more diverse bacterial population (using Shannon index) in the large intestine at weaning compared to outdoor-reared piglets. This has further been supported by Mulder et al. (2009), who found increased alpha diversity (Chao1) in the small intestine of indoor and isolated piglets (reared away from a sow, in an isolation pen and given antibiotics daily) compared to outdoor-reared piglets. This is surprising given the increased exposure that outdoor-reared pigs have to a wider variety of bacterial species as well as archaea, fungi and viruses in an outdoor environment. Typically, a highly diverse gut microbiome has been considered to be of most benefit as it confers greater flexibility on the community to respond to changes within the GIT ecosystem, which is of particular importance when weaning pigs (Mulder et al., 2009). However, Hillman (2004) emphasised the need for focus on an optimal, or most favourable, microbiome rather than the composition and diversity of genera, particularly as commensal and pathogenic bacteria co-exist in both healthy and diseased states.

It could be assumed that outdoor reared pigs are exposed to a greater variety of microbes in early life, directly from soil, compared to pigs reared in indoor rooms that are disinfected and cleaned between batches, reducing bacterial exposure of indoor reared pigs. This potential increase in exposure to a greater variety of microbes could then be expected to have a greater microbial diversity within their GIT. However, Mulder et al. (2009) and Pluske et al. (2007) did not identify that pigs exposed to an outdoor environment subsequently had a greater microbial diversity at weaning. Instead, Mulder et al. (2009) concluded that the outdoor pigs microbiome was dominated by fewer phyla that had proven health benefits, thus suggesting a more optimal microbiome, as described by Hillman (2004). One example is the marked increase in the phyla Firmicutes, and specifically in *Lactobacilli* within outdoor pigs. *Lactobacilli* are known for their health benefits including the direct limit on pathotypes of *E. coli* and *Salmonella* (Mulder et al.,

2009). This was further supported by the reduced presence of potentially pathogenic bacteria in outdoor-housed pigs, that were present in indoor and isolated pigs, albeit without signs of visible infection (Mulder et al., 2009).

Although differences in bacterial diversity were not seen between indoor and outdoor piglets at weaning, Pluske et al. (2007) did find that diversity was significantly increased one week after weaning in outdoor-reared piglets. This diversity continued to increase with time in outdoor pigs, which was not seen in indoor pigs, suggesting that rearing pigs outdoors caused prolonged shifts in bacterial composition. However, these continued differences shown by Pluske et al. (2007) could be as a result of their outdoor pigs being weaned into deep-litter housing compared to indoor-reared pigs remaining in conventional indoor systems, thus potentially enhancing the prolonged effects rather than results being as a direct, prolonged effect of birth environment. In a more recent study which used 16S rRNA sequencing to determine microbial differences, Vo et al. (2017) found no significant differences in microbial diversity of indoor-reared pigs that were raised with or without exposure specifically to topsoil during the pre-weaning stage. However, in support of Pluske et al. (2007), they found increased diversity measures and acceleration of gut maturation after weaning in pigs that were exposed to topsoil. This included a significant increase in *Prevotella* in piglets exposed to soil, which is known to be dominant in the gut microbiota of mammals and humans that consume a diet rich in plant polysaccharides and fibre, thus would be expected to be higher when exposed to soil. An increase in *Prevotella* could also be expected after weaning as a result of the shift from sow's milk to a more plant-based, formulated feed. The results presented by Vo et al. (2017) indicate the isolated affected of exposure to soil and its bacteria, indicating its importance in the outdoor environment.

Conversely, the change in bacterial diversity of outdoor-reared pigs could perhaps be a result of these pigs typically being heavier. Han et al. (2017) identified that microbial richness estimators within heavier pigs (average body weight between 16.70 - 22.75 kg) was significantly higher than those within lighter pigs (8.09 - 11.89 kg), with heavier pigs having significantly higher levels of Firmicutes. Outdoor-reared pigs are frequently found to be heavier at weaning compared to those reared indoors (Miller et al., 2009; Gentry et al., 2002). This

makes it harder to determine whether the microbial differences are a result of heavier pigs and/or their rearing environment.

The influence of rearing environment on GIT microbiome can also have subsequent effects on the development of the immune system, which can be linked to the modification of the bacterial composition (Christoforidou et al., 2018). In humans, the exposure to a farm environment, considered less hygienic, from birth, has been correlated with the protection against the development of allergies in childhood, indicating an influence of early environmental differences on immune development (Von Mutius and Vercelli, 2010). As a result, the post-natal period can be considered a critical window for development of not just the GIT microbiome but also the immune system. Mulder et al. (2011) used Affymetrix microarray analysis to determine transcriptome differences between groups of pigs born indoors or outdoors that either remained in these environments to weaning, or were transferred to isolator units 24 hours after birth. Results of their study found that indoor, isolator-reared piglets had higher levels of the IFN alpha/beta signalling pathway as well as IFN-induced genes five days after birth, indicating an influence of environment on the level of immune activation in the early stages of life (Mulder et al., 2011). Although it is unclear whether the protective effects of rearing environment are elicited as a result of maternal exposure to the environment during pregnancy, the neonatal period or later rearing conditions (Christoforidou et al., 2018). Nonetheless, earlier development of the GIT microbiome as well as their immune system can aid in the transition from the pre- to post-weaning stage of pig production. Combining 16S sequencing with investigation of the immune response of pigs reared in different settings could provide an interesting insight into the whole effect of rearing environment on pig health, to determine common factors that may prove essential for further investigation into their links with any improvements seen in performance.

1.3.3.4 Weaning and the pig’s gastrointestinal tract microbiome

Unlike in humans, the weaning process of commercial indoor pigs involves an abrupt dietary shift from sow’s milk to a complete-feed diet, with no gradual wean (Salcedo et al., 2016; Frese et al., 2015). In outdoor pigs, although the change from sow’s milk to hard feed is still abrupt, piglets are exposed to a wider variety of substrates that reportedly allow for the GIT to have a more gradual adjustment

to complex nutrients, such as carbohydrates and fibre within the soil (Vo et al., 2017). Nonetheless, weaning remains a critical period that can instigate compositional changes to the bacteria found within the GIT of pigs, regardless of rearing environment.

Weaning has been associated with a decrease in bacteria such as *Clostridia, Bacteroidia* and *Lactobacillus* alongside a loss of bacterial diversity within the GIT (Gresse et al., 2017). *Lactobacillus* plays an important role in disease prevention through competing with pathogenic bacteria for nutrients and epithelial binding sites and produces antimicrobial factors such as lactic acid (Konstantinov et al., 2006; Bäumler and Sperandio, 2016). As a result, the decrease of bacteria such as *Lactobacillus* can lead to an increase in pathogenic bacteria. After weaning, reports indicate an increase in facultative anaerobes such as *Enterobacteriaceae* (Bäumler and Sperandio, 2016). The family of *Enterobacteriaceae* include gram negative bacteria such as *Salmonella* and *E. coli;* although not all *E. coli* are pathogenic, pathotypes such as ETEC and enteropathogenic *E. coli* (EPEC), are associated with the onset of post-weaning diarrhoea (PWD) (Fairbrother et al., 2005). Specifically, ETEC is the most common pathotype associated with PWD, which is characterised by frequent discharge of watery faeces within the first two weeks after weaning (Heo et al., 2013). Of all pig deaths recorded after weaning, PWD has been reported as being responsible for 50% of these deaths worldwide, per year (Gresse et al., 2017). As a result, PWD is a serious threat to the swine industry, causing huge economic losses that need to be balanced against a delayed weaning time, for optimal production gains and number of pigs born per year (Zhou et al., 2016; Klose et al., 2010).

1.3.3.5 Dysbiosis and the effect of post-weaning diarrhoea on the pigs gastrointestinal tract microbiome

The abrupt changes at weaning have been associated with a disruption of the GIT microbiota, known as dysbiosis. Dysbiosis in pigs is typically defined as an imbalance within the gut microbiota; however, specific characteristics are not completely clear and the process leading to dysbiosis in piglets is not well documented. In mammals, dysbiosis is associated with intestinal inflammation and is characterised by a loss of bacterial diversity (Bäumler and Sperandio, 2016). Inflammation within the GIT appears to provide a favourable environment that may confer an advantage for the growth of *Enterobacteriaceae*, including

pathotypes such as ETEC and EPEC (Zeng et al., 2017). The host-response to gut inflammation produces nitric oxide (NO) which is transformed to nitrate in the intestinal lumen (Figure 1.3) (Gresse et al., 2017). A nitrate-rich environment confers growth advantages to *E. coli,* including pathotypes that possess nitrate reductase genes (Bäumler and Sperandio, 2016). These genes are absent in bacteria such as *Clostridia* and *Bacteroidia*, which may be the cause of their decrease in abundance post-weaning leading to an overall reduction in diversity (Gresse et al., 2017).

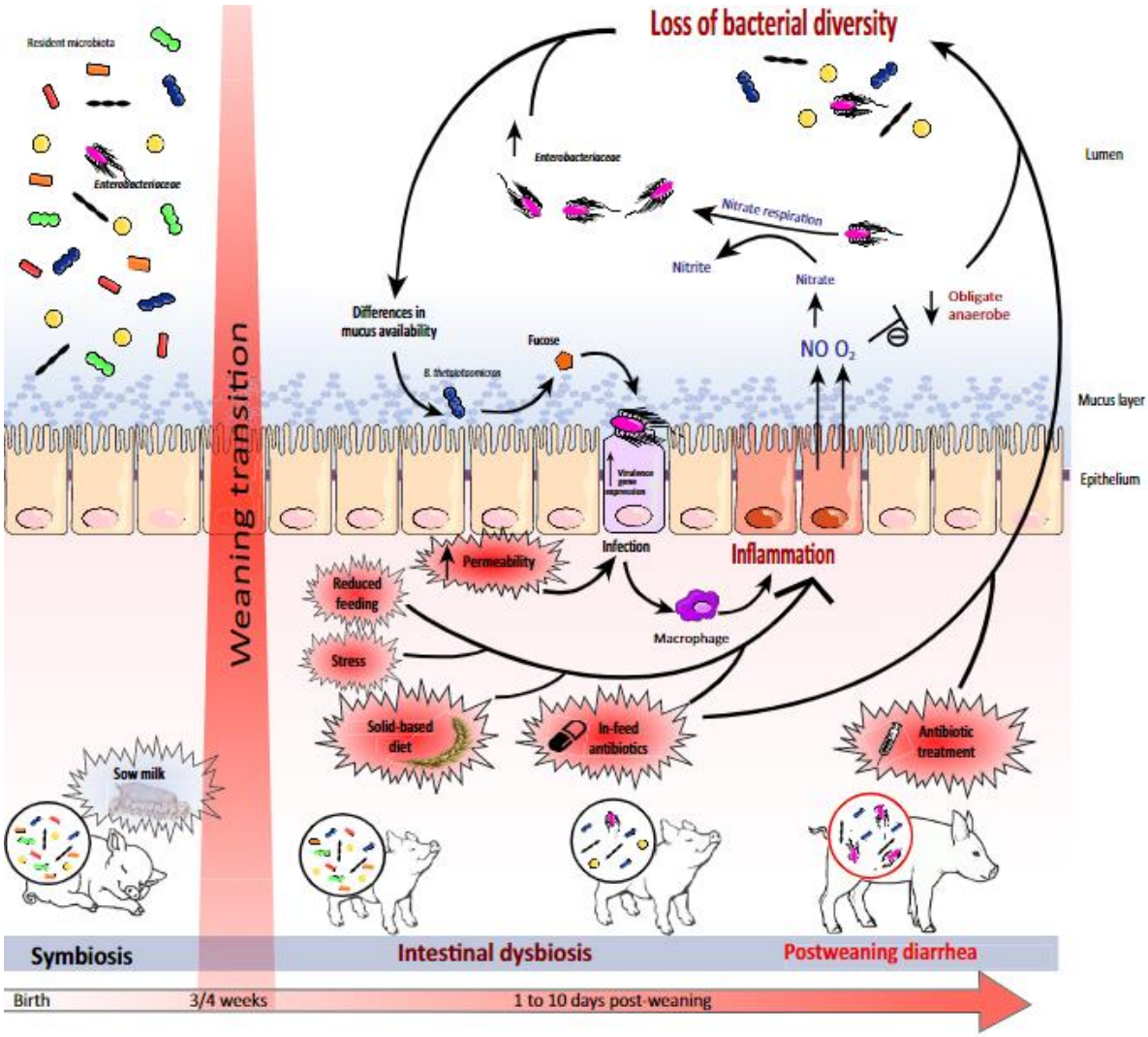

Figure 1.3 Impact of weaning on the pig GIT microbiome and intestinal barrier function leading to infection and PWD. Abrupt changes at weaning such as feed format and environment can result in reduced microbial diversity and an increase in GIT inflammation. This can increase the nitrate in the lumen, favouring growth of Enterobacteriaceae, which includes pathotypes of *Escherichia coli (E. coli)* such as enterotoxigenic *E. coli*. Source: Gresse et al. (2017); Holman et al. (2017).

As previously mentioned (Section 1.3.3.4), although not all *E. coli* are pathogenic, increases in pathotypes such ETEC are associated with the onset of PWD (Fairbrother et al., 2005). The pathogenesis of PWD is not fully understood, although ETEC fimbriae, specifically F4 and $F18^+$ are commonly found on ETEC during PWD in pigs (Fairbrother et al., 2005). It is believed that these fimbriae attach to glycoprotein receptors on the brush borders of villous enterocytes within the small intestine, which enhances colonisation of the GIT by the pathogen and increases permeability of the epithelium (Luppi et al., 2016; Heo et al., 2013). This colonisation results in diarrhoea through the release of enterotoxins; specifically, heat stable toxins, which activates the cyclic guanosine monophosphate system and inhibits the absorption of sodium and chloride ions from the lumen into epithelial cells (Pluske et al., 2002). Alternatively, heat-labile toxins bind irreversibly to mucosal cells and activate the cyclic adenosine monophosphate system, which induces secretion of chloride ions, sodium ions bicarbonate ions and water into the lumen (Pluske et al., 2002). Both toxins ultimately result in excess secretion of water and electrolytes into the lumen of the small intestine, which exceeds the absorptive capacity of the colon, resulting in the onset of PWD, as well as overall dehydration, a reduced feed intake, reduced growth and in many cases, death (Han et al., 2017; Heo et al., 2013). One of the most effective ways to encourage establishment of a healthy bacterial community and reduce the changes observed as a result of PWD is through dietary manipulation.

1.3.3.6 The effect of diet and feed additives on the pig's gastrointestinal tract microbiome

Although there are many factors (some of which have been discussed above) that can influence the microbiome of pigs, Frese et al. (2015) found that diet was responsible for more significant changes in the GIT microbiome, compared to age and genetics. Nutritional intervention have been used to promote feed intake and growth during the critical phase after weaning (Celi et al., 2017). Although there is a plethora of research detailing shifts in the microbiome after dietary changes, the causal link to any growth or health benefits is less definitive. Nonetheless, research detailing microbial changes have been seen after the addition of insoluble dietary fibres (De Lange et al., 2010), the use of pre- and probiotics (Heo et al., 2013), the historic use of antibiotic growth promotors (AGPs) or more

recent widespread use of pharmacological levels of zinc oxide (ZnO) (Pluske, 2013).

Probiotics are defined as a live microorganism, which when administered in adequate amounts, confer a health benefit on the host (Organization, 2002). The beneficial health effects to the host are obtained through manipulation of the GIT microbiota (Heo et al., 2013). The effects of probiotics on pig health are widely documented. Gram-positive lactic acid bacteria such as *Lactobacillus* and *Bifidobacterium* have largely been used as probiotics within animal production industries (Heo et al., 2013; De Lange et al., 2010). The underlying mechanisms of probiotics in protecting against enteric pathogens are largely still unclear, but one proposed mechanism is through direct antagonism, whereby the probiotics kill or inhibit the growth of pathogens to limit the spread of infection (Roussel et al., 2017). For example, Konstantinov et al. (2008) showed that the provision of 10^{10} colony-forming units of *Lactobacillus sobrius* as a probiotic reduced the prevalence of ETEC (after challenge with 10^{10} CFU ml^{-1} ETEC K88) in the ileum from a mean of 1 x 10^6 cells g^{-1} in control fed pigs compared to 4.2 x 10^4 cells g^{-1} in pigs fed a diet containing *Lactobacillus sobrius* ($p < 0.05$), as determined by real-time PCR. These results were believed to be due to the probiotic colonizing the gut mucosa, thus reducing attachment of ETEC in the intestine, rather than by competitive exclusion from a reduced pH in the GIT, which is a second proposed mechanism of probiotics (Roussel et al., 2017). Konstantinov et al. (2008) also found significant increases in daily liveweight gain from 101.3 g in control fed pigs to 176.2 g when supplemented with *Lactobacillus sobrius*, identifying direct benefits to farmers.

Alternatively, prebiotics are fibres defined as selectively fermented dietary ingredients that allow for specific changes in the composition and/or activity of the GIT microbiota, that confer a beneficial physiological effect on the host (Ducatelle et al., 2015). Most prebiotics belong to non-starch oligosaccharides such as Fructooligosaccharides (FOS) and trans-galactooligosaccharides (TOS) (Gresse et al., 2017). These are resistant to gastric acids, hydrolysis by enzymes and GIT absorption, and are reported to selectively stimulate the growth or activity of intestinal bacteria (Heo et al., 2013). Castillo et al. (2008) fed a mannanologosaccharide (MOS), derived from the outer cell wall of a selected strain of yeast, to pigs immediately post-weaning and compared performance and

intestinal microbiota within the jejunum and ileum of pigs 14 days after weaning. Their results showed no effect on growth performance but an improvement in overall gain:feed ratio from 0.63 kg/kg in control pigs to 0.68 kg/kg in pigs supplemented with MOS. Their research also identified a reduction of *Enterobacteria* from 9.13 log 16S rRNA gene copies/g of fresh matter in control pigs to 8.05 log 16S rRNA gene copies/g of fresh matter in MOS fed pigs. As previously discussed, not all *Enterobacteria* are pathogenic, although the significant improvement in faecal scores of pigs receiving MOS compared to control pigs suggest the reduction of pathogenic *Enterobacteria* was possible.

The most common feed additive to overcome the post-weaning growth check as a result of PWD was the use of AGPs, although this practice was banned by the EU in 2006 and has seen significant restrictions in many other countries (Maron et al., 2013). Nonetheless, AGPs have shown significant performance benefits, for example a meta-analysis of over 1000 growth experiments in pigs demonstrated an average of 16.4% improvement in weight of pigs immediately after weaning (Cromwell, 2002). Hence, AGPs were the most cost-effective way to improve the health and feed efficiency of production animals (Dibner and Richards, 2005). The improved performance seen is hypothesised to be as a result of reduced pathogen load and sub-clinical disease as well as reduced competition from microbes, for nutrients (Fouhse et al., 2016). The way in which the GIT microbiota is impacted appears to be specific to the antibiotic, with the commonly used Tylosin shown to reduce *Bacteroidetes* abundance (Looft et al., 2012). However, AGPs not only prevent colonisation of pathogenic bacteria but have also shown to prevent beneficial microbes colonising (Gresse et al., 2017).

Unfortunately, the long term use of AGPs can increase pathogenic colonisation as a result of changes in bacterial diversity which can increase the likelihood of dysbiosis and intestinal inflammation, as described in Section 1.3.3.5 (Gresse et al., 2017). In addition, AGPs can suppress the host immune defences, increasing susceptibility and incidence of disease (Fouhse et al., 2016). Furthermore, long-term use has caused the selection of antibiotic resistant bacteria and the spread of antibiotic resistance genes, between enteric bacteria within the GIT (Barton, 2014). There are now many bacteria that have been shown to be antimicrobial resistant in pigs, including *Enterococci, Salmonella*, and *E. coli.* This is proving problematic as resistance genes can, and are, being transferred via the food

chain to humans, leading to the compromise of treatment for human infections (Barton, 2014). In replacing the use of AGP after their ban in 2006, pharmacological levels of ZnO have been widely used in the pdiet of pigs after weaning.

1.4 The use of zinc oxide to overcome post-weaning diarrhoea and improve performance

Zinc (Zn) is an essential trace element for all species. It is a structural component and a catalytic cofactor for around 3000 proteins and 300 enzymes within the body (Karweina et al., 2015; Yin et al., 2009). In addition, Zn is essential for several enzymatic systems, membrane integrity and the displacement of redox-active metals (Karweina et al., 2015). According to the National Research Council (NRC), typical dietary requirements of Zn in pig diets immediately post-weaning are estimated at 100 mg Zn per kg feed (Konstantinov et al., 2006; Yin et al., 2009; Karweina et al., 2015; NRC, 2012). The physiological requirement of an element such as Zn can be defined as the amount of an element that the animal uses for maintenance of life, considering the amount lost during maintenance of organisms as well as that bound in blood and active tissue during growth (Blaabjerg and Poulsen, 2017). The disadvantage of these defined requirements is that they do not take into consideration when an animal is in a critical physiological period, such as that seen immediately after weaning, which is often characterised by reduced feed intake, compared to animals in a “steady” physiological state (Blaabjerg and Poulsen, 2017). Therefore, this could mean that defined requirements are not obtained by the animal during this time, potentially resulting in a deficiency. A deficiency of Zn in the diet can lead to a hinderance in growth and a depletion of enzyme activity in tissues (Heo et al., 2013). This is something that should be considered if pharmacological levels of ZnO are removed from the diet of pigs after weaning. Nonetheless, in pig production systems across the EU, levels of Zn in the form of ZnO currently range from 1000 – 3000 mg ZnO per kg feed (Konstantinov et al., 2006). This increased level, above NRC requirements, is due to the prophylactic effect that ZnO has, similar to that seen with previous AGPs in terms of reduced PWD and improved growth.

The beneficial effects of ZnO on pig performance after weaning has been widely disputed. A number of studies have reported significant improvements in average

daily gain (ADG), average daily feed intake (ADFI) and feed conversion ratio (FCR) immediately after weaning when using pharmacological levels of ZnO (Poulsen, 1995; Hill et al., 2001; Stensland et al., 2015; Hahn and Baker, 1993) as well as a reduction in PWD (Heo et al., 2013). For example, Stensland et al. (2015) found that the provision of ZnO for three weeks after weaning reduced the percentage of pigs exhibiting diarrhoea from 25% in control pigs to 4% in ZnO-fed pigs and significantly improved ADFI from 571 g/day to 707 g/day and ADG from 431 g/day to 525 g/day. However, other studies have not found significant improvements in performance when including ZnO in the post-weaning diet (Broom et al., 2006; Paschino et al., 2016). The differences obtained in results could be because of the overall health status of pigs, as higher health farms potentially see a reduced benefit of ZnO compared to those with higher disease levels. Nonetheless, the use of ZnO on UK and EU farms is widespread. Although the exact mode of action of ZnO remains unclear, there is evidence that ZnO enhances intestinal function due to improved morphology, restoration of the mucosal barrier integrity (Højberg et al., 2005), as well as shifts in microbial diversity within the GIT (Yu et al., 2017).

1.4.1 Risks associated with pharmacological levels of zinc oxide and the resulting European Union ban

Although ZnO has been widely used at high doses to prevent diarrhoea and improve growth of pigs after weaning for many years, starting from June 2022, pharmacological doses of ZnO will no longer be authorised in the EU (Directorate, 2017). The reasons for this upcoming ban are multifactorial, as depicted in Figure 1.4.

Figure 1.4 Risks related to pharmacological zinc oxide usage in pigs post-weaning, including toxicity, environmental pollution, effect on antibiotic resistance, heavy metal tolerance and modification of microbiota. Source: Bonetti et al. (2021)

1.4.1.1 Bioavailability of zinc oxide and leaching into the environment

Bioavailability of Zn in ZnO is low, with 20% being accessible to the pig; the remaining 80% is excreted via the faeces (Poulsen and Larsen, 1995). The 80% of Zn provided to pigs is not absorbed within the GIT as a result of low pH, which transforms a considerable amount of ZnO into free Zn^{2+} ions. The increased pH found within the GIT means only Zn^{2+} ions can have bactericidal effects, while remaining ZnO is rendered insoluble and is excreted via faeces into the slurry (Sales, 2013). The long-term spread of pig slurry on crops has progressively increased soil and groundwater concentration of Zn. Concentrations are reaching levels sufficient to affect soil organisms directly and/or give rise to concentrations in runoff and draining that give rise to toxic concentrations for aquatic and sediment organisms in receiving water (Monteiro et al., 2019). Regardless of risk mitigations such as manure dilution and distance to surface waters being increased, the European Medicines Agency (EMA, 2017) report concluded that

these precautions would only delay the accumulation of Zn, therefore making the continued high use of ZnO unsustainable within the industry (Milani et al., 2017).

Although the use of pharmacological levels of ZnO are the most frequently used source of Zn in pig diets, other forms are sometimes provided. Another source of Zn in animal husbandry is zinc sulphate ($ZnSO_4$). Zinc from $ZnSO_4$ has a higher bioavailability compared to ZnO, which can result in reduced leaching into the environment, as well as showing an antimicrobial action when given between 1.2 and 2.8 mg/ml *in vitro*, suggesting its potential use as an alternative to ZnO (Surjawidjaja et al., 2004; Bonetti et al., 2021). However, it is reported that feed manufacturers prefer to use oxide salts of trace minerals such as Zn because they are less reactive and contain up to twice the cation concentration as sulphate salts, thus occupying less 'space' within the trace-mineral premixes used (Edwards et al., 1999). Zinc chloride is a further inorganic compound that is used within animal nutrition. One of the most diffused forms of zinc chloride is tetrabasic zinc chloride (TBZC) which is considered more palatable and again, has been reported as being more bioavailable compared to ZnO (Zhang et al., 2020b). This could potentially mean that lower doses could be used in a diet compared to ZnO (Zhang et al., 2020a; Bonetti et al., 2021). The use of pharmacological levels of TBZC (~2000 mg Zn/kg) has also shown similar increases in weight gain and feed intake of pigs after weaning compared to pharmacological levels of ZnO, while also enhancing mRNA and protein expression of tight junction proteins such as occludin and zonula occludens protein-1 (ZO-1) in the ileal mucosa of pigs 14 days post-weaning (Zhang and Guo, 2009). Occludin is a membrane protein of the epithelial tight junction and is important for maintaining barrier function and integrity and ZO-1 is a linker protein in tight junctions (Zhang and Guo, 2009). Although TBZC shows benefits similar to ZnO and could be used as a replacement, it is higher in cost and would only reduce, rather than overcome the problems associated with Zn leaching into the environment.

1.4.1.2 Zinc overload

Although the bioavailability of Zn is relatively low when provided as ZnO, feed-grade sources vary widely in their content and bioavailability thus potentially leading to excessive Zn accumulation and overload in the host (Burrough et al., 2019). Zinc toxicity results in the loss of beneficial effects of ZnO and can lead to

reduced growth rate, anorexia and gastroenteritis (Burrough et al., 2019). Currently, ZnO is only permitted in the diet of pigs for two weeks after weaning in the UK/EU; however, in other countries, ZnO can be fed for longer. Martin et al. (2013) identified beneficial effects of pharmacological levels of ZnO on ADG and ADFI for two weeks after weaning, but thereafter effects were reversed, potentially indicating toxicity. This has been associated with increased concentrations of Zn in organs such as the liver and pancreas, leading to zinc overload (Martin et al., 2013; Komatsu et al., 2020).

1.4.1.3 The contribution of zinc oxide to antibiotic resistance

Antimicrobial growth promoters as feed additives in animal production were banned in the UK in 2006, but have been associated with the emergence of multi-resistant *Enterobacteriaceae* (Casewell et al., 2003). Since their ban, the use of ZnO at pharmacological levels has been widespread. However, the use of ZnO at these high levels have now also been suggested to contribute to the acquisition and spread of antibiotic resistant genes (Yazdankhah et al., 2014). Ciesinski et al. (2018) found that pharmacological levels of ZnO promoted higher proportions of multi-resistant *E. coli* isolates to antibiotics, in faeces and colonic digesta two weeks after weaning. This has also been identified by Bednorz et al. (2013), who found an increased proportion of multi-resistant *E. coli* from 0% in control fed pigs to 18.6% in pigs supplemented with 2500 ppm ZnO, which would likely increase overtime given that resistance is believed to accumulate. Furthermore, Ciesinski et al. (2018) also identified that the use of pharmacological levels of ZnO (2,103 ppm) compared to a control diet (72 ppm) did not significantly change the absolute number of *E. coli* in faeces or luminal digesta and mucosa from the colon; typically ZnO is believed to reduce pathotypes of *E. coli,* thus indicating the potential for resistant strains to be present.

Bacterial populations that are susceptible to antibiotics, become resistant through genetic mutation, or through horizontal transfer and expression of resistance genes from other strains (Huddleston, 2014). There are three major mechanisms that bacteria can transfer genes horizontally: conjugation, natural transformation and transduction (Huddleston, 2014). Conjugation is the main mechanisms of horizontal transfer and involves the transfer of DNA fragments through a multi-step process that requires cell to cell contact, mating pair formation and transfer of plasmid DNA through a conjugative pilus (Von Wintersdorff et al., 2016;

Huddleston, 2014). The high cell density of the mammalian GIT is ideal for the rise and spread of antibiotic resistance genes through bacterial populations. This can lead to increases in the number of bacterial species showing resistance, as well as an increase in what the bacteria are resistant too.

1.4.2 Proposed mechanisms of action for zinc oxide in the newly weaned pig

As previously mentioned, the precise mode of action of ZnO against the onset of PWD and the improved growth performance after weaning are not fully understood. However, there are a multitude of areas that have been researched to identify the effects of ZnO within the pig. One of the proposed modes of actions is through substantial enhancement in nutrient absorption and alteration of intestinal morphology (Pearce et al., 2015). The GIT morphology can significantly alter at weaning (see Section 1.3.1.1), with a reduction in villous height and an increase in crypt depth in the small intestine immediately post-weaning (Pluske et al., 1997; Xu et al., 2000; Miller et al., 1986). The provision of ZnO has been shown to ensure an increased villous height: crypt depth ratio (Zhu et al., 2017; Slade et al., 2011). Zhu et al. (2017) identified that ZnO supplemented pigs had an increased villous height within the duodenum and ileum of weaned pigs as well as a decreased crypt depth within the duodenum, but not ileum, compared to control-fed pigs. This has also been seen when using ZnO nanoparticles, which allow for an increased surface area and higher bioavailability of Zn (Pei et al., 2019). By increasing the villous height: crypt depth ratio, there is better protection against intestinal injury and an improvement in barrier integrity after weaning, leading to a reduction in the incidence of diarrhoea, which is mediated by intestinal epithelial barrier disruptions. There have also been suggestions that the provision of ZnO can reduce fermentation of digestible nutrients in the proximal GIT, rendering more energy available to the host, thus contributing to improved growth performance after weaning (Højberg et al., 2005).

1.4.3 The effect of zinc oxide on gastrointestinal tract barrier function and mucosal immunity

Pharmacological levels of ZnO has been shown to reduce intestinal permeability 14 days after weaning, as shown by oral administration of lactulose and mannitol, which can then be directly measured in urine of pigs after weaning (Zhang and

Guo, 2009). Zhang and Guo (2009) also identified an increase in the expression of mucosal tight junction RNA and proteins using qPCR and western blot analysis, respectively (Zhang and Guo, 2009). As previously described in Section 1.3.3.1, tight junction proteins connect the epithelial cells (Pacha, 2000) and a reduction in tightness favours the translocation of pro-inflammatory compounds and microbes from the gut lumen, leading to increased risk of inflammation and infection (Farré et al., 2020; Pluske et al., 2018b; Berg, 1995). Occludin is an integral membrane protein of the epithelial tight junction and is important in maintaining the integrity and barrier function. Zhang and Guo (2009) identified increased occludin expression with pharmacological ZnO, indicating improved integrity of the tight junction and thus reduced permeability to potential pathogens from the lumen. This was also supported by Zhu et al. (2017) in the jejunum mucosa of pigs receiving ZnO, although these pigs were weaned at 21 days of age, which has previously shown to result in reduced maturation of the intestinal barrier function, therefore may enhance beneficial effects of ZnO. Zinc is also an essential trace element for the development and correct function of the immune system (Bonetti et al., 2021). A disturbed Zn homeostasis has been correlated with an impaired balance of T-helper cells (required for adaptive immune responses) and an increase in apoptosis of immature T-cell precursors, increasing the risk for infection (Honscheid et al., 2009). The provision of ZnO to weaned pigs has shown improved adaptive immunity through the increased number of multiple T-cells and regulatory T-cells, which are pivotal in modulating the immune response and maintaining homeostasis (Kloubert et al., 2018).

As discussed previously, cytokines act as mediators in the regulation of the immune and inflammatory responses (Pié et al., 2004) and are up-regulated as a result of increased permeability of the GIT barrier, causing intestinal inflammation (Hu et al., 2014). *In vitro* infections of intestinal epithelial cells with ETEC F4 showed an upregulation of IL-8 and TNF-α and a downregulation of the anti-inflammatory cytokine, transforming growth factor-β, which was subsequently counteracted by the addition of 0.2 or 1 mmol/L ZnO (Roselli et al., 2003). Interleukin-8 has previously been described as one of the major molecules orchestrating intestinal mucosal inflammation (Pédron et al., 2003). Furthermore, Sargeant et al. (2011) used an intestinal porcine enterocyte (IPEC J2) cell from the small intestine as an *in vitro* model for infection with ETEC and reported

reduced expression of genes involved in the innate immune response, when cells were exposed to ZnO. However, the work carried out by Roselli et al. (2003) and Sargeant et al. (2011) was *in vitro* therefore exact comparison to the effect *in vivo* is limited. *In vivo* studies have since been carried out, with Zhu et al. (2017) also showing downregulation of mRNA expression of the pro-inflammatory cytokine IL-1β and IFN-ɣ as well as an upregulation of the anti-inflammatory transforming growth factor-β in the jejunum mucosa of piglet compare to control pigs. This supports the *in vitro* results shown by Roselli et al. (2003), which suggests that ZnO restores, or maintains intestinal barrier function and development after weaning, helping to reduce inflammation and incidence of PWD from colonisation of pathotypes of *E. coli*.

1.4.4 The effect of zinc oxide on the gastrointestinal tract bacterial composition

Changes in GIT microbial communities in response to ZnO have been widely reported with varying results (Vahjen et al., 2011; Katouli et al., 1999). Alpha diversity, the measure of within sample diversity, has been shown to significantly decrease in the small intestine of pigs fed higher levels of ZnO (Shen et al., 2014). However, Yu et al. (2017) identified that microbial richness (Chao1) and diversity increased with ZnO in the ileum, but decreased in the colon, which was also replicated in the ileum and colon of pigs treated with antibiotics, supporting the theory that ZnO has similar effects to AGPs.

When looking at the bacterial genera that change in abundance with ZnO, Yu et al. (2017) used 16S rRNA sequencing of the V3-V4 region and identified that pharmacological levels of ZnO had similar effects on ileal and colonic microbial richness and diversity than those observed in pigs receiving antibiotics (Chlortetracycline and colistin). They reported increases in both microbial richness and diversity in the ileum, compared to a control group, with richness and diversity decreasing in the colon. Increased diversity in the ileum of pigs receiving ZnO was also reported by Vahjen et al. (2011). Decreases in microbial richness and diversity has previously been associated with the onset of dysbiosis and subsequent PWD in pigs (see Section 1.3.3.5), therefore, the increases in diversity within the ileum in response to ZnO could be linked to the subsequent reduction in PWD frequently seen (Stensland et al., 2015; Heo et al., 2010).

Many previous studies have reported increases in *Enterobacteriaceae* in the ileum with high dietary ZnO (Vahjen et al., 2011; Yu et al., 2017; Højberg et al., 2005). The use of the in-feed antibiotic ASP250 (chlortetracycline, sulfamethazine and penicillin) has also shown increases specifically in *E. coli* populations within the ileum (Looft et al., 2014a). Yu et al. (2017) specifically saw increases in non-pathogenic *E. coli* such as K-12, and Slade et al. (2011) identified that the addition of 3100 mg/kg ZnO to the post-weaning diet reduced ETEC shedding in the faeces of pigs challenged with ETEC. Although an increase in overall *Enterobacteriaceae* are reported in the literature, an increase in diversity within this family may promote competition for pathotypes of *E. coli,* such as ETEC, thus reducing the abundance of pathogenic *E. coli* and subsequent PWD. Further beneficial effects of ZnO on the pig GIT microbiome have shown reduced abundance of *Helicobacter* and pathogenic *Campylobacter jejuni* genera, further replicating the effect of antibiotics on the pig microbiome (Xie et al., 2011; Yu et al., 2017).

1.4.5 Alternative ways to improve performance and health of pigs after weaning

The use of pharmacological levels of ZnO have been widely used across the EU pig industry to improve performance immediately after weaning and reduce the incidence of PWD. With the upcoming EU ban of pharmacological levels by 2022, identifying alternative ways to improve growth and health of pigs after weaning is essential. Dietary alternatives have been extensively researched, including the use of pre- and probiotics as discussed in Section 1.3.3.6 as well as the investigation of alternative forms of feeding ZnO, such as the use of ZnO nanoparticles, which has shown similar improvements in ADG, ADFI, villous height to crypt depth ratio within the small intestine and reduced incidence of diarrhoea compared to control fed pigs, indicating its potential use as an alternative, while still reducing levels of Zn excretion (Pei et al., 2019). Although dietary alternatives are an avenue of further investigation, further research into other aspects of pig production, such as rearing pigs in an outdoor environment, are less understood. With benefits historically seen when pigs are reared outdoors during the pre-weaning stage of production, alongside the unique structure of the UK pig industry, already housing 40% of the breeding herd

outdoors, further investigation and comparison of this management strategy is of benefit.

Previous work carried out at the University of Leeds firstly looked at the effect of rearing environment (indoor or outdoor), weaning age (four or six weeks of age) and post-weaning provision of a control or a ZnO and antibiotic (avilamycin) supplemented diet for one week after weaning (Miller et al., 2009). Results of this research showed the benefit of antimicrobial supplementation on performance data (ADG/ADFI), regardless of rearing environment and weaning age. Outdoor pigs grew faster than indoor-reared pigs for the first two weeks after weaning (295 vs 242 g/pig/day, $p < 0.001$), as did pigs weaned at six weeks versus four (324 vs 213 g/pig/day, $p < 0.001$), although weaning pigs at six weeks of age was detrimental overall on pig growth and ADG from four to eight weeks of age (not just two weeks post-wean). Subsequent research at the University of Leeds identified the effect of rearing pigs indoors or outdoors with the provision of control or just ZnO supplementation after weaning, on the response of pigs to a deliberate infection with ETEC (Slade et al., 2011). These results showed that rearing pigs outdoors and providing ZnO reduced ETEC excretion in faeces, while ZnO increased villous height in the small intestine, as well as lactic acid bacteria to coliform ratio in the lower small intestine and proximal colon, as determined by coliform count on agar plates. Both of these studies showed the beneficial effect of outdoor rearing and subsequent supplementation with ZnO, but with the advances in technology since both studies were conducted, identifying bacterial composition within the GIT in response to environment and treatment could provide a more in depth insight into bacteria that could subsequently be associated with the improved performance frequently seen, alongside further identification of the host immune response to each factor.

1.5 Aims, objectives and hypotheses of this research

The aims of this research were firstly to develop a method for analysis of 16S rRNA sequencing data from pigs housed at the National Pig Centre, University of Leeds; to identify influencing factors on the pig microbiome, including pharmacological levels of ZnO, in the absence of a deliberate pathogenic challenge. Secondly, the aim of this research was to determine lifetime performance benefits of rearing pigs in indoor commercial farrowing pens or outdoor paddocks and arks, and then provided control (~200 ppm) or

pharmacological levels of ZnO (~2500 ppm) for 14 days after weaning. Then, using the method of analysis developed for 16S rRNA sequencing, to identify the effect of rearing environment and dietary ZnO on the GIT microbiome, when no obvious disease challenge was present, alongside markers of inflammation in a variety of sample types.

Specific objectives:

- To identify a successful pipeline for 16S rRNA analysis of GIT samples and to identify the effect of time, GIT location, sample type (mucosa vs lumen), and dietary ZnO on measures of alpha and beta diversity in pigs reared at the National Pig Centre, Leeds
- To determine lifetime performance benefits of rearing pigs indoors or outdoors and whether these pigs respond differently to control (~200 ppm) or pharmacological levels of ZnO (~2500 ppm) for 14 days after weaning
- To determine the effect of rearing environment and dietary provision of ZnO on markers of inflammation in the GIT, blood and faeces
- To determine the effect of rearing environment and dietary provision of ZnO on bacterial alpha and beta diversity as well as overall bacteria composition

Primary Hypothesis

If pigs are reared outdoors prior to weaning, or provided pharmacological levels of ZnO after weaning, then they will show lifetime performance benefits, reducing their time to slaughter. Outdoor-reared pigs or those provided ZnO will have a more diverse bacterial composition within their GIT, increased abundance of beneficial bacteria and reduced inflammation, compared to indoor, control-fed pigs.

Secondary Hypotheses

- The bacterial composition of pigs housed at the National Pig Centre will alter in response to age, GIT location, tissue type and the provision of ZnO, in line with existing research
- If pigs are provided pharmacological levels of ZnO, regardless of their rearing environment, they will show performance benefits including increased feed intake and average daily gain
- Two weeks after weaning, if pigs have been reared outdoors and/or provided pharmacological levels of ZnO they will show similar shifts in bacterial

composition, differing from indoor control-fed pigs, as well as reduced inflammation

Chapter 2
General Methods

The farm trial associated with results presented in Chapter 3, referred to as Trial 1, was conducted at the National Pig Centre, University of Leeds in 2016 and was completed prior to the start of the work reported herein. The author is responsible for analysis of samples collected during this trial, which were used as preliminary data to assess microbial populations of the pigs at the National Pig Centre and to determine a method of analysis for subsequent work. For the purpose of clarity, the setup and sampling methods of Trial 1 are summarised in the following section.

2.1 Overview and ethical statement

All animal experimental protocols were approved by the Animal Welfare and Ethical Review Board of the University of Leeds prior to the trial commencing. All pig housing and husbandry procedures were compliant with the Council Directive 2008/120/EC standards and the Welfare of Farmed Animals, England, Regulation 2007. Additional procedures involving live animals, as detailed below, were carried out under a Home Office Project License (licence No. P615B6AD7), and procedures were undertaken by personnel holding a Home Office Personal Licence. Additional procedures carried out were all in accordance with the Animals (Scientific Procedures) Act 1986, as amended by the EU Directive 2012/3039/EU.

2.2 Animal husbandry

All animal experiments were conducted at the National Pig Centre, University of Leeds. Pigs were sourced directly from the National Pig Centre's breeding herd. Two trials were undertaken. Trial 1 consisted of one batch of pigs that were crossbred: Large White x Landrace sows with MAXGROW^{TM} boars (Hermitage Genetics, UK). Trial 2 consisted of two batches of pigs that were crossbred: Large White x Landrace sows with Danish Duroc boars (Rattlerow, UK).

2.2.1 Sow performance indicators, insemination and selection

Across both trials, sows were weighed, measured for backfat (P2) and artificially inseminated with boar semen (a total of 33 sows in Trial 1 and 50 sows in Trial 2). Sows were weighed by separating trial sows from the larger sow herd using

automatic feeder recognition of their ear tags. Sows were individually weighed using MS EasyScale scales (700 kg; accurate to two decimal points; MS Schipper, Netherlands). Once weighed and recorded, individual sows were marked using non-permanent spray (MS Schippers, Netherlands), and returned to the original sow herd. Sow backfat-depth (measured ~6.5 cm from the midline, over the last rib) was recorded using a back-fat scanner (Renco Lean Meter, Renco Corporation, USA) while each sow was enclosed on the weighing platform.

Sows were artificially inseminated using semen supplied by external companies (Hermitage Genetics and Rattlerow for Trial 1 and 2, respectively). Semen was supplied from multiple boars, with each semen bag consisting of semen from one boar only to provide single sire semen (rather than mixed, which would be normal commercial practice). Insemination was undertaken when sows were identified as being in ‘standing heat’, whereby the sow or gilt stands for service and had visible changes to her vulva (AHDB, 2017). Sows were held in individual serving stalls during insemination, whereby a spiral catheter was inserted into the cervix of the sow, individual semen bags were attached, and semen was deposited into the uterine horns before the sows returned to the herd. Sows were inseminated for up to three consecutive days to provide the optimum chance of conception. The semen used in Trial 1 was from four boars and each sow received the same semen at each insemination. In Trial 2, across both batches, a total of eight boars were used (four boars per batch). Boars were allocated to sows of the same parity, as shown in Table 2.1.

After artificial insemination, sows that were not successfully impregnated were returned to the breeding herd; all remaining sows were hereafter considered to be on trial. During Trial 1, all gestating sows remained within the normal gestating herd at the National Pig Centre, University of Leeds until approximately week 15 of gestation, after which they were moved to indoor farrowing accommodation. Indoor farrowing accommodation consisted of three farrowing rooms - identical in layout - with eight plastic, slatted farrowing pens per room (3 m x 1.5 m). Each pen contained identical farrowing crates for the sows and a heat lamp.

Table 2.1 Number of sows artificially inseminated and their parity, per boar in both batches of Trial 2.

Batch number	Boar number	Number of sows inseminated/ boar	Parity of sows inseminated
1	1	7	1
	2	5	2
	3	8	3
	4	10	4 and 5
2	5	6	1
	6	3	2
	7	6	3
	8	2	4 and 5

For Trial 2, which investigated the effect of rearing environment on the post-weaning response to ZnO, 32 of the 50 inseminated sows were selected and allocated to either an indoor (as described above for Trial 1) or outdoor farrowing environment based on: parity; previous litter size (if applicable, >11); weight; and backfat measure from insemination. Outdoor farrowing environments consisted of pasture paddocks measuring 4 m x 5 m and contained standard metal sow arks (John Harvey Engineering Ltd., UK) with fenders (attached for 14 d post-birth) and a separate sow crate for feeding, with an automatic drinker. In total, 32 sows were used, 16 sows per batch; eight farrowed indoors and eight farrowed outdoors. These numbers were based on a power calculation for the number of piglets required for the post-weaning stage of the trial using the following equation $n=2t^2(C.V)^2/d\%^2$, whereby n = the number of replicates per treatment required; t = students t value for the chosen probability; CV = coefficient of variation; $d\%$ = the size of difference to detect X 100, divided by the general mean value (Morris, 1999). This had to be completed across two batches due to the required sow numbers not being present within a single batch.

Gestating sows remained within the commercial indoor herd until week 11 of gestation. At this point, only sows allocated to an outdoor farrowing environment (eight per batch) were removed from the indoor commercial herd and were housed as a group in an outdoor paddock containing two gestating sow arks and access to water. All other sows remained within the indoor commercial gestating sow herd until week 15 of gestation. At week 15 of gestation, all sows were moved to their individual farrowing locations, in their allocated environments (the same farrowing rooms and outdoor paddocks were used across both batches within Trial 2). All sows, across both environments of Trial 2 were fed the same gestation feed (ABN, UK) and individually moved to the same lactation feed two days post-farrowing (ABN, UK). All sows were fed to appetite twice daily.

2.2.2 Standard farm practice

Unless otherwise stated, pigs used within both trials were treated according to standard farm practice during the trial periods. Pigs were reared at the National Pig Centre, University of Leeds, from birth to slaughter and were classified as 'on trial' for the duration of their lifetime in Trial 2. After farrowing, piglets remained with their sows for four weeks, which was considered the pre-weaning period. During this time piglets were only fed from the sow and had continuous access to additional water from automatic nipple drinkers (indoors) or automatic bowl drinkers (outdoors). Across both trials there was no cross fostering permitted into trial litters; however, piglets were removed from trial litters if there was a litter size in excess of sow teat numbers. Any piglets that were removed from these litters were placed onto designated sows that were within the same batch, but not on trial. If litters were born below the required size, they were taken off trial and were cross fostered.

All piglets had their teeth clipped, tail docked and ear tagged in accordance with DEFRA, Code of Practice for the Welfare of Pigs, 2020. All piglets also received a 200 mg/ml intra-muscular injection of iron (Gleptosil, Ceva Animal Health Ltd., UK) within 24 h of birth. At four days of age, all piglets received an oral dose of Baycox (Bayers, Germany) for the prevention of coccidiosis. At ~21 days of age, all piglets received a combined intra-muscular injection of 2 ml Suvaxyn Circo + *Mycoplasma hyopneumoniae* (MH) for the combined immunisation of pigs against Porcine Circovirus Type 2 (PCV2) and MH (Zoetis, 2020). Creep feed was not provided to any piglets across both rearing environments.

Pigs were weaned at ~28 days of age and moved into a weaner-grower facility (see Section 2.2.2.1). In this facility, pigs were housed in ventilated rooms containing 16 pens of five pigs per pen. Each pen (1.5 m x 1.3 m) contained two water nipples and an enrichment toy in accordance with The Welfare of Farmed Animals, England, Regulations 2007, amendment 2010.3033/EN. In addition, for the first 21 days post-weaning, a multi-space trough feeder was provided, which was then replaced by a single trough feeder from 21 days post-weaning until pigs were moved from the weaner-grower accommodation.

After a total of eight weeks after weaning, pigs were moved into finishing accommodation (1.75 m x 4 m), which included two water nipples, two single space trough feeders and two enrichment toys, in accordance with The Welfare of Farmed Animals, England, Regulation 2007, amendment 2010.3033/EN. Pigs remained here until they were sent to Cranswick Country Foods Abattoir (Hull, UK) for slaughter, at a weight of approximately 105 kg.

2.2.2.1 Post-weaning allocation

Trial 1 consisted of 88 piglets from eight litters; litters were selected based on their litter number exceeding 11 piglets (no cross-fostered piglets). Of the 11 piglets randomly selected per litter to move onto the weaner trial, one piglet was randomly selected for euthanasia, dissection and sample collection at weaning (see Section 2.5), while the remaining ten were allocated, based on weight, into two pens of five pigs per pen. One pen received the control diet after weaning, while the other pen received a matched diet containing pharmacological levels of ZnO (Table 2.2). All eight litters (16 pens) were weaned into the same post-weaning room of the weaner/grower accommodation at the National Pig Centre. In Trial 1, litters were weaned into two adjacent front and back pens to ensure single litters were close within the weaner/grower rooms (Appendix A.1).

In Trial 2, an unknown illness resulting in limb-locking and death was present within some of the outdoor-reared litters in batch 2; therefore, batch 2 only weaned half the number of piglets onto the post-weaning trial. This resulted in a total of 235 piglets weaned for trial purposes, including 128 from indoor-reared litters and 107 from outdoor-reared litters. Litters were selected for Trial 2 based on the number of pigs within the litter (four litters per environment with >11 piglets and four per environment with >10 piglets in batch 1 and only litters of >11 in

batch 2) as well as litters that did not include piglets that had received any form of medication during the pre-weaning stage, and the average weight of piglets. Within the litters of >11 piglets, six of these had blood and rectal swabs taken three days prior to weaning (see Section 2.5.3). When allocated to a post-weaning pen, three sampled pigs per litter were allocated to the control-fed pen while the remaining three were allocated to the pen receiving pharmacological levels of ZnO post-weaning, balanced for weight and sex. Weaning was carried out the same as in Trial 1, with strict hygiene procedures in place (see Section 2.2.4). The ten piglets per litter that went onto the post-weaning trial were selected based on two groups of five pigs balanced for body weight and sex. These two groups of five pigs were then weaned, with one group receiving the control diet and the other given a matched diet supplemented with pharmacological levels of ZnO (~2500 ppm), as in Trial 1. Room setup in Trial 2 involved splitting litters across the room, to house each treatment group together (Appendix A2). This allowed for reduced personal protective attire for hygiene purposes (see Section 0) as entire groups were expected to have similar microbial compositions, given they were from the same rearing environment and on the same treatment.

2.2.3 Feed

In both trials, pigs were provided with feed and water *ad libitum* throughout. For the first 14 days post-weaning, pigs across both trials were fed a standard first stage, post-weaning diet with either the reccomended levels of ZnO (~200 ppm; Control), or pharmacological levels of ZnO (~2500 ppm; Table 2.2 to Table 2.5). By day 15, all pigs across both trials were changed onto a standard second stage diet for a further 15 days. Both first stage diets and the second stage diets were formulated by a leading pig feed manufacturer and the industry sponsors, Primary Diets (Ripon, UK). After formulation, all diets were pelleted through a 3 mm die at 62 (±2)ºC and packaged into 25 kg feed bags in Trial 2. After day 29, all diets were formulated by ABN (Peterborough, UK) to meet or exceed the NRC Nutritional requirements for Swine (NRC, 2012). All feed provided was of a known weight and given as required. Diets were changed at days 34, 48, 57 and 81 post-weaning. Representative samples of all diets given were collected at multiple times throughout the entire trial and stored at -20ºC.

2.2.3.1 Feed analysis

Dietary samples of the first stage diet (control and ZnO diet fed for 14 days post-weaning) and the second stage diet (fed from day 15 to day 28 post-weaning) across both trials were sent to Sciantec Analytical services Ltd (Cawood, UK) for analysis of crude protein, crude fibre and minerals (Table 2.3; Table 2.5).

Table 2.2 Ingredient composition of the two experimental diets provided from weaning to day 14 and the second stage diet, given to all pigs, from days 15 to 29 (%, unless otherwise stated, as-fed basis) during Trial 1.

	Weaning to day 14		Day 15 to 28
Ingredients	Control diet	ZnO diet	2nd stage
Micronised Barley	10.0	10.0	10.0
Wheat whole meal	19.7	20.0	50.0
Wheatfeed	-	-	2.6
Micronized wheat meal	10.0	10.0	-
Micronized oats	10.0	10.0	-
Fishmeal	7.7	7.7	2.5
Soya hypro	16.5	16.5	24.0
Full fat soyabean	3.0	3.0	2.5
Vitamin/Mineral premix[1]	0.5	0.5	0.5
Dried skim milk	4.0	4.0	-
Whey powder	11.1	11.1	3.5
Flavouring	0.015	0.015	0.020
Sweetener	0.010	0.010	0.010
Benzoic acid	0.225	0.225	0.230
Pigzin (zinc oxide)	0.000	0.310	-
DCP	0.650	0.650	1.490
Salt	0.026	0.026	0.360
Soya oil	5.410	5.410	1.240

[1]Vitamin and mineral premix provided per kg of diet in the first stage was: 12,500 IU Vitamin A, 2,000 IU Vitamin D_3, 200 IU Vitamin E, 150 mg Iron, 140 mg Copper, 110 mg zinc, 40 mg Manganese, 1 mg Iodine, 0.25 mg Selenium.
DCP- Dicalcium phosphate

Table 2.3 Tested and calculated nutrient contents for both experimental diets given from weaning to day 14 and of the second stage diet, given to all pigs, from days 15 to 29 in Trial 1 (%, unless otherwise stated, as-fed basis).

	Weaning to day 14		Day 15 to 28
Tested Nutrient Content	Control diet	ZnO diet	2nd stage
Ash	5.9	6.1	5.6
Calcium	0.94	0.86	0.81
Copper (mg/kg)	149	147	142
Crude fibre	2.1	1.9	2.4
Crude protein	22.3	21.9	20.4
Manganese (mg/kg)	68	68	72
Moisture	9.8	9.9	11.4
Phosphorus	0.76	0.71	0.72
Potassium	0.99	1.01	0.97
Salt	0.74	0.76	0.78
Sodium	0.20	0.21	0.23
Total oil	8.96	9.39	4.45
Zinc (mg/kg)	153	2560	154
Dry matter and calculated nutrient content			
Dry matter	90.71	90.67	88.72
Vitamin A (IU/kg)	11500	11500	11500
Vitamin D3 (IU/kg)	2250	2250	2250
Vitamin E (IU/kg)	250	250	200
SID lysine	1.40	1.40	1.23
SID methionine	0.56	0.56	0.45
Total lysine	1.54	1.54	1.36
Total methionine	0.60	0.60	0.48

SID =standardised ileal digestibility

Table 2.4 Ingredient composition of the two experimental diets provided from weaning to day 14 and the second stage diet, given to all pigs, from days 15 to 29 (%, unless otherwise stated, as-fed basis) during Trial 2.

	Weaning to day 14		Day 15 to 28
Ingredients	Control diet	ZnO diet	2nd stage
Barley	15.0	15.0	15.0
Wheat whole meal	15.6	15.6	49.4
Wheatfeed	-	-	1.5
Micronized wheat meal	12.5	12.5	-
Micronized maize	2.5	2.5	-
Micronized oats	5.0	5.0	-
Fishmeal	6.0	6.0	1.5
Soya hypro	18.2	18.2	23.8
Full fat soyabean	2.5	2.5	2.5
Vitamin/Mineral premix[1]	0.5	0.5	0.5
Whey powder	13.9	13.9	-
Potato protein	1.6	1.6	-
Sugar	0.625	0.625	-
Flavouring	0.020	0.020	0.020
Sweetener	0.010	0.010	0.010
Benzoic acid	0.500	0.500	0.500
Pigzin (zinc oxide)	0.000	0.310	-
DCP	1.130	1.130	1.440
Salt	-	-	0.430
Sodium carbonate	0.050	0.050	0.040
Soya oil	3.400	3.400	1.600

[1]Vitamin and mineral premix provided per kg of diet in the first stage was: 12,500 IU Vitamin A, 2,000 IU Vitamin D3, 200 IU Vitamin E, 150 mg Iron, 140 mg Copper, 110 mg zinc, 40 mg Manganese, 1 mg Iodine, 0.25 mg Selenium.

Table 2.5 Tested and calculated nutrient contents for both experimental diets given from weaning to day 14 and of the second stage diet, given to all pigs, from days 15 to 29 in Trial 2 (%, unless otherwise stated, as-fed basis).

	Weaning to day 14		**Day 15 to 28**
Tested Nutrient Content	Control diet	ZnO diet	2nd stage
Ash	6.6	6.0	5.7
Acid insoluble Ash	0.51	0.48	0.34
Calcium	1.15	1.17	0.85
Copper (mg/kg)	148	157	131
Crude fibre	2.8	2.6	3.3
Crude protein	21.2	21.1	20.8
Manganese (mg/kg)	88	96	86
Moisture	9.3	10.1	10.4
Phosphorus	0.83	0.92	0.79
Selenium (mg/kg)	0.37	0.52	0.37
Sodium	0.21	0.23	0.24
Total oil	7.31	6.86	5.35
Zinc (mg/kg)	214	2292	328
Dry matter and calculated nutrient content			
Dry matter	89.96	89.96	88.03
NE piglet (MJ /kg)	10.16	10.16	9.40
Vitamin A (IU/kg)	12500	12500	12500
Vitamin D3 (IU/kg)	2000	2000	2000
Vitamin E (IU/kg)	300	300	200
SID lysine	1.350	1.350	1.250
SID methionine	0.540	0.540	0.500
Total lysine	1.497	1.497	1.376
Total methionine	0.581	0.581	0.533

SID =standardised ileal digestibility

2.2.4 Hygiene protocol

During both trials, a strict hygiene protocol was followed to reduce microbial contamination between treatment groups during the main experimental period (from farrowing until day 30 post-weaning). Before sows entered indoor farrowing rooms during both trials, all rooms were thoroughly cleaned and disinfected using Oxy-Des (MS Schippers, UK) to remove residual debris and microorganisms from the previous batch of pigs.

Prior to weaning, attire specific to the farrowing house was worn by personnel: full-body overalls, flat-soled boots and nitrile gloves and these were kept separate from the rest of the farm. In Trial 2, boot soles were disinfected with Distel High-level Medical surface disinfectant (Tristel, UK) between each pen, and gloves were changed. In addition, as Trial 2 included indoor and outdoor environments pre-weaning, full overalls, wellington boots and gloves were completely changed between both environments.

Weighing of pigs during the pre-weaning stage of Trial 2 (see Section 2.3) was conducted using separate plastic boxes between environments, these were disinfected with Distel (Tristel, UK) between each pen within each environment. Other equipment used during the pre-weaning stage for injections, teeth clipping, tail docking and ear tagging as well as all equipment used for sampling of pigs, was disinfected between pens using Distel (Tristel, UK; see Section 2.4.3).

At weaning, litters were weaned as a group, either within a plastic box (Trial 1) or feed barrow (Trial 2) disinfected with Distel (Tristel, UK) prior to use and between each new litter. The same litter was weaned as a group and immediately placed in the appropriate pen within the weaner/grower accommodation according to the allocation of each trial (Appendix A1; Appendix A2). Once weaned, additional protective attire (disposable boot covers, aprons, sleeve protectors and gloves) were worn across both trials and changed between every pen in Trial 1 or every treatment block, unless dirtied, in which case they were immediately changed in Trial 2. Weighing of pigs after weaning included the use of different, suitably sized boxes per pen in Trial 1 and a singular weigh crate in Trial 2 that was disinfected with Distel (Tristel, UK) between each treatment block, or if dirtied. During any other activity after weaning, including daily weighing of feed troughs (see Section

2.3.2) and health checking pigs (see Section 2.2.5), protective attire was completely changed between pens (Trial 1) or treatment blocks (Trial 2).

During blood and rectal sample collection in Trial 2, full protective attire was worn by all personnel involved, and changed between treatment blocks or when dirtied. Equipment required for sampling was disinfected between pens with Distel (Tristel, UK) and needles used for blood sampling were changed between every pig. The strict hygiene protocol ceased in both trials at day 30 post-weaning and practices hereafter were carried out using standard farm practices/biosecurity.

2.2.5 Daily health checks

Throughout Trial 2, pig health, faecal scores and administered medication were recorded daily by the same individual. Each pen of pigs was counted to ensure pigs had not escaped/jumped pens, had their health assessed on a scale of 0-5 or 0-10 (depending on whether there was a maximum number of five or ten pigs per pen, respectively). Ill-health was based on the number of pigs within a pen showing signs of lameness, obvious injury, coughing or symptoms of infection (0= no pigs showing signs of ill-health; 1= 1 pig showing some signs of ill-health; 2= 2 pigs showing signs of ill-health; 3= 3 pigs showing signs of ill-health etc. continuing to a maximum of ten where all pigs within pens of ten were showing signs of ill-health). Faecal scores were recorded on a scale of 0-4 (0= no faeces in pen; 1= Firm faeces; 2= Soft faeces; 3= very soft faeces; 4= watery faeces). Cleanliness of the pigs/pen was also graded on a scale of 1-4 (1= all pigs clean; 2= < 3 pigs in pen were dirty; 3= all pigs in pen were dirty; 4= all pigs were heavily dirty). In accordance with standard farm practice, any pigs that displayed clear signs of ill-health were given medication according to symptom; dosage, date and type of medication were recorded. If pigs continued showing signs of ill-health and needed further treatment, they were taken off trial and removed from the pen; pig weight and date were recorded. If an entire pen of pigs was ill, the entire pen was taken off trial and the pigs were given a standard farm diet. Water availability and room temperature were checked daily throughout the entire trial.

2.3 Performance data collection

In Trial 2, pigs were weighed at given time points before and after weaning to calculate average daily gain (ADG). All feed given after weaning was weighed and recorded to calculate average daily feed intake (ADFI). Both ADG and ADFI

were used to calculate feed conversion efficiency (FCE). During the pre-weaning stage and for ~21 days after weaning, pigs were weighed using the EasyScale Pro Pig Bluetooth scales (measuring to two decimal places; MS Schippers, UK) with a plastic box (pre-weaning) or a metal weigh crate (post-weaning) to secure the pig on the scales. Pigs were weighed in accordance with the hygiene protocol (see Section 0); pigs were manually lifted in accordance with AHDB guidelines (AHDB, 2011) and placed on the EasyScale Pro Pig Bluetooth scales (MS Schippers, UK), once stabilised the weight was recorded and the pig returned to the same pen. After ~ day 21 post weaning, pigs were moved using pig boards into an Easyscale Pro Select Bluetooth standing scales, measuring to two decimal places (MS Schippers, UK).

2.3.1 Pig weight

Pig weights were only recorded for Trial 2. During the pre-weaning stage, weights were recorded within 24 hours of birth, at ~ 7 days old, ~ 21 days old and at weaning (pigs averaged 25.15 days old). After weaning, all pigs were weighed at days 7, 15, 22, 29, 43, 57, 71, 85, 99 and prior to selection for slaughter at days 116, 124 and 131. Selection for slaughter was based on a pig's weight being >105 kg.

2.3.2 Feed intake data

Feed intake was only recorded for Trial 2 and this was recorded daily from the day of weaning until day 21 to calculate an exact daily feed intake per pen during the key part of the trial. During this time, the EasyScale Pro Pig Bluetooth scales (MS Schippers, UK) were used to weigh feed troughs (feed troughs had a known weight without food per pen). After day 22, feed was weighed on the same day as pigs were weighed (see Section above), this was completed by vacuuming feed out of troughs, weighing the feed on weighing platform scales (MS Schippers, UK) and then returning feed to the same troughs.

2.4 Performance data analysis

Pre-weaning data were analysed using a univariate general linear model (GLM) in IBM SPSS Statistics (v.26) using litter as the experimental unit. Environment was used as the fixed factor in all pre-weaning analysis with sow parity, batch and litter size included as a random factor within the model. Random factors were removed from the model if no significant effect was observed. Pre-weaning

variables that were analysed include: weight at birth, days 7, 21 and weaning, ADG from birth to weaning and the number of pigs born alive, stillborn, laid on at birth and those that died between birth and weaning. Average litter weight at birth was based on the average weight before any cross-fostering occurred, whilst average litter weight at days 7, 21 and at weaning was based on post cross-fostering averages. Levene's test (Levene, 1960), which tests the null hypothesis that the population variances were homogenous, was used to check for homogeneity of variance, and residuals were tested for normality using the Shapiro-Wilk test (Shapiro and Wilk, 1965). Where data were not normally distributed, a non-parametric Kruskal Wallis test was used to determine significant differences or a Chi-squared test was used for count data.

Post-weaning data were analysed based on pen as the experimental unit, with weight at each time point, ADG, ADFI and FCE analysed. Data were split into three key time periods post-weaning: weaner stage from weaning to 29 days post-weaning, the grower stage from day 29 to day 57 and the finisher stage from days 57 to 116. These periods were set due to number of pigs within a pen and changes in location within the pig farm. These periods correspond to when diets changed or when pigs were moved into different buildings to accommodate their growth. Residuals were first tested for normality using the Shapiro-Wilk test and homogeneity of variance was tested using Levene's test (Levene, 1960). Where normality and homogeneity were met, data were analysed using a linear mixed model in IBM SPSS Statistics (v.26). The model included environment and treatment as fixed factors with litter of origin and sow parity as a random factor for weaning until day 29 and then location (room/pen) as a random factor for the grower and finisher stage. Weaning weight was included as a covariate for analysis until day 29 and only included in the model where significant. Likewise, interaction between environment and treatment was tested, and also only included in the model where significant. Where data did not show normal distribution or displayed heteroscedasticity, a generalised linear model was used, which allows for a response variable to have a distribution other than normal. In these cases, environment and treatment were still included as fixed factors, with weaning weight as a covariate until day 29. Interactions were also investigated and only included where significant. Responses to treatment or rearing environment were considered significant when $p \leq 0.05$, and trends were noted

when $p \leq 0.10$. The number of pigs sent to slaughter at each recorded time point (days 116, 123 and 130 post-weaning) were analysed using a chi-squared test to determine the effect of rearing environment and provision of ZnO.

2.5 Dissections and sample collection

Both trials included dissections, which were carried out under the Animals (Scientific Procedures) Act 1986. In Trial 1, dissections were carried out at weaning (day 0) and days 14 and 28 after weaning. At weaning, one piglet from each litter was randomly selected for euthanasia, dissection and sample collection (total of eight pigs). At days 14 and 28 one pig per pen (two per litter) were randomly selected for euthanasia, dissection and sample collection, totalling 16 pigs at each time point. Pigs were euthanized by captive bolt penetration to the forehead followed by exsanguination and dissections were carried out immediately after euthanasia and all on the same day.

Trial 2 included dissections 14 days after weaning, where one pig per pen (two per pre-wean litter) within the sampling room were randomly selected for euthanasia. Pigs were weighed prior to captive bolt penetration to the forehead and exsanguination, followed by dissection and sample collection, within one day.

2.5.1 Blood and faecal sampling

In Trial 2, after euthanasia, peripheral blood samples were collected into EDTA vacutainers (Scientific Laboratory Supplies, UK) and immediately stored on ice. Samples were centrifuged for 15 min at 1000 *x g* at 4ºC within 30 min of collection. Supernatant was collected and aliquoted into two 2-ml Eppendorf tubes and stored at -20ºC.

Faecal samples were collected from all dissected pigs in Trial 2. Specifically, 0.1 g of faeces was suspended in 1 ml of Phosphate Buffered Saline (PBS) within a 2 ml centrifuge tube (Greiner Bio-one Ltd, UK) and centrifuged for 20 min at 1000 *x g*. The supernatant was transferred to a clean 2-ml centrifuge tube and stored at –20ºC. Remaining faecal samples were aliquoted into two 2-ml cryovials (Greiner Bio-one Ltd, UK) and stored at -80ºC.

2.5.2 Sampling of the gastrointestinal tract

After a ventral incision was made in the pig, the gastrointestinal tract (GIT) was identified, clamped at both the distal and dorsal ends and removed from the pig,

the GIT was then laid out on a dissection table. In Trial 1, samples were collected from the terminal ileum (identified as 90 cm from the caecum inset), caecum and the proximal colon (40 cm from the caecum). From these sections, luminal digesta was collected and stored at -20ºC. Mucosal scrapings were collected from each section by washing with PBS and using a sterile spoon to scrape the top layer of the mucosa into a 2-ml cryovial (Greiner Bio-one Ltd, UK) before snap freezing on dry ice and storing at -20ºC.

In Trial 2, samples were collected from the mid-jejunum (considered 50% of the small intestine), terminal ileum (65 cm prior to the ileocaecal valve, with the most terminal 25 cm discarded) and the proximal colon (30 cm after the caecum, discarding the first 10 cm closest to the caecum). Mucosal scrapings, luminal digesta and tissue biopsies were collected from all four GIT locations (as described above), additional mucosal scrapings (0.1 g) were taken into 1 ml of Trizol™ (Invitrogen, USA) from the jejunum, ileum and colon. These were all snap frozen in liquid nitrogen and stored at -80ºC.

2.5.3 Blood sampling, rectal swabs and temperature recording of live pigs

In Trial 2, in addition to dissections, 48 pigs per batch were selected for additional blood sampling, rectal swabs and rectal temperature recording at further time points throughout the pre- and post-weaning stages. Within each batch, four litters per environment were selected based on pig numbers >11 (see Section 2.2.2.1). Pigs used for sampling within these litters were selected based on piglet weight and sex. A minimum of 2 ml of blood was taken from the external jugular vein (or the ear vein if the jugular was not possible) into EDTA vacutainers (Scientific Laboratory Supplies, UK) and stored on ice until processing. Processing involved centrifuging for 15 min at 1000 *x g* at 4ºC within 30 min of collection. Supernatant was collected and aliquoted into two 2-ml centrifuge tube (Eppendorf; Greiner Bio-one Ltd, UK) and stored at -20ºC.

Rectal swabs were taken using sterile cotton swabs (Copan, Ireland) that were placed ~ 3 cm into the rectum of the piglet and rotated 360 º before being frozen at -20ºC. If faeces was naturally excreted during the sampling process, it was collected and stored in sterile 30-ml universal containers (Elkay Laboratory Products Ltd, UK) at -20ºC. Rectal temperature was recorded using an Accuvet digital Thermometer (Vet World, UK), accurate to 0.1ºC, which was placed 1 cm

into the rectum until a bleep was sounded by the thermometer and the temperature was recorded. If the temperature appeared excessive to the operator (+ 40.5ºC), the probe was re-inserted one additional time to validate the reading. These six pigs were weaned and balanced across two pens of the 'Sampling room' (see Section 2.2.2.1), the same pigs were repeat sampled during the post-weaning stage, using the same methodology explained in this section. If any pigs appeared ill, had received medication or had any signs of prolapse, they were not used for sampling. The number of pigs used was calculated to allow for pigs to be taken off trial and still obtain sufficient repeat samples, based on a power calculation (see Section 2.2.1).

2.6 Sample analysis

2.6.1 DNA extraction and 16S rRNA sequencing

In Trial 1, samples from four litters (four pigs at weaning and eight at days 14 and 28) were selected for use in subsequent 16S rRNA sequencing. This equated to 120 samples. Of these, 108 had > 0.2 g sample required for DNA extraction. Microbial genomic DNA was extracted from 108 samples in total from the ileum, caecum and colon using 0.2 g of material and the QIAamp stool mini kit (Qiagen, Germany) with some modifications. Briefly, 0.2 g of 0.1 mm zirconia/silica beads (Thistle Scientific, Scotland) were added to facilitate sample lysis by bead-beating for 5 min at maximum speed (50 rps; Tissue Lyser, Qiagen, Germany) and then incubated at an increased lysis temperature of 95°C. Extracted DNA samples were quantified using a Nanodrop ND-1000 (Thermo Scientific, USA) prior to amplification. Once genomic DNA was extracted, the quantity and quality was assessed using a NanoDrop-ND1000 Spectrophotometer (Thermo Scientific, USA). Samples with low DNA concentration (≤0.4 ng/µl) were excluded from further analysis at this point, leaving a total of 94 samples.

Samples were sent to the University of Leeds Next Generation Sequencing Facility (St James Hospital, UK) for amplification and sequencing on an Illumina MiSeq platform. Extracted DNA were diluted to 6 ng/µl in 10 mM Tris pH 8.5 and 2.5 µl was used for 25 µl PCR reaction mix. Five-microlitres of each universal bacterial primer for the V4 hypervariable region of the 16S rRNA gene were used at 1 µM (F 5'- AYTGGGYDTAAAGNG-3'; R 5'- TACNVGGGTATCTAATCC-3'). DNA quality was checked using an Agilent TapeStation D1000 ScreenTape

(Agilent Technologies, USA). Products of PCR were purified using 20 µl AMPure XP beads and 400 µl 80% (v/v) ethanol per sample. Index PCR was run using 5 µl of both Nextera XT Index Primer 1 (N7xx) and Primer 2 (S5xx) and 25 µl Illumina NEBNext® Q5 HiFi PCR Master Mix. All amplification conditions were 3 min at 95°C followed by 25 cycles of 30 s at 95°C, 30 s at 55°C, 30 s at 72°C and a final extension step of 5 min at 72°C. Subsequent DNA products were pooled and sequenced on the Illumina MiSeq platform (V2; 250 bp pair-end reads) at the University of Leeds Next Generation Sequencing Facility (St James Hospital, UK). Quality filtering and bioinformatics analysis was complete as described in Section 2.5.2.

In Trial 2, samples from the lumen and mucosa of the jejunum, ileum and colon as well as faeces were collected as described in section 2.4.2 and stored at -80ºC for subsequent 16S rRNA sequencing (n = 224). At the point of analysis, samples from three pigs (one in batch 1 and two in batch 2) were excluded due to incomplete or insufficient set of samples collected, therefore 203 samples (seven samples from 29 pigs) were used for DNA extraction. The PureLink™ Microbiome DNA Purification Kit (Thermo Fisher Scientific, USA) protocol was followed for DNA extraction. The 203 samples were thawed on ice and briefly vortexed before 0.2 ± 0.05 g was weighed into the provided bead tubes with ~600 µl Lysis Buffer (volume adjusted for each sample to make total mixture 800 µl) and vortexed. Lysis Enhancer was added (100 µl), vortexed, incubated for 10 mins at 65 ºC and then homogenized by bead beating for 10 mins at maximum speed (50 rps; Tissue Lyser, Qiagen). Samples were then centrifuged at 14,000 *x g* for 5 min. 400 µl of the supernatant was transferred to a clean microcentrifuge tube and 350 µl Cleanup Buffer was added and vortexed immediately followed by centrifuging at 14, 000 *x g* for 2 min. The supernatant (500 µl) was transferred to a clean microcentrifuge tube and 900 µl Binding Buffer was added and vortexed. From this, 700 µl of the sample mixture was then loaded into a spin-column tube and centrifuged at 14,000 *x g* for 1 min, the flow-through was discarded and a further 700 µl sample mixture loaded and centrifuged under the same conditions. The spin column was placed into a clean collection tube and 500 µl Wash buffer added and centrifuged under the same conditions for 1 min. The flow-through was discarded and centrifuged again. Finally, the spin column was placed in a 1.5 ml DNase/RNase free tube and 100 µl Elution buffer added and incubated at room

temperature for 1 min before a final centrifuge at 14,000 *x g* for 1 min. The column was discarded and purified DNA within the 1.5 ml tube was stored at -80 ºC until subsequent analysis. Once genomic DNA was extracted and quality/quantity checked using a NanoDrop-ND1000 Spectrophotometer (Thermo Scientific, USA), 190 samples were sent to Novogene (China) for amplification and 16S rRNA sequencing on a NovaSeq PE250 platform.

2.6.2 Quality-filtering and bioinformatics analysis of sequencing data

2.6.2.1 Mothur

In Trial 1 and 2, sequencing reads were processed, quality filtered and aligned using Mothur v.1.40.3 (Schloss et al., 2009) following the MiSeq standard operating procedure (SOP) (Kozich et al., 2013). The SOP was accessed in June 2018 and again in October 2020. Briefly, forward and reverse reads were combined to form contigs and those with ambiguous bases were removed. Unique sequences were identified and aligned against the SILVA database (v.132). Chimeras (hybrid products between multiple parent sequences that can be falsely interpreted as novel organisms (Haas et al., 2011)) were removed alongside any sequences that were identified from the 16S rRNA of archaea, chloroplasts, mitochondria or unknown sequences. Sequences were then clustered into operational taxonomic units (OTUs) with 97% similarity between a pair of sequences as described previously by others (Nguyen et al., 2016) and a BIOM file was generated to transfer the OTU table and associated taxonomy into a format suitable for analysis of alpha and beta diversity in R studio (v. 3.4.3). In Trial 2, the same method was used but due to the quantity of data, processing was complete on the High Performance Computers at the University of Leeds.

2.6.2.2 R studio

The following packages were installed and used for microbiome analysis in R studio (v. 3.4.3): Phloseq v1.30.0 (McMurdie and Holmes, 2013), ggplot2 v3.3.3 (Wickham, 2009), Vegan v2.5-7 (Oksanen et al., 2015), Lme4 v1.1-26 (Bates et al., 2011), LmerTest v3.1-3 (Kuznetsova et al., 2017), DeSeq2 v1.26.0 (Love et al., 2014) and dplyr v1.0.5 (Wickham et al., 2015). Alpha diversity is a measure of species richness and evenness within a community. This was measured using the Shannon-Wiener index (Shannon, 1948), Simpson's diversity index (Simpson, 1949) and the Chao1 index (Chao, 1984). A general linear model

(lme4) was used on unrarefied data to identify the interactive effects of GIT location, dietary treatment and time (Trial 1) or GIT location, sample type (mucosal/luminal), rearing environment and dietary treatment (Trial 2). Models were reduced using Analysis of Deviance (lmerTest) where interactive effects were not identified.

Beta diversity was plotted using a non-metric multidimensional scaling (NMDS) using the Bray Curtis distance, with the axis set at 2. A PERMANOVA (adonis) was used to identify significant differences ($p < 0.05$) between GIT location, dietary treatment and time point (Trial 1) or GIT location, sample type, rearing environment and dietary treatment (Trial 2) and any interactions between these factors. Where significant differences were seen, DeSeq2 was used on un-rarefied data to identify log2fold changes in shrunken OTU counts that significantly differed between factors (Love et al., 2014). P-values were adjusted for multiple comparisons using the Benjamin Hochberg correction. Where significant effects were seen in sample type and/or GIT location, significant effects of additional factors were investigated within each GIT sample type/location. In Trial 2, for DeSeq2 analysis, data were split into four groups to allow for pairwise comparisons between all groups, and these groups were: indoor-reared, control-fed pigs (indoor control); indoor-reared, ZnO-fed pigs (indoor ZnO); outdoor-reared, control-fed pigs (outdoor control); outdoor-reared, ZnO-fed pigs (outdoor ZnO).

2.6.2.3 SPSS

In Trial 2, relative abundance of phyla and the top 20 genera present in at least one sample above 0.1% were generated in R studio and statistically analysed in IMB SPSS Statistics (v26). Data were split into groups as follows: indoor-reared and control-fed pigs (indoor control); indoor-reared and ZnO-fed pigs (indoor ZnO); outdoor-reared and control-fed pigs (outdoor control) and; outdoor-reared and ZnO-fed pigs (outdoor ZnO). Differences in relative abundance between these four groups were analysed within the lumen and mucosa of the jejunum, ileum, colon and in faeces. Relative abundance data were tested for normality using the Shapiro-Wilk test and tested for homogeneity of variance using the Levene's test. Data displaying heteroscedasticity or non-normal data were analysed using the non-parametric Kruskal-Wallis test, while data that met

normality and homogeneity were analysed using a one-way ANOVA with Tukey's (HSD) *post-hoc* test. Comparisons were made between all four groups.

2.6.3 qPCR Sampling and RNA Isolation

During dissection at day 14 in Trial 2, mucosal scrapings from the ileum and colon were taken and stored in Trizol™ at -80 ºC, as described in Section 2.5.2, until qPCR analysis. Subsequent qPCR analysis was carried out on all ileal samples collected at day 14 (n = 32) and colonic samples from pigs reared indoors only (n = 16) due to cost limitations.

Selected samples for qPCR analysis were left to thaw on ice before 25 mg of each tissue sample (ileum or colon) were removed and placed into a nuclease free 2-ml Safe-Lock microtube with 0.8 ml of Trizol™ and 0.7 ml of 2 mm lysis beads. Samples were then homogenized by bead beating for 60 seconds at maximum speed (50 rps; Tissue Lyser, Qiagen, Germany). Particulates were removed from homogenised tissue by centrifugation at 12,000 *x g* for 1 min and 600 µl of the supernatant was transferred into a new nuclease free 2-ml Safe-Lock microtube (Fisher Scientific Ltd, UK).

Total RNA was isolated from the transferred supernatant of the homogenate using the Direct-zol™ RNA Miniprep with Zymo-Spin™ ICC Columns (Cambridge Bioscience, UK), following the 'Tough-to-lyse' tissue samples protocol. 600 µl of 99% Ethanol was added to the supernatant and vortexed for 5 sec before loading 700 ul onto a Zymo-Spin™ IIC Column. The column was centrifuged for 60 sec, reloaded and centrifuged for a further 60 sec. All centrifugation steps were complete at 12,000 *x g* using a benchtop micro-centrifuge. The protocol used incorporated an in-column DNase I (6U/µl) digestion step to minimise genomic DNA contamination. The remaining isolation followed the manufacturers protocol and RNA was eluted in 50 µl DNase/RNase free water.

The quality and quantity of isolated RNA was assessed using a NanoDrop-ND1000 Spectrophotometer (Thermo Scientific, USA). The integrity of RNA was also confirmed using gel electrophoresis on a 1% agarose gel in a 1 X TAE running buffer using 5 µl GelRed™ Nucleic Acid Gel Stain (Cambridge Bioscience, UK). Gels were loaded with 5 µl total volume which consisted of 2.5 µl sample and 2.5 µl Gel Loading Dye (New England BioLabs, USA) and run for 25 mins at 90 volts. A DNA ladder (1 Kb Plus, Thermo Scientific, USA) was loaded

onto the gel to serve as a positive control. After electrophoresis, a visual inspection of the RNA was conducted using a UV transilluminator to identify the ribosomal 28S and 18S subunits. Eight samples showed faded bands and had low concentration, these were placed on a heat block at 65 ºC for 30 min to evaporate off some DNase/RNase free water. All remaining RNA was frozen at -80ºC until subsequent analysis.

2.6.4 cDNA Synthesis

Previously isolated total RNA was converted into complimentary DNA (cDNA) using the First Strand cDNA Synthesis Kit (Thermo Scientific, USA) according to the manufacturer's instructions. Isolated total RNA was thawed on ice and 2 µg was added to 1 µl of random hexamer primer, 4 µl Reaction Buffer, 1µl RiboLock RNase Inhibitor, 2 µl 10mM dNTP mix, 2 µl M-MuL V Reverse Transcriptase and DNase/RNase free water to provide an end volume of 20 µl. The reaction mixture was mixed gently and incubated for 5 min at 25ºC followed by 60 min at 37ºC and terminated by heating at 70ºC for 5 min. For each sample, a minus reverse transcriptase (-RT) control was also run, which included DNase/RNase free water instead of reverse transcriptase enzyme, no template controls and positive controls were also run.

Resulting cDNA was diluted 10 X and stored at -80 ºC in working aliquots. cDNA synthesis product and the corresponding -RT for each sample was assessed on a 1% agarose gel following standard end point PCR to amplify a 496 bp section of the housekeeper gene, Glyceralehyde 3-phosphate dehydrogenase (GAPDH). GAPDH primers were provided within the cDNA synthesis kit and were: forward, 5' – CAAGGTCATCCATGACAACTTTG -3'; reverse 5'-GTCCACCACCCTGTTGCTGTAG -3'. PCR amplification was performed in a total of 25 µl; 12.5 µl of GoTaq® Green Master Mix (Promega, USA), 1 µl Forward and Reverse GAPDH primer, 1 µl cDNA synthesis product and 9.5 µl DNase/RNase free water. Polymerase chain reaction was then performed in a thermal cycler using the following conditions: an initial denaturation step at 94ºC for 3 min followed by 35 cycles of denaturation at 94ºC for 30 sec, annealing at 58ºC for 30 sec and an extension at 72ºC for 45 sec before being held at 4ºC. Amplification products were confirmed using gel electrophoresis on a 1% agarose gel in a 1 X TAE running buffer using 5 µl GelRed™ Nucleic Acid Gel Stain (Cambridge Bioscience, UK) at 50 volts for 40 mins.

2.6.5 Quantitative PCR

Primers for all genes of interest (IFN-ɣ, IL-6, IL-17) and housekeeper genes (GAPDH and beta-actin) were purchased from Bio-Rad Laboratories (USA) as predesigned PrimePCR™ Assays, with the addition of specific designed primers for IL-17A, using Primer3Plus based on a melting temperature of 60 ºC +/-1 ºC with a GC content between 40-60% (Table 2.6). Primer efficiency was calculated for all primers based on a series of dilutions of pooled cDNA. A serial dilution of 1:10, in DNase/RNase-free water was initially trialled in triplicate but provided higher than desired quantification cycle (Cq) values (+35) for genes of interest. Subsequently, a 1:6 dilution series, run in triplicate was carried out and provided primer efficiencies between 90 - 110 % and a linear correlation coefficient (R^2) value > 0.99 for all primers. To prepare the serial dilutions, 4 µl of pooled cDNA was added to 16 µl of master mix, which consisted of 10 µl 2x SsoAdvanced™ Universal SYBR® Green Supermix, 1 µl of PrimePCR Assay or pre-designed IL17A at 300 nM and 5 µl DNase/RNase free water per replicate. A total of 20 µl per well was briefly centrifuged and run on a CFX-96™ Real Time PCR Detection system (Bio-Rad Laboratories, USA) using the following conditions: activation at 95ºC for 2 min and then 40 cycles of denaturation at 95ºC for 5 sec and annealing/extension at 60ºC for 30 sec. At the end of each PCR run a melt curve analysis (65 to 95ºC, 0.5ºC incremental increases every 5 sec) was performed to assess primer specificity in Bio-Rad CFX Manager, v.3.1.

After primer efficiency was determined, the relative expression of selected genes of interest were measured in relation to two housekeeper genes by qPCR on a CFX-96™ Real Time PCR Detection system (Bio-Rad Laboratories, USA) using the same conditions as described above, without pooling cDNA. Amplification was carried out using white 96-well unskirted qPCR plates (Bio-Rad Laboratories, USA), sealed with an optically clear adhesive to prevent evaporation.

Table 2.6 Selected genes of interest and house keeper genes for inflammatory gene expression analysis.

	Function	Unique Assay ID	Amplicon Length
Reference Gene			
Beta Actin (ACTB)	Actin synthesis	qSscCED0016579	110
Glyceralehyde 3-phosphate dehydrogenase (GAPDH)	Catalyses glycolysis	qSscCED0017494	188
Genes of Interest			
Interleukin-6 (IL-6)	Pro-inflammatory cytokine	qSscCED0014488	65
Interferon gamma (receptor 1) (IFN-G)	Activates macrophages	qSscCED0018912	93
Interleukin-17A (IL-17A)	Inflammatory mediator	qSccCID0012676*	108

*Three designed primers for IL-17A include: (1) forward 5'- AGCTCCAGCTCATCCATCTG -3', reverse 5'- GTCCTCAGTTTTTGGGCATC -3'; (2) forward 5'- AGCTCCAGCTCATCCATCTG -3', reverse 5'- CTCAGTTTTTGGGCATCCTG -3; (3) forward 5'- TGACTCCTGTGAGATCCTCGT -3', reverse 5'- AGTCCATGGTGAGGTGAAGC -3'.

2.6.6 qPCR data analysis

All qPCR data analysis was performed using the qbase+ software, v.3.2 (Biogazelle, Zwijnaarde, Belgium). Stability of the housekeeper genes was determined using the geNorm expression stability value of the reference gene (M) and the coefficient of variation (CV) of the normalised reference gene relative quantities, which are automatically calculated within qbase+ (Vandesompele et al., 2002). The thresholds for both M and CV were automatically set to 0.5 and

0.2 respectively and were met. The genomic mean of the combination of housekeeper genes was used as the normalisation factor for subsequent genes of interest. The Cq values generated by qPCR were converted into normalised relative quantities according to the formulas described by (Hellemans et al., 2007). Data were then log-10 transformed within qBase+, to ensure symmetrical data distribution and exported for subsequent analysis in SPSS (v. 26). Data were then back-transformed for presentation in relevant tables.

Ileal and colonic samples were analysed separately in SPSS (v.26). Firstly, data were tested for normality using the Shapiro-Wilk test and homogeneity of variance using the Levene's test. Ileal and colonic samples were then separately analysed using a univariate general linear model. For ileal samples, treatment and environment were set as fixed factors while colonic samples only included treatment, as samples were only analysed from indoor-reared pigs. Batch was also included as a random factor within the model. Interactions between batch x treatment (ileum and colon) as well as batch x environment (ileum) and treatment x environment (ileum) were also investigated and removed from the model if no significant interactions were observed. Significant differences were determined by a p-value ≤ 0.05.

2.6.7 Enzyme-linked immunosorbent assays

Blood and faecal samples used for ELISAs were collected as described in Section 2.5.1 and 2.5.3. Samples were used from the pigs that were dissected at day 14. Blood samples were also analysed from three days prior to weaning and seven days after weaning. Faecal samples were also analysed from days 4, 7, 13 and 28 after weaning. Discrepancies in samples analysed at three days prior to weaning versus four days post weaning for blood and faecal samples, respectively, were due to insufficient faecal sample collection at three days prior to weaning during the first batch of pigs and restraints of the Home Office License preventing blood collection at day four post-weaning. The exclusion of additional blood samples at days 13 and 28 after weaning were based on results of faecal analysis and cost restrictions.

2.6.7.1 Faecal concentrations of calprotectin

Faecal concentrations of calprotectin were measured in samples collected at days 4, 7, 13, 14 and 28 after weaning using the MBS033848 Porcine

Calprotectin ELISA kit from MyBiosource (USA). The kit was stored at 4ºC until use and the manufacturer's instructions were followed for faecal samples during sample collection and subsequent analysis. Wash solution was diluted 1:20 µl with distilled water, whilst standards, HRP-Conjugate reaction, stop solution and Chromogen solutions A and B were ready-to-use solutions. All reagents were brought to room temperature prior to use. Faecal samples were thawed completely, brought to room temperature and vortexed on the day of analysis, prior to use. Neither samples nor standards required dilution.

Each sample, standard and blank (no sample/standard added) were run in duplicate. 50 µl of standards (S_1,S_2, S_3, S_4, S_5, S_6) and samples were added to designated standard and sample wells within a 96 well plate. Then, 100 µl of HRP-Conjugate Reagent was added to all wells, except blanks. The plate was covered with a closure plate membrane and incubated for 60 min at 37ºC. All wells were washed four times by aspiration with 1X wash solution and excess liquid was eliminated by tapping against absorbent paper. Once washed, 50 µl of Chromogen solution A was added to every well before 50 µl of Chromogen solution B was added to every well, which was protected from light. This created a dark blue colour solution, the intensity of which was directly proportional to the amount of calprotectin present in the faecal samples. Following this, 50 µl of stop solution was added to every well to stop the reaction and caused a colour change from a dark blue solution to a yellow solution.

The absorbance was measured at 450 nm using a SPECTAmax™ 340 (Molecular Devices, USA) after 5 min of adding the stop solution. The concentrations of calprotectin were calculated using the average absorbance values of samples against the standards (minus the blank from each absorbance) using a linear calibration curve in Excel. Inter- and intra-assay CVs were <15%. Results were analysed in SPSS v.26 as described in Section 4.2.4.1.

2.6.7.2 Plasma concentrations of pig major acute phase protein

Plasma concentration of pig-major acute phase protein (pig-MAP) was measured from samples collected three days prior to weaning and at days 7 and 14 after weaning using the PigMAP ELISA kit from Acuvet Biotec (Spain), following the manufacturer's instructions. Sample diluent buffer and wash buffer were both diluted 1:10 µl with distilled water, as instructed. The conjugate was diluted 1:20

µl with the provided conjugate dilution buffer and all stock was used on the day of preparation. Chromogen substrate and stop solution came as a ready to use solutions. All components of the kit were brought to room temperature prior to use. The pig-MAP standard used was supplied at 3.2 mg/ml concentration and diluted 1:500 µl with sample diluent buffer, before serial dilution of 1:2, 1:4, 1:8 µl for use as the calibration curve, according to the manufacturer's instructions.

Plasma samples were thawed to room temperature on the day of analysis and briefly vortexed prior to analysis. Samples and standards were diluted 1:500 µl, using sample diluent buffer on the day of analysis. After analysis, 25 samples were too concentrated and gave an optical density (OD) above the standards, these were reanalysed at a 1:1000 µl dilution. Each sample, standard and blanks (nothing added to wells) were run in duplicate. 100 µl of diluted sample and standards were each added to two wells of a 96 well plate. The plate was incubated at 22ºC for 30 min and then washed four times by aspiration with 1X wash buffer and excess liquid was eliminated by tapping against absorbent paper. After washing, 100 µl of 1X conjugate was added to each well and incubated for a further 30 min at 22ºC. The plate was washed a further four times and liquid eliminated through tapping against absorbent paper. Next, 100 µl of chromogen substrate was added to each well and incubated for a further 30 min at 22ºC, which changed the solution from a clear solution to a pink colour; the intensity of which was directly proportional to the amount of pig-MAP present in the plasma samples. After incubation, 100 µl of stop solution was added to each well to stop the reaction, changing the colour of the solution from pink to yellow.

The absorbance was measured at 450 nm using a SPECTAmax™ 340 (Molecular Devices, USA) within 15 mins of the stop solution being added. The concentrations of pig-MAP were calculated using the average absorbance value of the samples against the standards (minus the blank from each absorbance) using a linear calibration curve in Excel. Inter- and intra-assay CVs were < 10%. Results were analysed in SPSS v. 26 as described in Section 4.2.4.

2.6.7.3 Statistical analysis of pig-major acute phase protein and calprotectin concentrations

Although both calprotectin and pig-MAP were analysed in samples from the same pig through time, some missing data points resulted in analysis using a linear

mixed model in SPSS (v.26). The linear mixed model used compound symmetry as the repeated covariance type as variances for all data were homogenous. Both models included rearing environment and dietary treatment as main, fixed effects, with batch as a random effect within the model. Interactions between rearing environment and dietary treatment were included within the model if a significant interaction was observed. If no interaction was observed, main effects were reported without the interaction in the model.

To identify the differences in concentration of calprotectin and pig-MAP at each time point, a univariate general linear model was used, with rearing environment and dietary treatment as fixed effects, and batch as a random effect within each model. Interactions between rearing environment and dietary treatment were again included within the model if significant, but were removed from the model if no significance was reported.

Chapter 3

Determining factors that can influence the gastrointestinal tract microbiome of pigs, including pharmacological levels of zinc oxide, in the absence of a deliberate pathogenic challenge

3.1 Introduction

Next generation sequencing of the 16S rRNA gene, to identify similarities and differences in the bacterial composition of the gastrointestinal tract (GIT) in response to a variety of factors, is now widely used across many species, including humans and pigs (Takahashi et al., 2014; Kumar et al., 2019; Wang et al., 2019; De Rodas et al., 2018; Kim and Isaacson, 2015; Crespo-Piazuelo et al., 2018). The use of 16S rRNA sequencing enables bacterial species richness (i.e. the number of species present) as well as evenness (comparison of the uniformity of a population size of each species present), to be identified through analysis of alpha diversity measures (see Section 1.3.3.1) (Kim et al., 2017b). Furthermore, beta diversity, or between-area diversity, enables identification of differences in species composition between environments or ecosystems (Council, 1999; Koleff et al., 2003). Although 16S sequencing has enabled identification of a core bacterial composition and bacterial succession within the GIT from birth, there are a multitude of internal and external factors that can affect the bacterial composition of pigs (Holman et al., 2017; Petri et al., 2010; Nowland et al., 2019; Konstantinov et al., 2006).

Bacterial populations can vary longitudinally (small to large intestine) as well as radially (mucosa to lumen) within the GIT (Zhao et al., 2015; Crespo-Piazuelo et al., 2018; Kelly et al., 2017). External factors, such as host genetics, changes in dietary format and composition, the inclusion of in-feed antibiotics or a change in environment can also significantly alter the microbiome of pigs (Dethlefsen et al., 2008; Lallès and Montoya, 2021; Guevarra et al., 2019; Frese et al., 2015). These external, management factors can differ between pig farms, leading to variation between farms (Vigors et al., 2020). Age, or stage of production, can also have a significant effect on the microbiome of pigs (Wang et al., 2019; De Rodas et al., 2018). This is particularly evident around weaning of pigs, which has been associated with reduced bacterial diversity, imbalances of the GIT microbiome and inflammation, collectively known as dysbiosis (see Section 1.3.3.3) (Gresse et al., 2017). Dysbiosis can confer growth advantages for *Enterobacteriaceae*,

including pathotypes such as enterotoxigenic *Escherichia coli* (ETEC), the main infectious agent of post-weaning diarrhoea (PWD; see Section 1.3.3.3) (Gresse et al., 2017).

In overcoming the detrimental effects of weaning within the pig industry, nutritional interventions have frequently been investigated (Celi et al., 2017). Historically, antibiotic growth promoters (AGPs) were used to improve growth performance and reduce PWD, but their ban in 2006 led to an increase in the alternative use of pharmacological levels of ZnO (Vondruskova et al., 2010; Cromwell, 2002; Looft et al., 2014a). The use of 16S rRNA sequencing has identified the similar effect of ZnO and antibiotics, such as Chlortetracycline and colistin sulphate, on the pigs microbiome, with increased microbial richness and diversity in the ileum, compared to control pigs (Yu et al., 2017; Vahjen et al., 2011). This increase in bacterial diversity could be the cause of reduced PWD observed when providing pharmacological levels of ZnO to pigs after weaning, as it could reduce dysbiosis within the GIT (see Section 1.3.3.5) (Stensland et al., 2015; Heo et al., 2010). Further research has also reported that pharmacological levels of ZnO reduced ETEC shedding in the faeces of pigs deliberately challenged with ETEC (0149) (Slade et al., 2011), as well as reduced abundance of the genera *Helicobacter* and pathogenic *Campylobacter jejuni*, further replicating the effect of antibiotics on the pig microbiome (Xie et al., 2011; Yu et al., 2017).

A substantial amount of research looking at the effect of ZnO on the pigs microbiome has included a deliberate pathogenic challenge, to elicit an immune response and focused on the effects seen up to day 14 after weaning (Slade et al., 2011; Sargeant et al., 2011; Stensland et al., 2015; Heo et al., 2010). However, there is less research determining differences in GIT microbiome as a result of ZnO, without a deliberate challenge, and whether these effects are seen beyond its inclusion to day 14. The use of 16S sequencing to determine influential factors on the bacterial composition of pigs housed at the National Pig Centre, University of Leeds, had not previously been complete. Therefore, using historically collected samples, a method for analysis was developed to determine factors that changed the GIT bacterial composition of pigs housed at this unit. This included the effect of pharmacological levels of ZnO, without a deliberate pathogenic challenge, on the pigs microbiome at days 14 and 28 after weaning.

3.1.1 Aims

The initial aim of this chapter was to develop a method of analysis of 16S rRNA sequencing to determine whether age, GIT location and sample type (mucosa/lumen) influenced the bacterial composition of pigs housed at the National Pig Centre, University of Leeds. Then, while taking these factors into consideration within the analysis model, to identify whether the use of pharmacological levels of ZnO altered the bacterial composition of pigs 14 and 28 days after weaning, when no deliberate pathogenic challenge had been given.

3.1.2 Primary hypothesis

1. If pigs are provided pharmacological levels of ZnO after weaning, without being given a deliberate disease challenge, then bacterial richness and diversity will increase in the small intestine but have less of an effect in the large intestine at days 14 and 28 after weaning.

Secondary hypotheses

2. The bacterial composition of the small intestine will show higher levels of facultative anaerobes compared to the large intestine, at all time points.
3. Within each gastrointestinal tract location, the mucosa-attached bacteria will have a higher number of oxygen-tolerant bacteria compared to the lumen.
4. As pigs age, bacterial species richness will increase and the ratio of Firmicutes to Bacteroidetes in each GIT location will increase.

3.2 Materials and Methods

3.2.1 Animals and Management

Trial details can be found within the General Methods, Chapter 2. Samples analysed were from pigs housed at the National Pig Centre, University of Leeds in 2016, with the trial being conducted prior to the start of the current PhD. The samples used for analysis within this chapter were inherited but a brief explanation of the trial they were inherited from is given for context. In brief, one pig per litter (from four litters), reared in commercial indoor facilities, was selected for euthanasia, dissection and sample collection at weaning. Remaining pigs within a litter were weaned into two pens of five pigs per pen, in indoor commercial accommodation and provided control (~150 ppm) or pharmacological (~2500 ppm) levels of ZnO for 14 days after weaning (Table 2.3 and 2.4). After day 14 all pigs received the same commercial second stage diet for a further 14 days

(Table 2.3 and 2.4). On days 14 and 28 after weaning, one pig per pen (eight per time point) were randomly selected for euthanasia, dissection and sample collection. All animals (*n* = 20) were humanely killed under Schedule 1 of the Animals (Scientific Procedures) Act 1986.

3.2.2 Microbiome Sampling and DNA Extraction

Samples from the lumen and mucosa of the ileum, caecum and colon were collected at weaning (day 0), day 14 and day 28 as described in Section 2.4.2. Microbial genomic DNA was extracted from 0.2 g of sample using the method described by the QIAamp stool mini kit (Qiagen, Germany) with some modifications, as described in Section 2.5.1. A total of 94 samples were sent to the University of Leeds, Next Generation Sequencing Facility (St James Hospital, UK) for amplification and sequencing on an Illumina MiSeq platform (Section 2.5.1).

3.2.3 Microbial Analysis

Raw sequence reads were quality filtered to remove unwanted sequences (such as those representing Archaea) and processed using Mothur v1.40.3, following the MiSeq standard operation procedure (Schloss et al., 2009). Unique sequences were identified, aligned against the SILVA (v.132) database, filtered and clustered into operational taxonomic units (OTUs) with 97% similarity. The effect of GIT location, sample type, age and dietary treatment on alpha and beta diversity were analysed in R Studio (v. 3.4.3). Alpha diversity measures (Shannon, Simpson, Chao1) were analysed using a general linear model (lme4). In addition, beta diversity was plotted using non-metric multidimensional scaling (NMDS) using the Bray-curtis distance and a PERMANOVA (adonis) was used to determine factor effects and interactions, as described in Section 2.5.2.2. DeSeq2 analysis was conducted to determine pairwise comparisons of differences in OTU counts (see Section 2.5.2.2).

3.3 Results

3.3.1 Bacterial sequencing data

A total of 1,054,897 reads from all 94 samples were generated through 16S rRNA sequencing of the V4 hypervariable region. The total number of OTUs was 467 from 94 samples. There were consistently no differences in alpha or beta diversity between mucosal and luminal samples within each GIT location at all time points; therefore, they were combined for all subsequent analysis. All identified sequences were classified into 18 different phyla, with Firmicutes (88.16 ± 10.27%), Bacteroidetes (4.17 ± 4.98%) and Tenericutes (1.66 ± 2.61%) being the top three abundant phyla. All identified genera, split for GIT location, treatment and time point are shown in Figure 3.1.

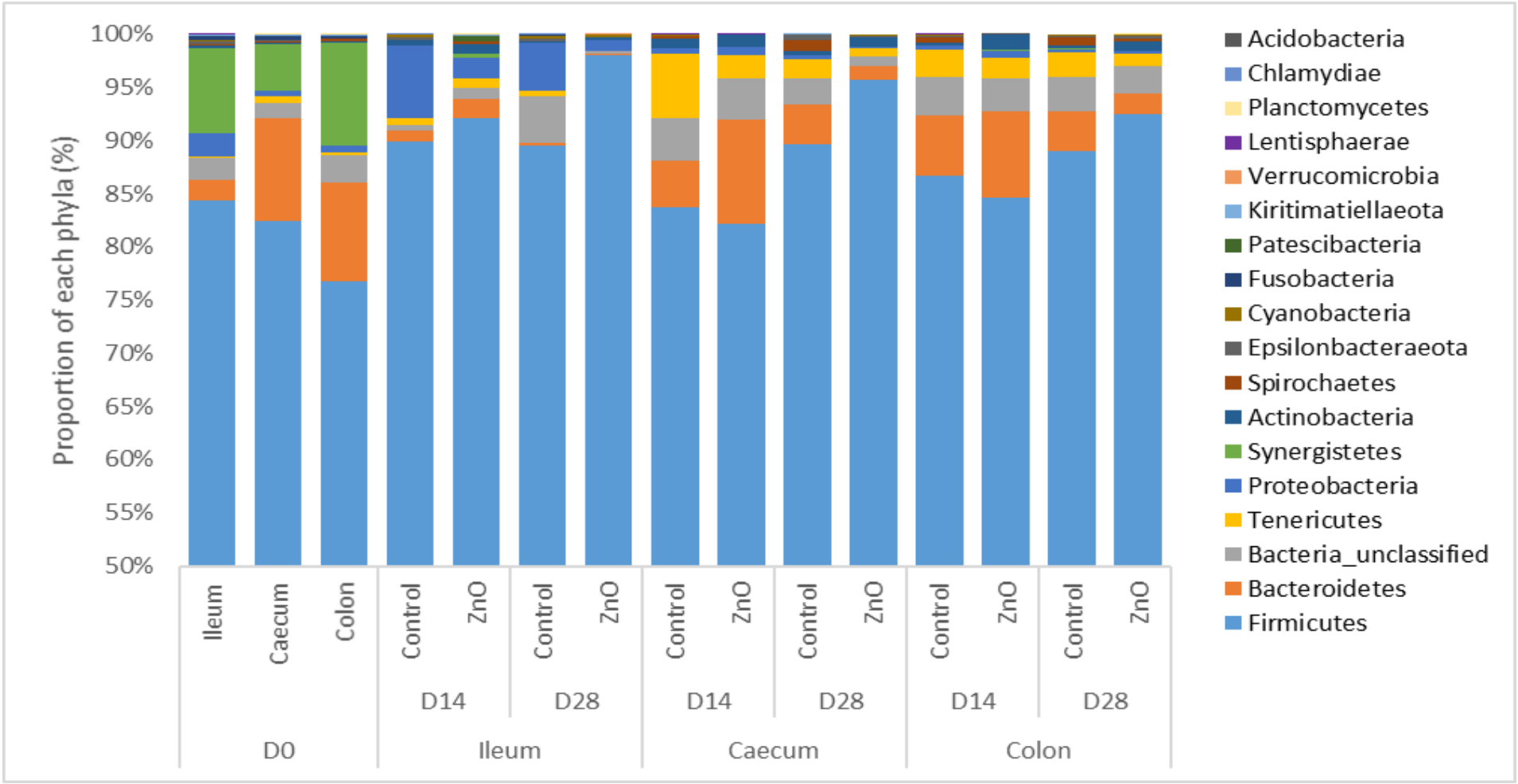

Figure 3.1 Percentage relative abundance of phyla present above 0.1% in at least one sample at days 0, 14, and 28 within the ileum, caecum and colon of pigs at fed a control (~150 ppm) or pharmacological (~2500 ppm) levels of ZnO post-weaning. Note: percentage abundance axis starts at 50%.

3.3.2 Gastrointestinal tract location, but not sample type, affected bacterial diversity and composition of control pigs at day 28

The effect of GIT location on the microbiome of control pigs at day 28 was investigated first and showed no significant differences in alpha diversity (Table 3.1). The most abundant phyla across the ileum, caecum and colon of control pigs at day 28 are shown in Figure 3.2. Beta diversity was significantly different between the ileum and both the caecum and colon (F-statistic = 11.086, p = 0.001; F = 12.901, p = 0.001, respectively). DeSeq2 analysis also supported these differences in terms of composition (Table 3.2).

Table 3.1 Alpha diversity measures for the ileum, caecum and colon of pigs 28 days post-weaning.

	Ileum	Caecum	Colon	F-statistic	p value
Simpson	0.828	0.760	0.790	2.425	0.103
Shannon	2.371	2.252	2.354	2.935	*0.066*
Chao1	70.41	81.33	89.92	2.566	*0.091*

The top five OTUs that significantly increased or decreased in the caecum and colon compared to the ileum at day 28 are shown in Table 3.2. In addition to those shown in Table 3.2, all remaining OTUs that significantly changed in abundance are shown in Appendix B1 and Appendix B2. Of the remaining OTUs that increased, 78% were *Firmicutes*, including *Intestinibacter* (Log2 Fold Change -7.14, $p < 0.001$) and *Lactobacillus* (Log2 Fold Change -6.29, $p < 0.001$). Of the OTUs that significantly increased in the colon compared to the ileum, nine were also seen to increase in the caecum compared to the ileum.

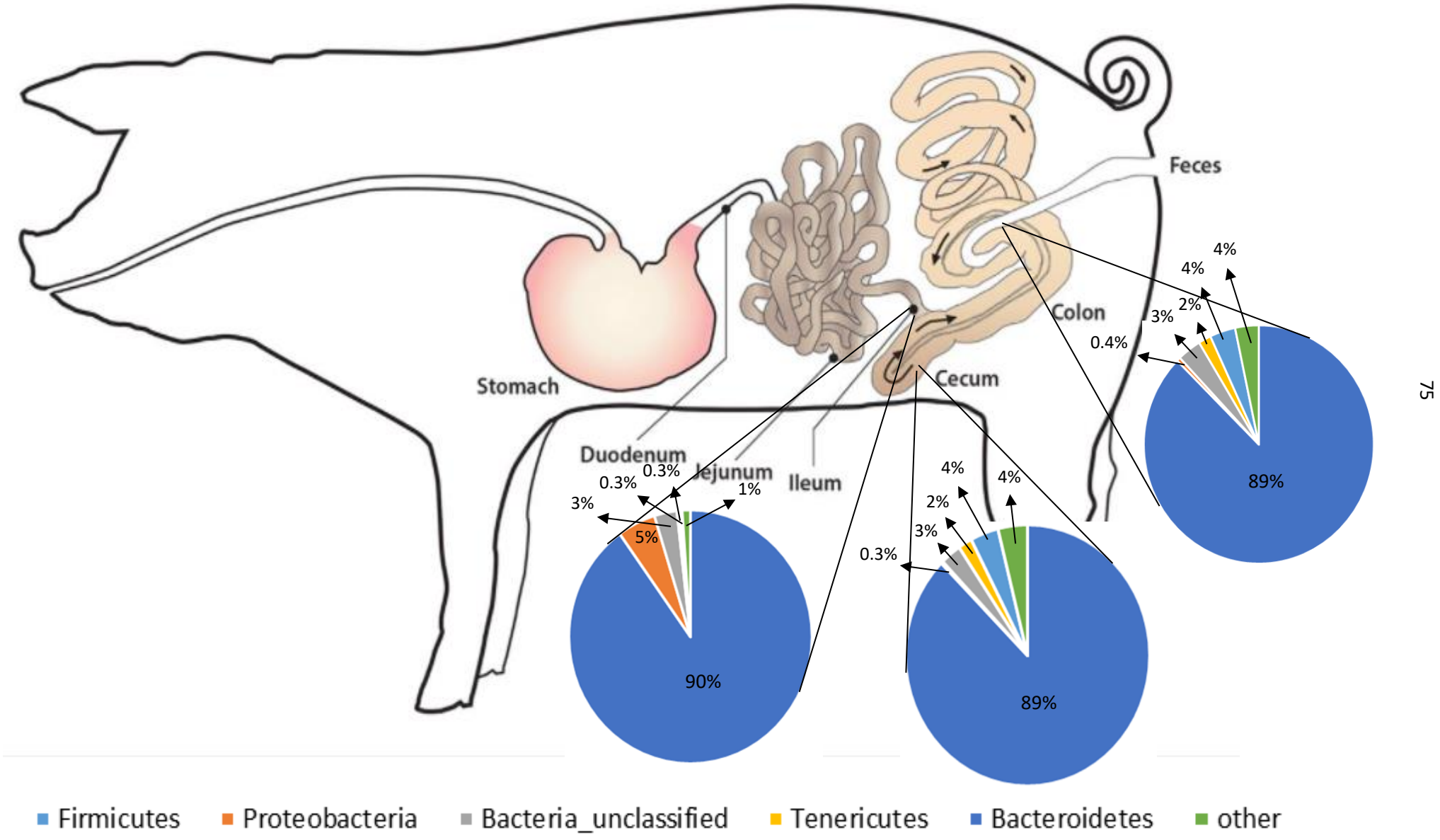

Figure 3.2 Community composition of the most dominant phyla (Firmicutes, Proteobacteria, unclassified bacteria, Tenericutes, Bacteroidetes and 'other' phyla in the ileum, caecum and colon of control fed pigs, 28 days post-weaning. Image adapted from Holman et al. (2017).

Table 3.2 DeSeq2 analysis of the operational taxonomic units (OTU), showing the top 5 OTUs that changed in the caecum and colon relative to the ileum at day 28, in control pigs. OTUs were classified to the genus level. P values are adjusted for multiple testing using the Benjamin-Hochberg correction. Fold changes represent Log2 fold changes.

OTU Number	Genera	Fold Change	p value
Increase from Ileum to Caecum			
OTU 12	*Muribaculaceae, unclassified*	2.45	0.002
OTU 23	*Ruminococcaceae*	3.13	0.002
OTU 16	*Muribaculaceae*	2.29	0.003
OTU 11	*Veillonellaceae, unclassified*	2.12	0.004
OTU 01	*Megasphaera*	1.69	0.005
Decrease from Ileum to Caecum			
OTU 19	*Terrisporobacter*	-9.46	<0.001
OTU 83	*Lactococcus*	-8.20	<0.001
OTU 18	*Clostridium sensu stricto 1*	-6.54	<0.001
OTU 25	*Pasteurellaceae, unclassified*	-6.85	<0.001
OTU 39	*Clostridiaceae1, unclassified*	-6.16	<0.001
Increase from Ileum to Colon			
OTU 16	*Muribaculaceae*	2.86	<0.001
OTU 12	*Muribaculaceae, unclassified*	2.69	<0.001
OTU 11	*Veillonellaceae, unclassified*	2.08	0.005
OTU 04	*Bacteria, unclassified*	1.29	0.005
OTU 05	*Ruminococcaceae, unclassified*	1.91	0.006
Decrease from Ileum to Colon			
OTU 19	*Terrisporobacter*	-9.39	<0.001
OTU 83	*Lactococcus*	-9.73	<0.001
OTU 18	*Clostridium sensu stricto 1*	-7.41	<0.001
OTU 39	*Clostridiaceae1, unclassified*	-7.23	<0.001
OTU 25	*Pasteurellaceae, unclassified*	-7.38	<0.001

3.3.3 Gastrointestinal tract location differs in richness, diversity and composition at all ages

Alpha diversity, as measured by Chao1, in the ileum of pigs at weaning was lower than the caecum and colon (64.18 *vs* 98.63 and 96.96 respectively, F= 4.265, p = 0.05; Table 3.8), as expected. Although as the pig matured, no differences were seen in alpha diversity at days 14 or 28.

The gross microbiome analysis at the phyla and genera level along the GIT at each time point showed the microbiome continuously differed along the GIT, particularly from the ileum to both the caecum and colon, with few differences between the latter two locations. Analysis of beta diversity showed differences at day 0 (F = 3.223, p = 0.007), with DeSeq2 identifying 47 OTUs that increased in the caecum compared to the ileum, of which, 36 also significantly increased in the colon compared to the ileum (Table 3.3 and Appendix B4; Appendix B5). At day 14, as there were no interactions between GIT location and dietary treatment, the main effect of GIT location can be reported; F = 5.133, p = 0.001. The pattern of differences along the GIT at day 14 mimic those of weaning and day 28; the ileum differed in diversity and composition compared to both the caecum and colon (F = 6.901, p = 0.001; F = 8.680, p = 0.001, respectively), with no differences between the latter locations. The differences seen in GIT location, alongside dietary treatment, can be seen in the left-hand side of the NMDS plot in Figure 3.3; this used the Bray-Curtis distance on two axes. DeSeq2 analysis at day 14 indicated 32 OTUs that changed between the ileum and caecum (Table 3.4 and Appendix B.6). The same genera that changed from the ileum to the caecum, were replicated when comparing the ileum to colon, with an additional eight OTUs (Appendix B.7).

Table 3.3 DeSeq2 analysis of the operational taxonomic units (OTU) in the caecum and colon relative to the ileum at day 0. P values adjusted for multiple testing with Benjamin-Hochberg correction. Fold changes represent Log2 fold changes.

OTU Number	Genera	Fold Change	p value
Increase from Ileum to Caecum			
OTU 20	*Prevotellaceae UCG-003*	6.84	<0.001
OTU 43	*Pyramidobacter*	9.58	<0.001
OTU 23	*Ruminococcaceae*	6.62	<0.001
OTU 15	*Uncultured Firmicutes*	5.02	<0.001
OTU 92	*Anaerofilum*	7.47	<0.001
Decrease from Ileum to Caecum			
OTU 35	*Veillonella*	-5.43	<0.001
OTU 39	*Clostridiaceae1 unclassified*	-4.37	0.001
OTU 18	*Clostridium sensu stricto 1*	-6.06	0.001
OTU 68	*Actinobacillus*	-5.30	0.001
OTU 36	*Romboutsia*	-3.70	0.001
Increase from Ileum to Colon			
OTU 123	*Hydrogenoanaerobacterium*	8.92	<0.001
OTU 20	*Prevotellaceae UCG-003*	6.04	<0.001
OTU 23	*Ruminococcaceae*	6.20	<0.001
OTU 43	*Pyramidobacter*	8.31	<0.001
OTU 15	*Uncultured*	4.66	<0.001
Decrease from Ileum to Colon			
OTU 39	*Clostridiaceae1 unclassified*	-5.16	<0.001
OTU 35	*Veillonella*	-5.27	0.0002
OTU 36	*Romboutsia*	-3.86	0.0010
OTU 53	*Peptostreptococcaceae unclassified*	-5.01	0.0014
OTU 18	*Clostridium sensu stricto 1*	-5.05	0.0030

Table 3.4 DeSeq2 analysis of the operational taxonomic units (OTU) in the caecum and colon relative to the ileum at day 14, irrespective of dietary treatment. P values are adjusted for multiple testing using the Benjamin-Hochberg correction. Fold changes represent Log2 fold changes.

OTU Number	Genera	Fold Change	p value
Increase from Ileum to Caecum			
OTU 12	*Muribaculaceae (unclassified)*	2.83	<0.001
OTU 16	*Muribaculaceae*	2.78	<0.001
OTU 23	*Ruminococcaceae*	2.59	<0.001
OTU 54	*Enterorhabdus*	3.55	0.002
OTU 17	*Subdoligranulum*	2.36	0.002
Decrease from Ileum to Caecum			
OTU 19	*Terrisporobacter*	-8.00	<0.001
OTU 18	*Clostridium sensu stricto 1*	-6.33	<0.001
OTU 35	*Veillonella*	-7.95	<0.001
OTU 36	*Romboutsia*	-7.64	<0.001
OTU 53	*Peptostreptococcaceae (unclassified)*	-6.06	<0.001
Increase from Ileum to Colon			
OTU 12	*Muribaculaceae (unclassified)*	3.10	<0.001
OTU 16	*Muribaculaceae*	3.14	<0.001
OTU 54	*Enterorhabdus*	3.58	<0.001
OTU 17	*Subdoligranulum*	2.16	0.006
OTU 69	*Family XIII UCG-001*	2.52	0.006
Decrease from Ileum to Colon			
OTU 18	*Clostridium sensu stricto 1*	-7.60	<0.001
OTU 08	*Terrisporobacter*	-7.97	<0.001
OTU 19	*Romboutsia*	-9.59	<0.001
OTU 35	*Peptostreptococcaceae (unclassified)*	-7.51	<0.001
OTU 53	*Pasteurellaceae (unclassified)*	-8.95	<0.001

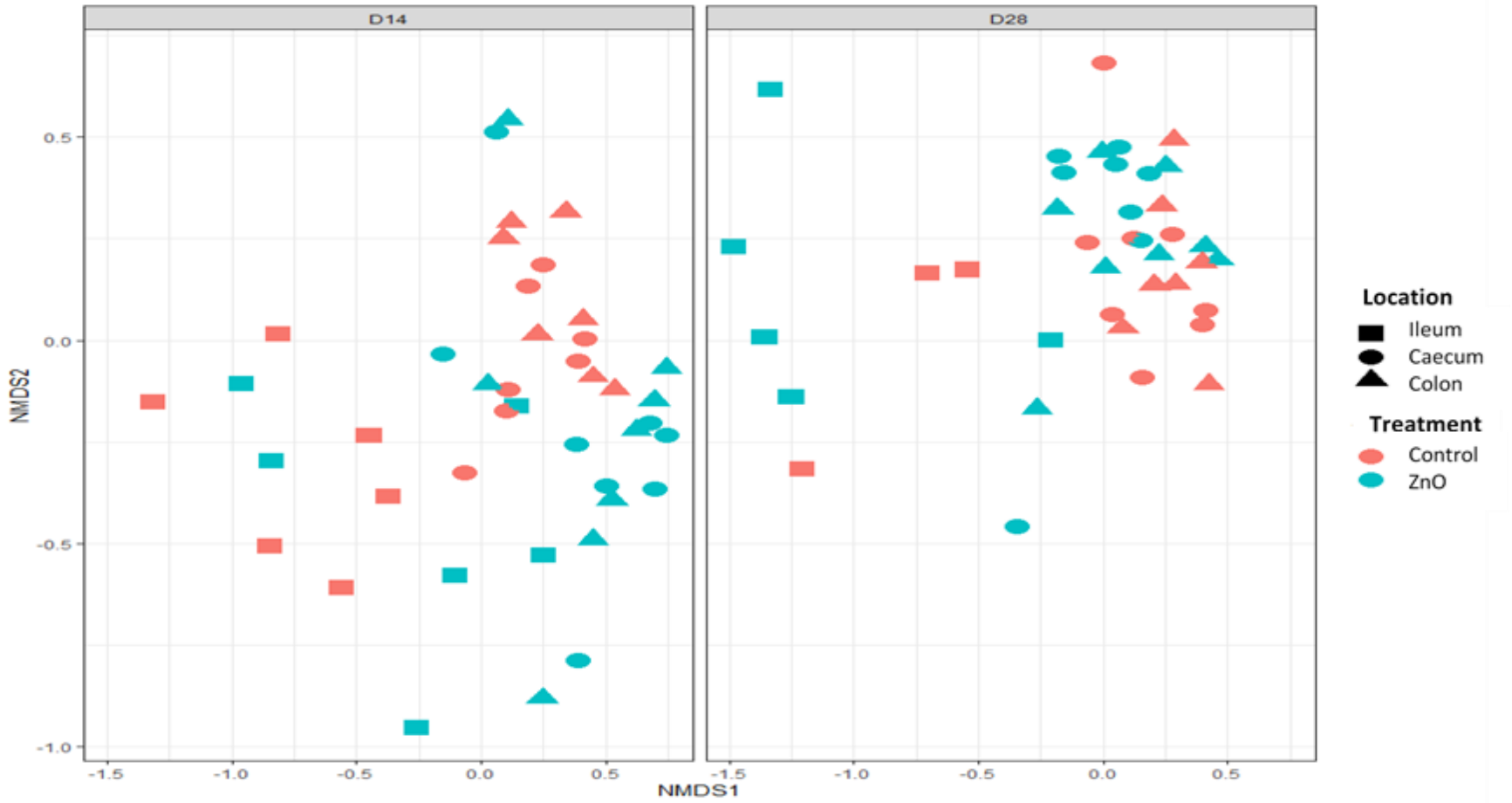

Figure 3.3 NMDS plot showing the distribution of samples for location within the gastrointestinal tract and treatment at both day 14 (left) and day 28 (right). Separation of the control (blue) and ZnO (red) colours can be seen at day 14. Distribution of ileal samples (squares) are also seen at both day 14 and day 28, with clear separation from the other GIT locations.

3.3.4 Bacterial richness, diversity and composition changes with age from weaning to days 14 and 28 after weaning

As there were no significant interactions between age, GIT location and dietary treatment, the main effect of age on Shannon, Simpson and Chao1 measures of alpha diversity, are shown in Table 3.5. No differences were observed between weaning (day 0) and day 14 for any alpha diversity measures, but diversity decreased from day 14 to day 28 when looking at Simpson and Shannon indices.

Table 3.5 Alpha diversity indices of all samples collected at days 0, 14 and 28 post-weaning, irrespective of GIT location or dietary treatment.

	Day 0	Day 14	Day 28	F- statistic	p value
Simpson	0.77^{ab}	0.84^{a}	0.75^{b}	5.830	**0.004**
Shannon	2.21^{ab}	2.55^{a}	2.10^{b}	9.370	**<0.001**
Chao1	71.20	77.80	71.47	0.870	0.420

Rows with different superscripts (a-b) indicate significant differences.

An interaction between GIT location and age was observed for beta diversity ($F_{4,84}$ = 1.573, p = 0.05). DeSeq2 revealed differences in count OTU data were seen in all locations between days 0 and 14, while differences between days 14 and 28 were predominantly seen in the caecum and colon. Table 3.6 and Table 3.7 show the top five OTUs that significantly changed within each GIT location between days 0 and 14 and days 14 and 28, respectively. Additional OTUs that significantly increased between days 0 and 14 are shown in Appendix B3. No additional OTUs decreased between days 0 and 14 nor did any additional OTUs change between days 14 and 28 to those presented in Table 3.7.

Table 3.6 Top five OTUs that were identified by DeSeq2 as changing in counts between day 0 and day 14 within the ileum, caecum and colon, irrespective of dietary treatment. OTUs were classified to the genus level. P values are adjusted for multiple testing using the Benjamin-Hochberg correction. Fold changes represent Log2 fold changes.

OTU Number	Genera	Fold Change	p value
Ileum			
Increased day 0 to day 14			
OTU 28	*Negativibacillus*	8.61	<0.001
OTU 09	*Anaerovibrio*	6.66	<0.001
OTU 34	*Dialister*	7.52	<0.001
OTU 83	*Lactococcus*	4.69	0.004
OTU 102	*Sanguibacter*	6.99	0.007
Decreased day 0 to day 14			
OTU 11	*Veillonellaceae (unclassified)*	-4.74	<0.001
OTU 35	*Veillonella*	-6.02	<0.001
OTU 49	*Enterobacteriaceae (unclassified)*	-3.60	0.014
OTU 89	*Fusobacteriaceae (unclassified)*	-5.78	0.033
OTU 10	*Firmicutes (unclassified)*	-3.03	0.047
Caecum			
Increased day 0 to day 14			
OTU 17	*Subdoligranulum*	4.58	<0.001
OTU 30	*Faecalibacterium*	5.83	<0.001
OTU 18	*Clostridium sensu stricto 1*	6.33	0.002
OTU 38	*Allisonella*	4.53	0.006
OTU 54	*Enterorhabdus*	5.53	0.006
Decreased day 0 to day 14			
OTU 43	*Pyramidobacter*	-10.33	<0.001
OTU 35	*Veillonella*	-6.38	0.002
OTU 15	*uncultured*	-3.14	0.005
OTU 93	*WCHB1-41*	-5.14	0.013
OTU 88	*Fusobacterium*	-5.49	0.015
Colon			
Increased day 0 day 14			
OTU 34	*Dialister*	24.34	<0.001
OTU 17	*Subdoligranulum*	4.24	<0.001
OTU 30	*Faecalibacterium*	8.68	<0.001
OTU 38	*Allisonella*	5.85	<0.001
OTU 28	*Negativibacillus*	4.02	<0.001
Decreased day 0 to day 14			
OTU 43	*Pyramidobacter*	-11.59	<0.001
OTU 35	*Veillonella*	-5.96	<0.001
OTU 88	*Fusobacterium*	-5.82	0.011
OTU 36	*Romboutsia*	-4.46	0.021

Table 3.7 All OTUs that were identified by DeSeq2 as changing in counts between day 14 and day 28 within the ileum, caecum and colon, irrespective of dietary treatment. OTUs were classified to the genus level. P values are adjusted for multiple testing using the Benjamin-Hochberg correction. Fold changes represent Log2 fold changes.

OTU Number	Genera	Fold Change	p value
Ileum- *Decreased day 14 to day 28*			
OTU 80	*Selenomonas*	-15.94	<0.001
OTU 28	*Negativibacillus*	-4.70	0.010
OTU 46	*Candidatus Soleaferrea*	-4.53	0.013
Caecum *Increased day 14 to day 28*			
OTU 59	*Campylobacter*	3.81	0.002
OTU 02	*Streptococcus*	2.92	0.006
OTU 60	*Gastranaerophilales*	2.39	0.017
Decreased day 14 to day 28			
OTU 28	*Negativibacillus*	-3.59	<0.001
OTU 82	*Ruminiclostridium 9*	-3.35	0.006
OTU 16	*Muribaculaceae*	-1.55	0.008
OTU 37	*Ruminococcaceae UCG-002*	-1.90	0.008
OTU 05	*Ruminococcaceae (unclassified)*	-1.50	0.017
OTU 66	*Ruminococcaceae UCG-005*	-2.29	0.019
OTU 90	*Blautia*	-2.71	0.023
OTU 106	*Bacteroides*	-3.48	0.023
OTU 121	*Phocea*	-3.52	0.023
OTU 92	*Anaerofilum*	-2.39	0.032
OTU 12	*Muribaculaceae (unclassified)*	-1.46	0.049
Colon *Increased day 14 to day 28*			
OTU 72	*Helicobacter*	3.51	0.006
OTU 36	*Romboutsia*	3.88	0.013
OTU 60	*Gastranaerophilales*	2.38	0.013
OTU 78	*Agathobacter*	3.24	0.013
OTU 87	*Anaerobiospirillum*	2.58	0.032
OTU 62	*Ruminococcus 1*	1.73	0.033
OTU 59	*Campylobacter*	2.45	0.033
OTU 57	*Desulfovibrio*	1.79	0.036
OTU 127	*Butyricimonas*	3.28	0.046
Decreased day 14 to day 28			
OTU 28	*Negativibacillus*	-3.63	<0.001
OTU 82	*Ruminiclostridium_9*	-4.11	0.002
OTU 92	*Anaerofilum*	-2.89	0.013
OTU 58	*Prevotellaceae_UCG-001*	-2.96	0.028
OTU 99	*Coprococcus_3*	-3.01	0.029
OTU 98	*Ruminococcaceae_NK4A214_group*	-2.19	0.040
OTU 134	*UBA1819*	-4.07	0.044

3.3.5 The effect of pharmacological levels of zinc oxide, within each gastrointestinal location, on bacterial richness, diversity and composition at day 14

Including dietary treatment (control *vs*. ZnO) in the statistical model when assessing alpha diversity indices at day 14 showed no effect of ZnO on alpha diversity measures of species richness and evenness within any GIT location (Table 3.8). However, beta diversity and composition were affected by the inclusion of ZnO at day 14 ($F_{1,40}$ = 2.159, p = 0.05; Figure 3.3,Figure 3.4). Although the ileum and caecum showed minimal changes in response to ZnO, more prominent effects were seen within the colon (Table 3.9). However, the effect of ZnO on all locations of the GIT was not long-lived, and no differences were observed in alpha or beta diversity at day 28 (beta diversity: F = 1.745, p = 0.10; Figure 3.3), although four OTUs differed in the caecum of pigs at day 28 in response to ZnO (Table 3.9).

Table 3.8 Shannon, Simpson and Chao1 measures of alpha diversity for the ileum, caecum and colon at weaning (day 0) and day 14.

	GIT Location	Weaning (day 0)	Day 14		Weaning (day 0)		Day 14						
							F-value$_{df}$			p value			
			Control	ZnO	F-value$_{df}$	p value	Location	Treatment	Location* Treatment	Location	Treatment	Location* Treatment
Simpson	Ileum	0.67	0.85	0.82	$2.531_{9,11}$	0.134	$0.823_{39,41}$	$0.082_{40,41}$	$0.154_{36,38}$	0.447	0.776	0.858
	Caecum	0.82	0.86	0.86								
	Colon	0.84	0.83	0.83								
Shannon	Ileum	1.86	2.55	2.48	$2.197_{9,11}$	0.167	$1.739_{39,41}$	$0.138_{40,41}$	$0.074_{36,38}$	0.189	0.713	0.929
	Caecum	2.39	2.75	2.65								
	Colon	2.45	2.43	2.44								
Chao1	Ileum	64.18[a]	73.84	85.66	$4.265_{9,11}$	***0.050***	$0.206_{39,41}$	$1.644_{40,41}$	$2.025_{36,38}$	0.815	0.207	0.147
	Caecum	98.63[b]	86.75	73.62								
	Colon	96.96[b]	85.75	66.10								

a-b Means of each indices within a column showing different superscripts indicate significant differences (P<0.05)

Table 3.9 All DeSeq2 analysis of operational taxonomic units (OTUs) in the ileum, caecum and colon of ZnO fed pigs relative to control pigs at days 14 and 28. P values are adjusted for multiple testing using the Benjamin-Hochberg correction. Fold changes represent Log2 fold changes.

OTU Number	Genera	Fold Change	p value
DAY 14 - Ileum			
Decreased with ZnO			
OTU 35	*Veillonella*	-4.397	0.017
OTU 55	*Intestinibacter*	-4.353	0.026
Caecum			
Increased with ZnO			
OTU 121	*Phocea*	4.224	0.039
Decreased with ZnO			
OTU 66	*Ruminococcaceae UCG-005*	-3.881	0.005
OTU 07	*Mitsuokella*	-3.573	0.045
Colon			
Increased with ZnO			
OTU 92	*Anaerofilum*	3.90	0.002
OTU 54	*Enterorhabdus*	3.14	0.003
OTU 61	*GCA-900066225*	5.10	0.004
OTU 49	*Enterobacteriaceae (unclassified)*	6.33	0.011
OTU 52	*Olsenella*	2.07	0.020
OTU 17	*Subdoligranulum*	2.26	0.027
OTU 134	*UBA1819*	4.60	0.027
OTU 121	*Phocea*	3.74	0.043
Decreased with ZnO			
OTU 42	*Treponema_2*	-5.60	<0.001
OTU 84	*Sphaerochaeta*	-6.14	<0.001
OTU 45	*Christensenellaceae (R-7 group)*	-4.07	0.002
OTU 66	*Ruminococcaceae (UCG-005)*	-4.47	0.002
OTU 57	*Desulfovibrio*	-3.95	0.005
OTU 115	*Ruminococcaceae (UCG-009)*	-4.61	0.005
OTU 09	*Anaerovibrio*	-3.34	0.008
OTU 26	*Alloprevotella*	-2.23	0.012
OTU 59	*Campylobacter*	-4.86	0.016
OTU 64	*Rikenellaceae (RC9 gut group)*	-2.27	0.023
OTU 58	*Prevotellaceae (UCG-001)*	-3.49	0.027
DAY 28 - Caecum			
Increased with ZnO			
OTU	*Treponema 2*	6.79	<0.001
OTU	*Negativibacillus*	2.54	<0.001
Decreased with ZnO			
OTU	*Streptococcus*	-3.23	<0.001
OTU	*Lactobacillales, unclassified*	-3.51	<0.001

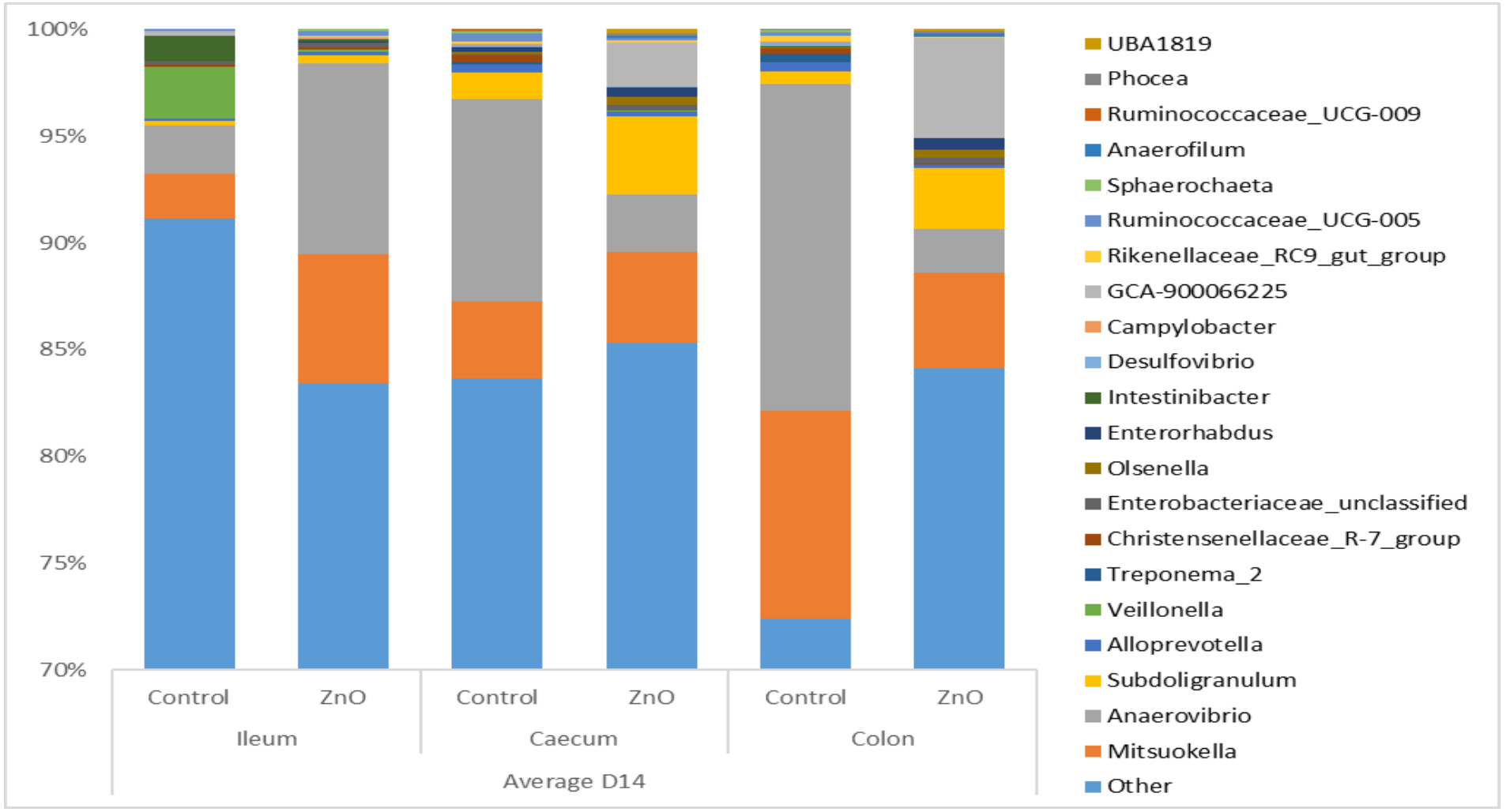

Figure 3.4 Percentage relative abundance of the top 20 genera that were present in at least one same above 0.1% at day 14. Samples include the ileum, caecum and colon of pigs fed control (~150 ppm) or pharmacological (~2500 ppm) levels of ZnO

Note: Percentage abundance axis starts at 70% due to high number of 'other' genera' in overall composition.

3.4 Discussion

The primary hypothesis of the work presented in this chapter stated that bacterial richness and diversity would increase within the small intestine and have a reduced effect in the large intestine of pigs provided pharmacological levels of ZnO for 14 days after weaning, in the absence of a deliberate pathogenic challenge. Although species richness did not increase with ZnO at any time point, the provision of ZnO did alter beta diversity, leading to shifts in the bacterial composition of the GIT at day 14, but not day 28, partially confirming the primary hypothesis of this study. Previous research has more frequently determined the effect of ZnO on GIT bacterial composition, in response to a deliberate pathogenic challenge, resulting in an immune response (Slade et al., 2011; Sargeant et al., 2011; Stensland et al., 2015; Heo et al., 2010). Although some studies have still identified differences in the bacterial composition of some GIT locations without a pathogenic challenge (Yu et al., 2017), differences through time are less documented. Furthermore, given the effect of GIT location, sample type, age, genetics and dietary treatment on bacterial richness, diversity and composition in pigs, which has been readily documented, varying effects are seen between studies (Holman et al., 2017; Zhao et al., 2015; Wang et al., 2019; Yu et al., 2017; Xiao et al., 2018; Crespo-Piazuelo et al., 2018). These differences between studies are likely as a result of different herds of pigs used for sampling, which is likely due to some of the effects described above, as well as herd heath on different farms (Vigors et al., 2020). As a result of this, the research presented in this chapter also aimed to identify the effects of GIT location, age and sample type (mucosa or lumen) on pigs without a deliberate disease challenge, housed at the National Pig Centre, University of Leeds, prior to assessing the effect of pharmacological levels of ZnO. To achieve this, an analysis method was developed in RStudio to determine the effects of these factors on the pig microbiome and then factors that significantly influenced the microbiome were included in the analysis when determining the effect of ZnO on the pigs GIT bacterial composition.

Across all samples, the current research identified Firmicutes (88.16 ± 10.27%), Bacteroidetes (4.17 ± 4.98%) and Tenericutes (1.66 ± 2.61%) as being the most dominating phyla, which have all been defined as part of the core microbiome of pigs (Mach et al., 2015; Kelly et al., 2017; Yu et al., 2017; Zhang et al., 2018; Holman et al., 2017) and humans, giving confidence in sample collection and analysis process

(Lynch and Pedersen, 2016). Before determining the effect of pharmacological levels of ZnO on the bacterial composition of the GIT, the effect of GIT location, sample type and age of pigs was determined.

3.4.1 Gastrointestinal tract location, but not sample type within each location, affected bacterial richness, diversity and composition

The GIT is responsible for breaking down and metabolising food, absorption of water and minerals, excretion of waste metabolites and protection against both non-infectious and infectious stressors (Hornbuckle and Tennant, 1997; Maslowski and Mackay, 2011; Kogut and Arsenault, 2016). Results presented in this chapter support previous findings, in that the small intestine continuously had a different bacterial composition to the caecum and colon; while the latter two did not differ (Zhang et al., 2018; Kelly et al., 2017; Zhao et al., 2015). In terms of alpha diversity, Chao1 showed significantly lower species richness in the ileum, compared to both the caecum and colon at weaning. These results were analogous with previous studies that identified lower richness (Chao1) within the ileum compared to the caecum (Yang et al., 2016) and colon (Quan et al., 2018) in pigs, albeit at different stages of production.

The small intestine is critically important for the absorption of nutrients but also must be able to protect against pathogenic infections (see Section 1.3) (Santaolalla and Abreu, 2012). Higher oxygen concentrations compared to the large intestine are observed; therefore, allowing facultative anaerobes to colonise (Donaldson et al., 2016). Accordingly, results presented in this chapter saw facultative anaerobes, that can be present both in the presence or absence of oxygen and are therefore oxygen-tolerant, were significantly more abundant within the ileum compared to the caecum and colon, in support of the first of the secondary hypotheses. For example, members of the Proteobacteria phyla were more abundant within the ileum at every time point, including the genera *Actinobacillus*, unclassified *Enterobacteriaceae* and unclassified *Pasteurellaceae.*

An increase in abundance of Proteobacteria is also evident in the relative percentage abundance data, as illustrated by Figure 3.2, decreasing from 5% in the ileum to 0.3% in both the caecum and colon. Higher abundance of this phyla within the ileum has been seen previously in both pigs (Crespo-Piazuelo et al., 2018; Zhao et al., 2015; Zhang et al., 2018), and humans (Donaldson et al., 2016). Although the percentage of

Proteobacteria was higher in the ileum, the percentage abundance seen was still lower than that reported by Zhao et al. (2015), who found Proteobacteria occupied 75.5% and 72.6% of the jejunum and ileum, respectively, reducing to 12.7% and 13.2% in the caecum and colon, respectively. Pigs used by Zhao et al. (2015) were slaughtered at six months of age, when the GIT is likely to be more stable compared to the age of pigs sampled within the current trial. At the genera level, members of the *Clostridiaceae 1* family, including both unclassified *Clostridiaceae* and *Clostridium sensu stricto 1* significantly reduced along the GIT at every age, which supports previous work (Crespo-Piazuelo et al., 2018; Zhang et al., 2018). Specifically, *Clostridium sensu stricto* is hypothesised to strongly influence the host immune system and would therefore be expected to be most abundant within the ileum, whereby there is more crossover between the microbiota and the immune response (see Section 1.3.2) (Lopetuso et al., 2013).

The large intestine has lower oxygen concentrations compared to the small intestine and correlations have been identified between these low oxygen concentrations and the associated microbiota, such as an increase in strict anaerobes, like Bacteroidetes (Albenberg et al., 2014). The increase of Bacteroidetes in the caecum and colon, compared to the ileum was evident at day 28 in the current research (Figure 3.2) and at all other time points, as detailed in DeSeq2 analysis. This supports previous research of the GIT microbiome in pigs (Zhao et al., 2015; Quan et al., 2018). Two genera from the family *Muribaculaceae* (*Muribaculaceae ge* and unclassified *Muribaculaceae*) significantly increased in the caecum and colon compared to the ileum at each time point. The *Muribaculaceae* family, previously known as S24-7, has been associated with the degradation of complex carbohydrates, which are expected to be more available within the large intestine (Lagkouvardos et al., 2019; Ormerod et al., 2016).

Conditions between the mucosa and lumen of the GIT alter as proximity to the mucosal layer exposes bacteria to host-derived oxygen, thus promoting the growth of oxygen tolerant bacteria, such as Proteobacteria, which led to one of the secondary hypotheses (Hypothesis 3) of this chapter (Marteyn et al., 2011; Kelly et al., 2017). Differences in bacterial composition and diversity between the mucosa and lumen of the GIT in pigs has previously been reported (Zhang et al., 2018; Looft et al., 2014b). However, results obtained from the current study do not support this previous work,

rejecting Hypothesis 3, as no significant differences were seen between luminal and mucosal samples used. A meta-analysis of 20 publicly available data sets looked at overall differences along the entire GIT microbiome of pigs and found that there were significant differences observed between mucosal and luminal samples within the upper GIT (jejunum/ileum) (Holman et al., 2017). These differences in results obtained could be due to fewer mucosal, compared to luminal, samples used due to insufficient volumes of sample, and potentially different sampling methods compared to other studies that have identified significant differences in mucosal-attached bacteria (Zhang et al., 2018; Kelly et al., 2017). As no differences were observed between the mucosa and lumen, these samples were combined for all subsequent analysis.

3.4.2 The microbiome changes with age of pigs

In both humans (Mariat et al., 2009) and pigs (Jurburg and Bossers, 2021; Holman et al., 2017) it is well documented that the GIT microbiota shifts with age, and reaches a point of maturity, known as a climax community, that is more stable and aids in responding to disease (Kim and Isaacson, 2015). In support of previous findings significant changes were seen in alpha and beta diversity as pigs aged. However, the reduction in species richness and evenness between days 14 and 28 of the current research is surprising, and disputes aspects of Hypothesis 4 of this study, as previous reports have indicated an increase in species richness with age in faecal samples (Frese et al., 2015; Wang et al., 2019) and within the GIT (De Rodas et al., 2018). While Chao1 indices did not differ overtime, the low values presented for all measures of alpha diversity in this research compared to previous reports are of concern (De Rodas et al., 2018; Zhao et al., 2015; Wang et al., 2019). This could be as a result of long-term storage at -20ºC, as prolonged storage at temperatures above -80ºC has been shown to affect bacterial composition (Choo et al., 2015; Panek et al., 2018). Nonetheless, composition of bacteria, as determined by DeSeq2, generally follows the expected trend, as previously discussed. At the phyla level, shifts in the ratio of Bacteroidetes, such as *Prevotellaceae*, and the number of Firmicutes were observed between days 0 and 14, supporting previous work by Zhao et al. (2015) and partially accepting the final secondary hypotheses of this research (Hypothesis 4). Zhao et al. (2015) reported the ratio of Firmicutes to Bacteroidetes increased over 10fold from one month old (equivalent to day 0 in the current study) to two months old pigs (equivalent to day 28 in the current study). Our findings show the ratio of Firmicutes to

Bacteroidetes change from 2:1 at day 0 to 7:1 at day 14. These changes in Firmicute to Bacteroidetes ratio with age have also been reported in humans (Mariat et al., 2009).

Beta diversity was also significantly affected by age and time point within the current work, however these differences were observed between all time points, indicating beta diversity is more susceptible to changes with age, than alpha diversity. When comparing the number of OTUs that significantly altered within the GIT at days 0, 14 and 28, there was a decrease in the number of OTUs that changed within the GIT, with age. This is likely as a result of a less mature GIT at weaning, when pigs still have an immature immune system and are dependent on sows milk to prevent overgrowth of opportunistic pathogens, although identification to the species level of classification is not possible with 16S sequencing; therefore it is not known if bacteria were pathogenic at this age (Guevarra et al., 2019). The reduced differences in OTU count within the GIT through time, as shown in Table 3.6 and Table 3.7 indicates the establishment of a microbiota that is less susceptible to change.

Changes in the composition of the microbiome from weaning to day 14 support the expected shift in bacteria as a result of a change in dietary composition. Nursing pigs, such as those sampled at weaning, prior to moving away from the sow, show a milk-oriented microbiome with increased abundance of *Fusobacteria* and *Enterobacteriaceae* which then shifts to become more dominated by *Prevotellaceae* and *Ruminococcaceae* following weaning (De Rodas et al., 2018; Frese et al., 2015; Mach et al., 2015). Increases in *Prevotellaceae* are seen in the microbiome of mammals and humans that consume diets rich in plant polysaccharides and fibre as this bacterium is linked to their fermentation (Ivarsson et al., 2014). Pigs were fed a grain-based diet which would therefore have been relatively high in fermentable carbohydrate concentrations, and certainly higher than their previous milk diet (Holman et al., 2017). Operational taxonomic units associated with the family *Ruminococaceae* have also been identified as a core microbial component of the GIT, of which, multiple genera of this family increased as age increased (Appendix B.3 and Appendix B.4). *Prevotella* species can produce acetate in the gut, providing a source of energy for butyrate-producing bacteria (Looft et al., 2014b). *Ruminococcaceae* are a butyrate-producing bacteria, often producing butyrate from acetate; an increase in butyrate can decrease inflammation within the GIT and can therefore be beneficial to

host health (Looft et al., 2014a). Increases in both of these bacteria were seen as age increased.

Fewer differences were observed in OTU counts between days 14 and 28, indicating a move towards a more stable composition at this age. *Negativibacillus* previously increased in abundance from day 0 to 14 within the ileum, but between days 14 and 28, its abundance decreased in all GIT locations. Previously, *Negativibacillus* has been seen to significantly increase in human patients with ulcerative colitis, an inflammatory bowel disease, which causes inflammation within the GIT (Gryaznova et al., 2021). This suggests that potentially an increase in inflammation was observed between weaning and day 14, which reduced by day 28. This could be expected given the abrupt changes observed at weaning, frequently leading to ill-health. The increase of butyrate producing bacteria described above, could be associated with the decrease in potential inflammation between days 14 and 28 and the subsequent demise of *Negativibacillus*. Markers of inflammation were not recorded within the current study and therefore validation of this association is not possible. The inclusion of markers of the immune systems inflammatory response in conjunction with microbiome data could be of benefit in future work.

3.4.3 Pharmacological levels of zinc oxide affected bacterial diversity and composition at 14 but not 28 days after weaning

Given initial analysis within this research identified that both GIT location and age of pig had a significant effect on the bacterial composition of their GIT, the effect of pharmacological levels of ZnO was identified at each time point, within each GIT location. This increases confidence in differences observed being directly as a result of ZnO in the diet and shows changes in pigs are still observed when a deliberate pathogenic challenge is not given, supporting previous work (Yu et al., 2017).

In partial support of the primary hypothesis of this research, results presented in this chapter showed ZnO affected bacterial composition of the GIT at day 14. However, few longer-term alterations were identified at day 28, disputing aspects of the primary hypothesis and previous work (Yu et al., 2017). Although neither alpha or beta diversity was affected by ZnO at day 28, the reduction of two genera from the order *Lactobacillales* in ZnO-fed pigs, supports previous work (Starke et al., 2014). However, Starke et al. (2014) saw lasting reductions of three *Lactobacillus* species within the

small intestine, which was further supported by the reduction of lactic acid concentrations within the intestine. They concluded that lasting reductions of certain *Lactobacillus* species could be due to their inability to adapt to high dietary Zn.

Alpha diversity, which looks at measures of species richness and evenness, was not affected by ZnO at either time point; disputing results by Yu et al. (2017) who identified increased richness in the ileum and decreased richness in the colon of pigs receiving ZnO or antibiotics. Zinc oxide primarily affected bacterial composition in the large intestine within the research presented in this chapter, although there was no significant interaction between GIT locations and ZnO provision. This is perhaps surprising, given that the low pH in the stomach transforms insoluble ZnO into free Zn^{2+} ions, which are available within the small intestine, where the absorption of Zn almost exclusively occurs (Starke et al., 2014; Moltedo et al., 2000). A proposed mode of action of ZnO has suggested free Zn^{2+} ions reduces the incidence of diarrhoea in rats by inhibiting the cyclic adenosine monophosphate (cAMP)-induced Cl secretions by the intestinal mucosa, through blocking basolateral membrane potassium channels in the ileum (Hoque et al., 2005). Transepithelial Cl- secretion is an essential transport process to determine intestinal fluid secretions, which is stimulated mostly via the cAMP (Pongkorpsakol et al., 2014).

As the pH along the GIT increases, the majority of remaining Zn is rendered insoluble in the large intestine and little is absorbed, thus it could be expected that fewer differences in bacterial composition would be observed beyond the small intestine, in response to ZnO (Starke et al., 2014). However, in the current research, the only difference observed in bacterial composition in the ileum was the decrease of *Veillonella;* a lactic acid fermenter, and *Intestinibacter*, of which the sole species, *Intestinibacter bartlettii* (previously *Clostridium bartlettii*), has been associated with higher levels in pigs more susceptible to ETEC and appears to be resistant to oxidative stress (Messori et al., 2013; Forslund et al., 2015). This would suggest a potentially beneficial effect within the ileum, although as the pigs used within the current study were not deliberately challenged with ETEC, direct associations to the pigs susceptibility cannot be made.

Although the main effect of ZnO within the GIT was expected within the ileum, differences in colonic and faecal microbiomes have still been reported, indicating fewer

Zn^{2+} can still influence the microbial population (Yu et al., 2017; Pieper et al., 2020; Wei et al., 2020). At day 14, there were decreases in *Treponema* and *Sphaerochaeta* within the family *Spirochaetaceae*, while at day 28, *Treponema* increased within the caecum. The family *Spirochaetaceae* has been associated with improved body weight gain in pigs (Unno et al., 2015). An increase in this family at day 28 could indicate a prolonged, or potentially delayed weight gain improvement in response to ZnO in the current research, although this cannot be confirmed as performance data is not available. Several genera associated with gut dysbiosis and inflammation decreased within the colon of ZnO-fed pigs (*Desulfovibrio* and the family *Prevotellaceae)*, while bacteria associated with the production of butyrate, and therefore linked to the reduction of inflammation, were increased (*Subdoligranulum)* (Panasevich et al., 2017; Looft et al., 2014a). Butyrate has also been reported as the preferred energy source for colonocytes, potentially contributing to optimal growth and nutrient absorption within the colon, thus providing further benefit of this genera in the large intestine (Kubasova et al., 2018).

Enterobacteriaceae is reported to favour conditions during inflammation within the GIT (Zeng et al., 2017); although it must be noted that not all species within this family are pathogenic. Many previous studies have reported an increase of *Enterobacteriaceae* within the GIT of pigs receiving pharmacological levels of ZnO (Vahjen et al., 2011; Yu et al., 2017; Højberg et al., 2005). Yu et al. (2017) specifically reported an increase in non-pathogenic *E. coli* such as K-12, while Slade et al. (2011) identified a reduction of the pathotype ETEC in faeces of pigs fed ZnO. Therefore, the increase in overall *Enterobacteriaceae* is likely to be an increase in diversity within the family, which could promote competition for pathogenic *E. coli*; reducing their abundance and increasing non-pathogenic *E. coli*. This is further supported by the association between *Intestinibacter*, which reduced in the ileum in the present research, and susceptibility to ETEC, that has previously been reported (Messori et al., 2013). The changes observed with ZnO support a shift in the microbiome generally in line with previous research and confer a beneficial effect on the host, although future inclusion of performance data would be beneficial to assess benefits to the pig (Yu et al., 2017; Starke et al., 2014; Messori et al., 2013). The shifts seen also replicate similar effects previously seen with the use of AGPs, such as chlortetracycline, sulfamethazine and penicillin (Gresse et al., 2017; Yu et al., 2017; Looft et al., 2014a).

3.5 Conclusion

The provision of pharmacological levels of ZnO affected beta diversity in all GIT locations of pigs at 14, but not 28 days after weaning, partially accepting the primary hypothesis of this study. This shows that a deliberate pathogenic challenge is not required to identify changes in bacterial composition along the length of the GIT in response to ZnO at day 14, in support of the primary hypothesis of this work. However, contrary to expectations, differences were predominantly observed within the large intestine rather than the small intestine. Nonetheless, differences along the GIT indicate the importance of considering sampling site in future work. Bacterial composition did not differ between the mucosa or lumen of each GIT location, rejecting Hypothesis 3. Although this suggests sample type is less influential on the microbiome of pigs, previous studies have reported differences and interpretation of results should still acknowledge the potential for differences between these environments. Differences were observed as pigs aged, with and without pharmacological levels of ZnO, supporting the importance of identifying an appropriate age point for investigation and/or considering its natural effect of the pigs GIT microbiome. Future work looking at the effect of ZnO on the pigs microbiome should also consider pig performance and assessment of the pigs immune response to determine the health status of pigs used, regardless of a deliberate pathogenic challenge.

Chapter 4

The effects of pre-weaning rearing environment and post-weaning supplementation of zinc oxide on lifetime pig performance

4.1 Introduction

Across the UK pig industry, 40% of the UKs sow herd is maintained outdoors, while the remining 60% are kept indoors (AHDB, 2017). While a large percent of the sow herd is maintained outdoors, the growing and finishing stages of pig production are typically completed within indoor production systems (Edwards, 2005). Indoor environments can be better controlled; however, these systems typically involve the sow being restrained for the duration of the pre-weaning stage in farrowing crates, which is a major animal welfare concern for consumer groups (Baxter et al., 2012). However, farrowing crates are used in many UK and EU indoor-pig farms to achieve optimum piglet survival and performance prior to weaning, whilst sometimes reducing running and labour costs (Baxter et al., 2012). The consumer typically perceives rearing of piglets in an outdoor environment as improved piglet welfare, as a result of increased space and ability to show natural foraging, rooting and explorative behaviours (Pietrosemoli and Tang, 2020). However, pre-weaning mortality in outdoor-reared piglets is often higher due to sow crushing, which can result in fewer piglets weaned and directly affect overall profitability for farmers, as well as being a major welfare concern (Vande Pol et al., 2021; Park et al., 2017; Dawkins, 2017).

Litter size at birth and weaning are reproductive traits that can have a significant economic impact on the profitability of pig production (Camargo et al., 2020). Larger litter sizes can pose challenges, including increased variation in weight within litters (Wülbers-Mindermann et al., 2002). High weight variation can lead to a greater number of piglets with low birth weight, which reduces mobility and the potential for successful suckling (Kobek-Kjeldager et al., 2020). Although pre-weaning mortality is often higher for piglets reared outdoors, these piglets have been show to grow faster post-weaning (Wülbers-Mindermann et al., 2002; Gentry et al., 2004). However, some previous reports have indicated that the increase in weight is associated with an increase in backfat and a decrease in lean meat compared to indoor-reared pigs, which is less desirable for the consumer (Lebret, 2008).

Weaning of pigs is associated with removal from the sow, a change in pen environment and (often) pen-mates, and a change in diet with the withdrawal of the sow's milk and provision of a solid, plant-based diet (Lau et al., 2015). The accumulation of challenges faced often result in a period, immediately after weaning, of low feed intake and poor weight gain, or in some cases, weight loss, diarrhoea and death. Previous research suggests that piglets weaned from outdoor environments cope better with the weaning process than those from indoor environments (Cox and Cooper, 2001; Payne et al., 2003). Miller et al. (2009) identified that piglets reared outdoors wean significantly heavier at four weeks of age, with increased average daily gain (ADG), average daily feed intake (ADFI) and feed conversion compared to indoor-reared pigs.

For conventional, indoor rearing systems, overcoming post-weaning diarrhoea (PWD) and performance losses has been an ongoing area of research. The use of pharmacological levels of ZnO in the diet for 14 days after weaning has been used since the late 1980s and is still permitted for dietary inclusion by EU legislation until 2022. The beneficial effects of ZnO have been widely disputed, with several studies identifying significant improvements in ADG, ADFI and feed conversion immediately after weaning (Poulsen, 1995; Stensland et al., 2015) as well as a reduction in the incidence of PWD (Heo et al., 2010). However, others have not identified performance benefits (Broom et al., 2006; Paschino et al., 2016). Differences seen in response to ZnO could be due to the general health status of the entire farm, with higher health farms potentially seeing less of an improvement compared to lower health herds. Nonetheless, the use of ZnO on UK farms is widespread, with an estimated 70 – 90% of pig starter diets across the UK in 2017 containing ZnO at pharmacological levels (NPA, 2017). Although the benefit of providing ZnO for 14 days after weaning to pigs reared indoors is documented in the literature, the combined effect of both pre-weaning rearing environment and subsequent provision of pharmacological levels of ZnO, on lifetime pig performance to slaughter is less defined. Therefore, the primary aim of this research was to determine lifetime performance benefits of pre-weaning rearing environment and pharmacological levels of ZnO fed for the first two weeks after weaning. Secondary aims of this research were to also identify any performance differences during the pre-weaning stage of production and in the period immediately after weaning, when ZnO was provided.

4.1.1 Primary Hypothesis

1. Rearing pigs outdoors before weaning and providing ZnO for two weeks after weaning will increase pig weights throughout their production life, leading to heavier pigs, that can be sent to slaughter earlier than indoor pigs that received a control diet.

Secondary Hypotheses

2. Rearing piglets outdoors will reduce the number of piglets stillborn at birth, but increase the number of piglet deaths, particularly recorded as being crushed by the sow, between birth and weaning
3. Rearing pigs outdoors prior to weaning will increase ADG, ADFI and feed conversion efficiency (FCE) for the first two weeks after weaning, resulting in heavier pigs through to slaughter
4. The provision of pharmacological levels of ZnO will increase ADG, ADFI and FCE for the first two weeks after weaning and result in heavier pigs through to slaughter

4.2 Materials and methods

4.2.1 Animals and management

Full trial details can be found within Chapter 2. This trial was conducted at the University of Leeds, National Pig Centre in 2019. Twenty-four Large White X Landrace sows and their subsequent litters were allocated to a rearing environment (factor 1; indoor or outdoor) based on: parity, previous litter size (if applicable, >11 piglets), weight and backfat measure at insemination. The indoor rearing environment involved sows farrowing in indoor farrowing crates and pens, while outdoor environments included a larger outdoor farrowing paddock with farrowing ark and feeding crates (see Section 2.2.1).

At farrowing, the number of piglets born alive, stillborn or laid on at birth were recorded for each sow. During the pre-weaning stage of the trial, any additional piglet deaths were recorded and reasons, if known, provided. Cross-fostering was not permitted into indoor litters. Outdoor litters had minimal cross-fostering but where necessary, cross-fostering was permitted within trial litters and recorded. No creep feed was provided to any indoor or outdoor litters. Piglet weights were recorded within 24 hours of birth, when piglets averaged 7 and 21 days of age and on the day of weaning (average age of 25.15 days old). Weights were used to calculate pre-weaning ADG.

At weaning, ten pigs per litter (a total of 235 piglets; 128 indoor vs 107 outdoor-reared; see Chapter 2, Section 2.2.2.1) were selected based on weight and sex, weaned as a group of littermates and split into two groups of five pigs per pen within the indoor commercial facilities, balanced for body weight and sex across the two pens. From weaning, each pen was randomly allocated to the second factor (diet) of this trial. Diets contained either control (~200 ppm) or pharmacological levels of ZnO (~2500 ppm; ZnO diet) as seen in Table 2.4. After day 14, pigs remained on the main trial (for sampling purposes) until day 28, during this time all pigs were fed the same commercial second stage diet (Table 2.4 and 2.5). Hereafter, all pigs were given standard commercial diets and were all transitioned to the new feed at the same time, based on age. Pigs were weighed at days 15, 29 and then every two weeks until slaughter, and feed intake was recorded per pen (see Section 2.3). Average pig weights and pen feed intakes were used to calculate ADG, ADFI and FCE.

4.2.2 Statistical analysis

Full details of statistical analysis can be found in Section 2.4. In brief, pre-weaning data were analysed using a univariate general linear model (GLM) in IBM SPSS Statistics (v.26). Litter was the experimental unit with environment as a fixed factor. Sow parity, batch and average litter size were included as random factors within the model but removed from the model if they have no significant effect. Data that was not normally distributed was analysed using a Kruskal-Wallis non-parametric test. Estimated marginal means are shown with individual SEM given the high variation between groups.

Post-weaning data were analysed based on pen as the experimental unit, with weight at each time point, ADG, ADFI and FCE analysed. Where normality and homogeneity of data were met, data were analysed using a linear mixed model in IBM SPSS Statistics (v.26). The model included environment and treatment as fixed factors with litter of origin as a random factor for weaning until day 29 and then location (room/pen) as a random factor for the grower and finisher stage. Where data did not show normal distribution or displayed heteroscedasticity, a generalised linear model was used. Interactions were also investigated and only included where significant. Responses to treatment or rearing environment were considered significant when $p \leq 0.05$, and trends were noted when $p \leq 0.10$. Data

are expressed as estimated marginal means (EMM) along with their pooled standard error of the mean (SEM).

The number of pigs sent to slaughter at each recorded time point (days 116, 123 and 130 post-weaning) were analysed using a chi-squared test to determine the effect of rearing environment and provision of ZnO on when pigs reached slaughter weight of >105 kg. Data are then presented as number of pigs from the following groups: indoor control-fed pigs, indoor ZnO-fed pigs, outdoor control-fed pigs, and outdoor ZnO-fed pigs. Percentages shown are based on the number of pigs from each of the four groups out of the total number of pigs at slaughter weight at each time point.

4.3 Results

4.3.1 Pre-weaning performance

Rearing environment had no effect on the average number of piglets born alive and their birth weight (Table 4.1). Although the number of piglets stillborn was higher in indoor-reared litters than those outdoors, the difference was not significant (p=0.226). Similarly, although the number of piglets recorded as being laid on by the sow was numerically higher for outdoor reared piglets, this was not significant (p= 0.617). There was a tendency for outdoor reared litters to have a higher number of piglet deaths between birth and weaning (p=0.060) and subsequently there was a tendency for indoor sows to wean a higher number of pigs per litter (p=0.099). Weight of piglets before weaning did not significantly differ at any time point from birth to weaning, neither did total ADG from birth until weaning.

Table 4.1 Results of statistical analysis on pre-weaning data, comparing indoor and outdoor rearing environments, showing EMM. Average numbers per litter shown for born alive, stillborn, laid on and died birth to weaning.

	Environment			
	Indoor	**Outdoor**	**Test statistic$_{df}$**	**p values**
Born Alive[1]	15.74±0.96	15.56±1.11	$0.019_{1,17}$	0.893
Stillborn[1]	0.75±0.34	0.23±0.39	$1.468_{1,23}$	0.226
Laid on[1]	0.78±0.36	1.21±0.42	$0.250_{1,23}$	0.617
Number of piglet deaths birth-wean[2]	2.09±0.66 (13%)	3.87±0.77 (20%)	$4.077_{1,17}$	*0.060*
Numbers weaned[3]	11.84±0.34	11.01±0.37	$2.727_{1,21}$	*0.099*
Average pig weight, kg				
Birth[4]	1.52±0.06	1.45±0.07	$0.761_{1,20}$	0.393
Day 7[5]	2.62±0.10	2.72±0.11	$0.482_{1,20}$	0.495
Day 21[5]	5.92±0.18	6.15±0.19	$0.822_{1,20}$	0.375
Weaning[5]	7.09±0.26	7.62±0.27	$2.046_{1,20}$	0.168
ADG Birth-Wean[5]	0.20±0.01	0.22±0.01	$2.130_{1,20}$	0.160

[1] Non-parametric Kruskal Wallis test used, H test statistic shown
[2] Univariate general linear model accounting for sow parity and batch with numbers born alive as covariate
[3] Generalised linear model accounting for numbers born alive, showing Wald Chi-square test statistic.
[4] Univariate general linear model, based on litter weight before cross fostering
[5] Univariate general linear model accounting for batch with birth weight (after cross fostering) as covariate

4.3.2 Post-weaning performance

During the immediate post-weaning phase of production, a significant interaction was identified between pre-weaning rearing environment and the provision of ZnO on ADFI from weaning until day 15 after weaning ($p = 0.048$; Table 4.2). This interaction showed that the provision of pharmacological levels of ZnO was necessary to improve feed intake of pigs reared indoors (0.16 vs 0.22 kg for indoor control and ZnO fed pigs, respectively) but not those reared outdoors (0.30 kg for both treatments outdoors). An interactive effect was also seen for FCE during the first two weeks after weaning ($p = 0.007$), with ZnO and outdoor rearing

of pigs increasing FCE compared to indoor control-fed pigs. Indoor, ZnO-fed pigs had a similar FCE to outdoor-control fed pigs and outdoor-ZnO fed pigs had the highest FCE. Although rearing environment and post-weaning diet had an interactive effect on ADFI and FCE, there was only a tendency towards an interactive effect on ADG and weight at day 15. Nonetheless, as main effects, environment and diet significantly affected ADG between weaning and day 15 and subsequently weight of pigs at day 15. Specifically, outdoor-reared pigs had higher ADG and a higher weight at day 15 compared to indoor-reared pigs (12.19 vs 10.02 kg ± 0.21 respectively, $p < 0.001$), and the provision of pharmacological levels of ZnO increased ADG and weight of pigs at day 15 compared to control-fed pigs (11.7 vs 10.49 kg ± 0.19, $p < 0.001$).

After day 15, when all pigs received the same commercial feed for a further 14 days, ADFI was no longer affected by either rearing environment or earlier dietary treatment. However, ADG was still increased for pigs reared outdoors compared to those reared indoors ($p = 0.002$) but was no longer affected by dietary treatment ($p = 0.782$). The average weight of outdoor pigs at day 29 was also increased compared to indoor-reared pigs (20.15 vs 16.78 kg ± 0.43 respectively, $p = <0.001$). In addition, the earlier provision of ZnO increased piglet weight compared to control fed pigs at day 29 (19.03 vs 17.90 kg ± 0.38, $p = 0.026$). Although improvements in weight were seen, FCE was not affected by either rearing environment or dietary treatment between days 15 and 29 (Table 4.2).

The number of pigs that died during the entire post-weaning phase were not different between groups; eight pigs died from indoor control, outdoor control and outdoor ZnO treatment pens and nine from indoor ZnO pens.

Table 4.2 Results of a linear mixed model comparing environment and treatment on performance parameters for the first 29 days post-weaning. Values shown are EMM with pooled SEM and are shown in kg.

Rearing Env.	**Indoor**		**Outdoor**			**Test Statistic$_{df}$[1]**			**p values**		
Treatment	Con	ZnO	Con	ZnO	SEM	*Environment*	*Treatment*	*Environment *Treatment*	*Environment*	*Treatment*	*Environment *Treatment*
Weaning Weight[2,3]	7.75	7.79	7.95	7.99	0.25	0.511	0.018	n/a	0.475	0.894	n/s
D15 Weight[4,5]	9.14	10.88	11.82	12.56	0.27	$53.208_{1,29}$	$25.493_{1,20}$	$4.100_{1,20}$	***<0.001***	***<0.001***	*0.057*
D29 Weight[4]	16.22	17.34	19.59	20.71	0.49	$31.178_{1,19}$	$5.728_{1,21}$	n/a	***<0.001***	***0.026***	n/s
ADG 0-15[5]	0.09	0.20	0.26	0.32	0.02	$55.642_{1,20}$	$31.183_{1,20}$	$4.003_{1,20}$	***<0.001***	***<0.001***	*0.059*
ADG 16-29[3]	0.57	0.56	0.68	0.67	0.29	9.741	0.077	n/a	**0.002**	0.782	n/s
ADFI 0-15[5]	0.16^{a}	0.22^{b}	0.30^{c}	0.30^{c}	0.01	$41.373_{1,20}$	$6.735_{1,20}$	$4.446_{1,20}$	***<0.001***	***0.017***	***0.048***
ADFI 16-29	0.80	0.85	0.95	0.99	0.09	$1.584_{1,20}$	$1.577_{1,21}$	n/a	0.223	0.223	n/s
FCE 0-15[3,5]	0.49^{a}	0.91^{bc}	0.89^{b}	1.06^{c}	0.05	36.720	42.516	7.152	***<0.001***	***<0.001***	**0.007**
FCE 16-29[3]	0.72	0.67	0.70	0.65	0.06	0.074	0.579	n/a	0.785	0.447	n/s

[1] F test statistic shown unless a generalised linear model used whereby the Wald Chi-Square test is shown
[2]Weight based on pigs selected to go onto the post-weaning trial, not entire litter as seen in Table 4.1.
[3]Data shown based on Generalised Linear Model output and Wald Chi-Square test statistic
[4]Weaning weight included as a covariate
[5] Environment X Treatment interaction included within the model
Rows with different superscripts indicate significant differences within the interaction.
NOTE: Weaning weight differs compared to Table 4.1 as average weights presented in this table are based on the pigs selected to go onto the post-weaning trial, compared to all pigs in the pre-weaning environment shown in Table 4.1.

4.3.3 Grower and finisher performance

Throughout the grower phase neither ADFI, ADG or FCE were affected by rearing environment or earlier provision of pharmacological levels of ZnO, between days 30 and 43 and days 44 to 57 (Table 4.3), as well as during the entire grower period (days 30 to 57; Table 4.5). However, at both days 43 and 57, rearing environment significantly affected average weight of pigs, with outdoor-reared pigs maintaining heavier weights compared to indoor-reared pigs. Furthermore, the earlier provision of ZnO tended to improve average weight at day 43, but this was not maintained at any subsequent time (Table 4.3). During the complete grower phase (days 30 to 57; Table 4.5), there were no overall significant interactions between rearing environment or diet on ADG, ADFI and FCE.

During the finisher stage, from day 57, average weight at each time point continued to be affected by rearing environment, with outdoor-reared pigs being heavier than those reared indoors and had more pigs reach slaughter weight (~105 kg) sooner, compared to indoor-reared pigs (Table 4.4). For the first two weeks of the finisher period, between days 58 and 71, there was a tendency for outdoor-reared pigs to have higher ADFI. This became a significant increase in ADFI between days 72 and 85 as well as days 86 to 99 for outdoor-reared pigs (days 72 to 85: 2.64 vs 2.40 kg ± 0.06 respectively, $p = 0.009$; days 86 to 99: 2.95 vs 2.72 kg ± 0.07 respectively, $p = 0.021$). Although ADFI was affected by rearing environment, it was not impacted by earlier dietary treatment and neither ADG or FCE were affected by rearing environment or dietary treatment during these time points (Table 4.4). During the entire finisher period (days 57 to 116; Table 4.5), outdoor-reared pigs ate more than those reared indoors (2.74 vs 2.49 kg ± 0.06 respectively, $p = 0.009$). However, as there were no significant differences seen in ADG during this time, outdoor-reared pigs had a lower FCE than indoor-reared pigs (0.38 vs 0.40 kg ± 0.01 respectively, $p = 0.002$). Treatment did not affect any performance parameters during the finisher period.

Although carcass characteristics were not recorded within this study, the percentage of pigs that reached a slaughter weight >105 kg was recorded. The first group of pigs sent to slaughter was on day 116 post-weaning, followed by days 123 and 130. Overall, at day 116 there was a tendency towards a higher number of outdoor-reared pigs to have reached slaughter weight ($p = 0.076$; Table 4.5). The earlier provision of ZnO had no effect on the number of pigs that

reached slaughter weight at the first time point ($p = 0.550$). At days 123 and 130, neither rearing environment or the earlier provision of ZnO effected the number of pigs that were at a slaughter weight of >105 kg ($p>0.05$). Of the pigs that were sent to slaughter at days 116, 123 and 132, the percentage of each treatment group is shown in Figure 4.1. Feed cost per pig within each treatment group was also looked at from weaning until day 116 after weaning, based on the cost of feed at the time of running the trial (2019). This was calculated based on the average feed intake per group and showed that indoor control-fed pigs cost, on average, £45.83 to feed, indoor ZnO-fed pigs cost £50.17, outdoor control-fed pigs cost £54.69 and outdoor ZnO fed pig cost £53.41.

Table 4.3 Results of linear mixed model run to determine the effect of both environment and treatment on performance parameters during the grower phase (days 30 to 57). Values shown in kg.

Rearing Environment	**Indoor**		**Outdoor**			**Test Statistic$_{df}$[1]**		**p values[2]**	
Treatment	Con	ZnO	Con	ZnO	SEM	*Environment*	*Treatment*	*Environment*	*Treatment*
Day 43 Weight[3]	25.53	27.99	29.99	30.59	0.75	16.542	3.101	***0.001***	*0.078*
Day 57 Weight	35.47	38.40	40.80	41.49	0.97	$14.106_{1,21}$	$2.604_{1,21}$	***0.001***	0.122
ADG 30-43	0.71	0.74	0.70	0.73	0.03	$0.028_{1,21}$	$0.841_{1,21}$	0.868	0.370
ADG 44-57	0.71	0.74	0.77	0.78	0.03	$1.757_{1,21}$	$0.297_{1,21}$	0.199	0.592
ADFI 30-43	0.93	0.98	0.92	0.91	0.05	$0.635_{1,21}$	$0.115_{1,21}$	0.434	0.738
ADFI 44-57	1.36	1.44	1.43	1.44	0.06	$0.301_{1,21}$	$0.428_{1,21}$	0.589	0.520
FCE 30-43	0.76	0.77	0.80	0.80	0.04	$0.595_{1,21}$	$0.021_{1,21}$	0.449	0.886
FCE 44-57	0.52	0.52	0.55	0.55	0.03	$0.933_{1,21}$	$0.003_{1,21}$	0.345	0.958

[1] F test statistic shown unless data is based on a Generalised Linear model whereby a Wald Chi-Square test is show
[2]All models excluded interaction from the model as it was consistently not significant
[3]Data shown based on Generalised Linear Model output and Wald Chi-Square test statistic

Table 4.4 Results of linear mixed model to identify effect of environment and treatment on performance parameters during the finisher stage (days 71 to 116). Values shown in kg.

Rearing Environment	**Indoor**		**Outdoor**		**SEM**	**Test Statistic$_{df}$[1]**		**p values[2]**	
Treatment	Con	ZnO	Con	ZnO		*Environment*	*Treatment*	*Environment*	*Treatment*
Day 71 Weight	48.95	51.10	54.04	54.77	1.26	$9.011_{1,21}$	$0.971_{1,21}$	***0.007***	0.336
Day 85 Weight	63.28	66.67	69.60	70.29	1.25	$11.815_{1,21}$	$1.984_{1,21}$	***0.002***	0.174
Day 99 Weight	76.99	79.86	84.08	84.00	1.33	$13.289_{1,21}$	$0.819_{1,21}$	***0.002***	0.376
Day 116 Weight	93.89	96.96	101.65	101.02	1.95	$6.916_{1,21}$	$0.296_{1,21}$	***0.016***	0.592
ADG 58-71	0.94	0.94	0.95	0.95	0.04	$0.092_{1,21}$	$0.000_{1,21}$	0.765	0.992
ADG 72-85	1.03	1.11	1.12	1.12	0.03	$2.782_{1,21}$	$1.882_{1,21}$	0.110	0.185
ADG 86-99	0.98	0.94	1.03	0.98	0.04	$1.266_{1,21}$	$1,257_{1,21}$	0.273	0.275
ADG 100-116	0.99	1.01	1.06	1.00	0.05	$0.239_{1,21}$	$0.132_{1,21}$	0.630	0.720
ADFI 58-71	1.81	1.95	2.03	1.98	0.06	$3.768_{1,21}$	$0.491_{1,21}$	*0.066*	0.491
ADFI 72-85	2.33	2.48	2.69	2.60	0.07	$8.187_{1,21}$	$0.156_{1,21}$	***0.009***	0.697
ADFI 86-99	2.70	2.75	3.08	2.82	0.08	$6.246_{1,21}$	$1.331_{1,21}$	***0.021***	0.262
ADFI 100-116	2.87	2.89	3.21	3.08	0.14	$2.617_{1,21}$	$0.131_{1,21}$	0.121	0.721
FCE 58-71	0.50	0.49	0.48	0.47	0.02	$1.637_{1,21}$	$0.517_{1,21}$	0.215	0.480
FCE 72-85	0.44	0.45	0.42	0.43	0.01	$2.691_{1,21}$	$0.834_{1,21}$	0.116	0.372
FCE 86-99	0.36	0.35	0.35	0.34	0.01	$0.514_{1,21}$	$0.142_{1,21}$	0.481	0.710
FCE 100-116	0.35	0.35	0.33	0.33	0.01	$3.881_{1,21}$	$0.015_{1,21}$	*0.062*	0.905

[1] Test statistic is F test.
[2]All models excluded interaction from the model as it was consistently not significant

Table 4.5 Summary of linear mixed model results for performance parameters during the entire grower (days 30 to 57) and finisher (days 57 to 116) periods. Values shown in kg.

Rearing Environment	**Indoor**		**Outdoor**			**Test statistic$_{df}$[1]**		**p value[2]**	
Treatment	Con	ZnO	Con	ZnO	SEM	*Environment*	*Treatment*	*Environment*	*Treatment*
ADG 30-57[3]	0.96	0.98	0.98	1.00	0.12	0.019	0.020	0.890	0.887
ADFI 30-57[3]	1.87	1.98	1.91	1.91	0.16	0.007	0.094	0.933	0.760
FCE 30-57[3]	0.59	0.59	0.60	0.60	0.13	0.003	0.002	0.955	0.964
ADG 57-116	1.00	1.00	1.04	1.01	0.03	$0.795_{1,21}$	$0.147_{1,21}$	0.383	0.706
ADFI 57-116	2.44	2.54	2.84	2.65	0.12	$8.147_{1,21}$	$0.340_{1,21}$	***0.009***	0.566
FCE 57-116	0.40	0.40	0.38	0.38	0.01	$5.173_{1,21}$	$0.002_{1,21}$	***0.034***	0.964
Number pigs >105 kg, day 116[4]	6 (13%)	12 (26%)	15 (38%)	20 (45%)		$11.429_{6,24}$	$4.952_{6,24}$	*0.076*	0.550

[1] Test statistic is F test unless a generalised linear model has been used whereby the test statistic shown is the Wald Chi-Square.
[2]All models exclude interaction as it was consistently not significant
[3]Data shown based on Generalised Linear Model output and Wald Chi-Square test statistic
[4]Data shown is number of pigs sent to slaughter and the percentage of each treatment group bracket. Analysis based on Chi-Squared test.

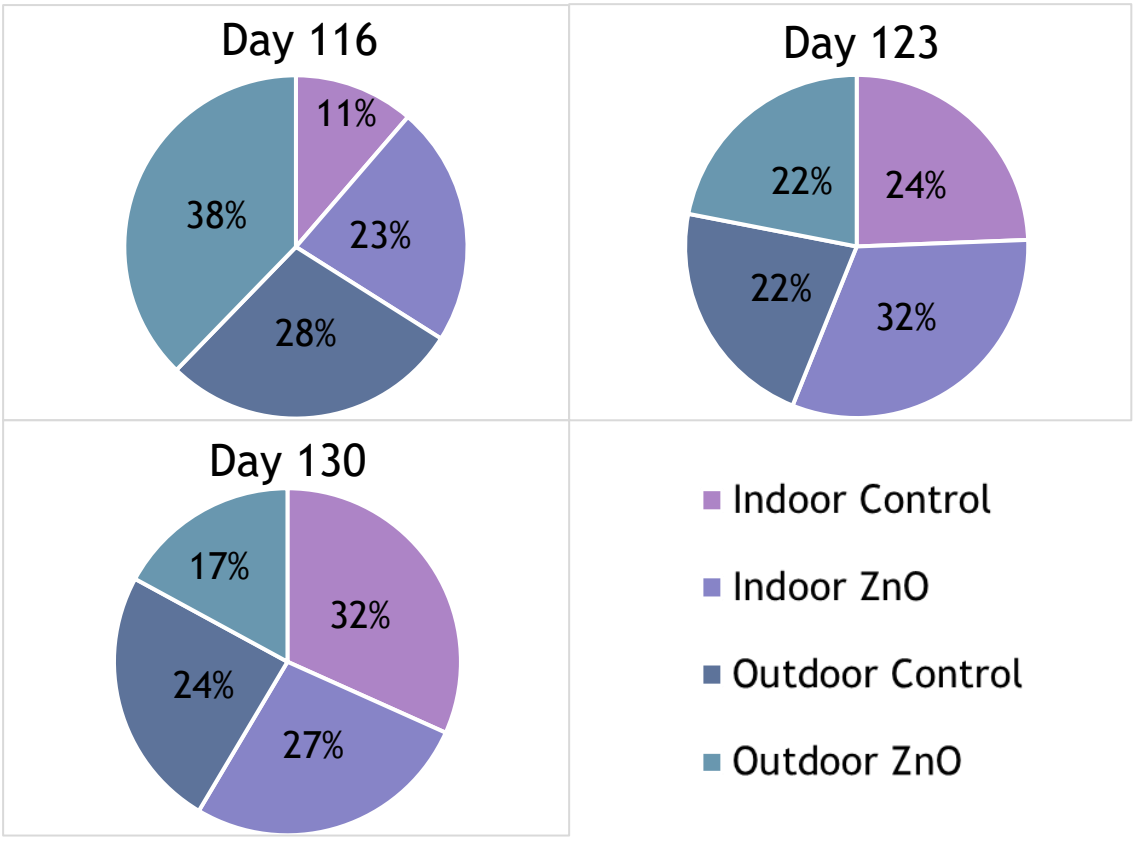

Figure 4.1 Percentage of pigs from each treatment group that went to slaughter at each time point from: indoor control, indoor ZnO, outdoor control and outdoor ZnO-fed at days 116, 123 and 130 post-weaning. Note: Different percentages to Table 4.5 as these are percentages of just the pigs sent to slaughter, not percentage of all pigs within each treatment group as shown in the table.

4.4 Discussion

The overall aim of this chapter was to identify whether there were lifetime performance benefits of rearing piglets indoors or outdoors before weaning and whether the provision of pharmacological levels of ZnO would improve pig performance of both indoor- and outdoor-reared pigs after weaning. The primary hypothesis of this work stated that pigs reared outdoors and provided pharmacological levels of ZnO would show lifetime performance improvements, enabling them to reach slaughter weight sooner than indoor, control-fed pigs. In partial support of this hypothesis, rearing of pigs outdoors prior to weaning resulted in significantly heavier pigs at every weigh point from weaning until day 116 after weaning, when the first group of pigs reached 105 kg, at which they could be sent to slaughter. As a result of being heavier throughout the post-weaning period, a higher percentage of outdoor pigs were sent to slaughter at day 116 post-weaning compared to pigs that had been reared indoors, reducing their overall time within the production system. Rearing environment had more long-lasting performance benefits compared to the provision of ZnO. However, the cost of feed per pig, per treatment, up to day 116 showed that outdoor reared pigs were more expensive to feed, which is likely as a result of the sudden increase seen in ADFI between days 72 and 99 after weaning. This may negate benefits of being able to send more outdoor pigs to slaughter at an earlier age. To identify lifetime performance differences between pigs, it was imperative to identify whether rearing environment affected piglets' weight, numbers born and mortality rates before weaning, as part of the secondary aims of this research, given that varying number of pigs through the system could further outweigh any benefit of increased weight. Furthermore, the effect of rearing environment and dietary treatment on performance of pigs immediately after weaning, when the biggest growth-check often occurs was also essential, before assessing grower and finisher performance differences.

4.4.1 The effect of rearing environment on litter size, weight and mortality before weaning

In accordance with previous research by Miller et al. (2009), there was no difference in the number of piglets born alive across both rearing environments. This is unsurprising given the sow and boar genetic lines used across both environments were the same. Sows across both environments were also

balanced for parity, which can cause discrepancies in litter size, with first parity, or high parity sows often having a lower number of piglets born alive (Schild et al., 2020).

The current study hypothesised that indoor sows would have a higher number of stillborn piglets compared to those farrowing outdoors, but that piglet mortality, including crushing by the sow, would be higher for outdoor litters than those indoors. Although there was a pattern for increased stillborn pigs indoors, this was not significant, partially rejecting Hypothesis 2. However, there was a tendency for outdoor reared pigs to have a higher mortality, although not specifically recorded as being crushed by the sow, accepting aspects of one of the secondary hypotheses of this study (Hypothesis 2). Previous research has shown increases in the number of piglets stillborn for indoor versus outdoor-reared (KilBride et al., 2012). However, this could be because of increased risk of dead piglets disappearing outdoors- either from predation, being eaten by the sow or getting lost within the straw bedding. Although piglets were checked at least once daily during the trial presented, all these factors cannot be completely discounted.

There is a general agreement that crushing of piglets by the sow is the leading cause of pre-weaning mortality around the world, regardless of the use of farrowing crates for indoor sows (Koketsu et al., 2006; Vaillancourt et al., 1990; Muns et al., 2016; KilBride et al., 2012). However, some previous work has identified a greater risk of crushing in piglets reared in outdoors or non-crated systems (Cronin et al., 2000; Weber et al., 2007). In the current study, 38% of all live-born piglet deaths were recorded due to sow crushing, in both environments. Although in most cases the symptoms of piglets dying as a result of crushing are apparent, there may be some deaths recorded as unknown that were still due to this cause. Furthermore, as with the recording of stillborn piglets, accurate recording of any piglet death in an outdoor environment is harder to manage, given the increased difficulty and risk of accessing a litter frequently. Mortality rates from birth to weaning of piglets reared indoors were in line with the UK average at the time of conducting the experiment (12.2%) (AHDB, 2021b). However, there was a tendency for more pigs to die when reared outdoors (20%), which is higher than the UK average. Factors specific to outdoor rearing of litters that could cause an increase in overall mortality before weaning include an

increased exposure to predators and pests, increasing the likelihood of disease in piglets (KilBride et al., 2012). An increase in piglet mortality is a major cause of economic loss at farm level as well as being a major welfare concern across the industry (Mellor and Stafford, 2004). This is often conflicted by the perceived improved welfare of pigs reared outdoors, where both piglets and sows are able to express more of their natural behaviours (KilBride et al., 2014). It is likely that this tendency towards an increased mortality per litter in outdoor reared litters is linked to the tendency for a reduced number of pigs weaned per litter in outdoor rearing, which is also an important economic factor to consider. Economic factors associated with mortality could be partially overcome with improved growth performance and feed efficiency of the piglets that do survive through to weaning.

4.4.1.1 Piglet weights before weaning

There was no significant difference in average piglet weight between indoor and outdoor rearing environments at any time point during the pre-weaning stage, disagreeing with expectations that outdoor-reared piglets would be heavier. Weaning is arguably the most critical stage in a production pig's life as they face multiple physiological and social challenges that can result in subsequent disease and production losses (Campbell et al., 2013). The findings from previous research into the effect of rearing environment on piglet performance has been varied, with some studies finding no significant effect of environment on weaning weight (Slade et al., 2011; Carroll et al., 2007). On the other hand, other studies have identified significantly heavier piglets outdoors compared to those indoors (Gentry et al., 2002; Payne et al., 2003). Discrepancies in studies looking at pre-weaning performance could be as a result of differing indoor rearing facilities, as facilities with highly accurate control of lighting, ventilation and temperature would be different to indoor facilities that do not have the capacity for such accurate controls, which could be a disadvantage of the current study. However Miller et al. (2009), who conducted their research at the same location as the current study, did find significantly heavier pigs in outdoor environments.

4.4.2 The interactive effect of rearing environment and pharmacological levels of zinc oxide on pig performance for 14 days after weaning

In support of the secondary hypotheses (Hypothesis 3 and 4), both rearing environment and the provision of ZnO improved pig performance immediately

after weaning. Results showed a significant interaction between rearing environment and dietary treatment on ADFI and FCE between weaning and day 15 ($p = 0.048$ and $p = 0.007$, respectively; Table 4.2). This interaction showed that ZnO improved ADFI for indoor-, but not outdoor-reared pigs. Furthermore, both ZnO and outdoor rearing improved FCE, with indoor-ZnO fed pigs and outdoor-control fed pigs having a similar benefit. The provision of ZnO did not improve outdoor-reared piglets ADFI from weaning to day 15. Even though creep feed was not provided to either environments before weaning, it is likely that outdoor pigs were naturally exposed to more complex nutrients, such as carbohydrates and fibre within the soil, as well as microbes, thus better preparing them for the more complex diet provided after weaning (Vo et al., 2017). This could explain why outdoor-reared pigs ate significantly more after weaning, regardless of the provision of ZnO. Lau et al. (2015) found that piglets raised outdoors spent more time eating when presented with solid feed for the first time at weaning than indoor-reared piglets, who spent more time exploring their new environment. This could be as a result of increased social learning from the sow, in outdoor piglets, as a result of increased stimulus and ability to express these behaviours (Lau et al., 2015; Cox and Cooper, 2001). However, previous work carried out at the University of Leeds revealed no effect of rearing environment on ADG after challenge with ETEC K88, a strain of ETEC that causes PWD, and found that ZnO improved outdoor pigs ADG more than indoor-reared pigs (Sargeant et al., 2010). These results oppose those presented in this chapter, however the indoor pigs used for Sargeant et al. (2010) received creep feed which is likely to have improved uptake of feed after weaning for indoor-reared pigs.

Although the current study showed that ZnO was not necessary to improve outdoor pig ADFI after weaning, for indoor-reared piglets, the provision of ZnO was necessary to increase feed intake. However, this improvement was not to the levels seen for all outdoor-reared pigs. Nonetheless, this significant improvement in ADFI with ZnO is likely to have resulted in the significant increase also seen in ADG and FCE for the provision of ZnO during this period. Enabling pigs to eat sooner after weaning is critical; Lalles et al. (2007) found typically only 50% of piglets consumed their first meal within 24 hours after weaning, and 10% don’t eat until 48 hours later. This delay in eating puts these pigs at a significant

disadvantage in adapting to the post-weaning environment and maintaining overall health. Although ADFI was significantly different for treatments, with indoor, control-fed pigs eating significantly less, mortality rates were not significantly affected during the entire phase, meaning that although these piglets did not eat as much, this did not result in ill-health and mortalities. At day 15, outdoor-reared pigs given ZnO post-weaning were heavier than all other groups of pigs, indicating a combined benefit of both, whilst control-fed pigs housed indoors were lighter compared to all other combinations.

4.4.3 The effect of pre-weaning environment on pig weight, ADG, ADFI and FCE for the first 29 days after weaning

Average daily gain was significantly improved for the first four weeks after weaning when pigs were reared outdoors. These results support previous work and enable one of the secondary hypotheses (Hypothesis 3) to be accepted (Miller et al., 2009; Gentry et al., 2004; Lebret et al., 2006). Given weaning weights were balanced prior to going onto the post-weaning stage of the trial (Table 4.2), the improvement in post-weaning performance of outdoor-reared pigs suggests that they are more resilient to the challenges faced at weaning than indoor-reared pigs. On the one hand, this is surprising as the change in environment for outdoor-reared pigs moving to indoor accommodation after weaning is more drastic than the change for indoor-reared pigs moving from one indoor room to another. However, outdoor-reared piglets exhibit more explorative behaviours, such as rooting and grazing and spend more time away from the sow and less time suckling, compared to piglets reared indoors (Hötzel et al., 2004). These different experiences prior to weaning teach piglets behavioural strategies to overcome environmental challenges, which benefit them post-weaning (Cox and Cooper, 2001).

4.4.4 The effect of pharmacological levels of zinc oxide on pig weight, ADG, ADFI and FCE in the first 29 days after weaning

For conventional, indoor-reared pigs, the provision of pharmacological levels of ZnO after weaning has been widely used for many years as previous research has shown significant improvements in post-weaning performance and reduced incidence of diarrhoea (Sales, 2013; Heo et al., 2010; Slade et al., 2011). In support of this previous research and enabling acceptance of Hypothesis 4,

results presented in this chapter also show benefits of ZnO, particularly to indoor-reared pigs, whereby increased ADFI and FCE were seen within the first two weeks after weaning. Further supporting previous research, these findings also showed improved ADG for both indoor- and outdoor-reared pigs that received ZnO. Slade et al. (2011) also investigated the effect of rearing environment and ZnO and found a significant increase in ADFI, ADG and gain:feed ratio when pigs received ZnO for seven days after weaning but found no effect of rearing environment. Furthermore, their results also showed an interactive effect of environment and ZnO on ADG, with ZnO increasing ADG for outdoor but not indoor-reared pigs, although these results were based on the pigs' response to a deliberate challenge with enterotoxigenic *Escherichia coli* (ETEC), a common cause of PWD in pigs (Slade et al., 2011). Beyond day 15 there was no benefit of the earlier provision of ZnO on ADFI, ADG or FCE, however average pig weights at day 29 were still heavier compared to control-fed pigs.

4.4.5 The effect of rearing environment and pharmacological levels of zinc oxide on grower and finisher pig performance

The primary hypothesis of this experiment was that rearing pigs outdoors and subsequently providing pharmacological levels of ZnO would provide lifetime performance improvements. As a result, it was expected that grower and finisher performance would be improved by these factors. When looking at the average weight of pigs at each time point through the grower (days 30 to 57; Table 4.3) and finisher (days 58 to 116; Table 4.4) phases, pigs reared outdoors were consistently heavier at every weight point. Evidently, rearing pigs outdoors before weaning, has lifetime benefits on performance and produces heavier pigs at an earlier age, thus potentially reducing feed and management costs and increasing turnover of pigs. These findings support the work by Gentry et al. (2004) and Gentry et al. (2002) in which both studies found pigs reared outdoors were significantly heavier at 28, 56 and 112 days after weaning compared to indoor-reared pigs, albeit with different genetics.

Average daily feed intake, ADG and FCE were not significantly affected by rearing environment during the entire grower phase (Table 4.3 and Table 4.5) differing from previous results (Gentry et al., 2002; Payne et al., 2003). Results presented in this chapter indicated that outdoor-reared pigs gained weight faster during the stage immediately post-weaning and thereafter grew at the same rate

as indoor-reared pigs, but due to already being heavier, they remained heavier through to slaughter. At the start of the grower phase, pigs were mixed from pens of five to ten for management purposes. Mixing of pigs has been shown to increase stress, often seen initially at weaning, which may have introduced some aggressive behaviours as pigs re-established a new hierarchy (Colson et al., 2012; Hötzel et al., 2011). This may be the reason results were not significant, although this would potentially have only affected the first two weeks after mixing and was applied to all pigs on trial.

During the finisher stage (days 57 to 116; Table 4.4), as previously mentioned, average weight of outdoor pigs was consistently heavier than indoor pigs at each time point. In the first two weeks in the finisher accommodation, ADFI showed a tendency to be increased in outdoor-reared pigs, with significant improvements then being seen between days 72 and 85 and days 86 to 99. This is in contrast to the Gentry et al. (2002) study, who found an improvement in ADG but not in ADFI during the grower/finisher period when pigs had been reared outdoors. Although ADFI was increased for outdoor-reared pigs during the majority of the finisher stage, ADG and FCE data did not improve, indicating the increased feed did not result in an increase in weight, which is arguably detrimental to farmers as a result of increase feed cost with no subsequent increase in weight. Furthermore, this increase in feed intake is the likely cause of an increase in average feed cost per outdoor reared pig compared to those reared indoors. However, in support of the lifetime benefit of rearing pigs outdoors; a higher percentage of pigs sent to slaughter at day 116 were from an outdoor environment, thus reducing their time within the production system, compared to indoor reared pigs. Given this, the additional feed provided to more indoor-reared pigs after day 116 could balance out feed costs between environments. Nonetheless, it is not clear whether the increase in weight was a direct increase in lean muscle or fat, as it was not possible to measure carcass weight and quality within the current research; this is something that would be beneficial to consider in future research within this area.

There were no long-lasting benefits of providing pharmacological levels of ZnO earlier in the production system. These findings support previous work that only saw significant improvements immediately after weaning rather than through to the grower (Milani et al., 2017) or finisher stage (Broom et al., 2003).

4.4.6 Could rearing piglets outdoors replace the use of zinc oxide supplementation after weaning?

Given that outdoor-reared pigs were consistently heavier at each weigh point from weaning to slaughter, regardless of diet, and pharmacological levels of ZnO only affected pig performance immediately after weaning, rearing of piglets outdoors could be an alternative to providing ZnO. Although not significant, pre-weaning mortality of outdoor reared pigs tended to be higher, resulting in fewer pigs weaned per sow, which is something that would need to be considered as this can have a considerable impact, commercially. Furthermore, the expectation that all farmers could move to an outdoor rearing system is, perhaps, unrealistic given the increased space requirement of outdoor production and the initial cost to shift production systems.

The improved growth performance of outdoor-reared pigs within this trial resulted in a higher percentage of outdoor-reared pigs reaching slaughter weight (> 105 kg) at day 116. Reducing time to slaughter can decrease the relative environmental impact per 1 kg of live pig (Ottosen et al., 2021) as well as potentially equating to a cost-saving for the farmer. However, the increased ADFI for outdoor-reared pigs during the finisher period of this trial, without a subsequent increase in ADG and FCE could reduce this potential cost saving. Nevertheless, sending pigs to slaughter sooner reduces time within the production system, which could result in higher numbers produced per year. Increasing the number of pigs produced per year is essential for the pork industry to attempt to keep pace with the growing human population and subsequent demand for pork. Given the upcoming ban of ZnO across the EU by 2022, rearing piglets outdoors is an option that should be considered further, given the lifetime benefits observed. To further this research, identifying differences in the health of these pigs, including their GIT microbiome, could prove beneficial.

4.5 Conclusion

Rearing piglets in an indoor or outdoor environment, did not significantly alter the number of piglets born or their weight from birth to weaning. However, the tendency for increased mortality of outdoor litters, leading to the reduced number of piglets weaned outdoors is an important economic factor that needs to be considered. Immediately after weaning, the provision of ZnO was effective at

improving ADFI of indoor-reared, but not outdoor-reared pigs and showed improved FCE for pigs reared in both environments. However, after day 15 there were no long-lasting benefits of providing pharmacological levels of ZnO on ADG, ADFI and FCE. Conversely, outdoor reared pigs had improved ADG for the first month after weaning, which resulted in significantly heavier pigs at every weigh-point until day 116. At day 116, a higher percentage of pigs that reached slaughter weight of 105 kg had been reared in an outdoor environment, reducing their time within the production system. However, an increase in ADFI of outdoor-reared pigs within the finisher stage led to an increase in overall feed costs compared to indoor-reared pigs, which may negate the cost benefit of sending these pigs to slaughter sooner. Nonetheless, rearing pigs outdoors before weaning shows benefits to the weight of pigs and warrants further investigation as a potential alternative strategy to the provision of ZnO, which in the current study did not show lifetime performance benefits. Although, it could be unrealistic to expect all pigs in the UK to be reared outside given the cost to farmers to source and set-up land to rear pigs outdoors.

Chapter 5
The effect on rearing environment and dietary zinc oxide on the gastrointestinal tract microbiome and immune markers of pigs 14 days post-weaning

5.1 Introduction

Rearing pigs in an outdoor environment prior to weaning can improve pig performance during the post-weaning stage of production, as seen in Chapter 4 of this thesis and previous research (Cox and Cooper, 2001; Miller et al., 2009). It is possible that the improvements seen in performance of pigs reared outdoors are as a result of being exposed to nutrients and microbes in the soil, pasture and straw, that are not accessible to indoor-reared pigs (Vo et al., 2017). This exposure to nutrients and microbes from the outdoor environment is likely to alter the bacterial composition of the gastrointestinal tract (GIT), compared to indoor-reared pigs, from an early age. A pigs GIT is considered sterile prior to birth, so their birth environment, along with birthing method and colostrum intake can affect the development of the GIT microbiome and immune system (Nowland et al., 2019; Kim and Isaacson, 2015). Previous research has identified a delayed impact of exposure to soil on bacterial diversity, with increased bacterial diversity reported two weeks after weaning (Pluske et al., 2007; Vo et al., 2017). Furthermore, exposure to a less hygienic environment, such as outdoors, has been shown to influence development of the immune system, with reduced immune activation and inflammatory response of outdoor-reared pigs in the early stages of life (Mulder et al., 2011). Rearing pigs outdoors has been reported to reduce the detrimental effect of weaning that can be seen in indoor-reared pigs.

As previously discussed in Section 1.4 and Chapter 3, the provision of zinc oxide (Gryaznova et al.) to indoor-reared pigs after weaning is frequently used to reduce post-weaning diarrhoea (PWD) and improve performance immediately after weaning (Stensland et al., 2015; Heo et al., 2013). Zinc oxide has also been reported to cause shifts in the bacterial composition of the GIT of pigs after weaning. This shift has included an increase in bacteria that produce short-chain fatty acids (SCFA), such as *Subdoligranulum* in the colon, as seen in Chapter 3 and previous research (Vahjen et al., 2010; Katouli et al., 1999; Starke et al., 2014; Pieper et al., 2020). Short-chain fatty acids can have beneficial effects for the host, including the production of host defence peptides to improve gut health

and immune function as well as increased energy source for colonocytes (Thomas et al., 2011; Zeng et al., 2013; Koh et al., 2016). Increases in SCFA production, as a result of the shift in bacterial composition, have also been associated with regulating several leukocyte functions, including the production of cytokines such as interleukin-6 (IL-6) and interferon-gamma (IFN-ɣ) (Vinolo et al., 2011).

Cytokines play a critical role in the modulation of an immune and inflammatory response within the GIT (Gao et al., 2013; Pié et al., 2004). Pro-inflammatory cytokines are released as part of an acute phase response (APR) that can occur in response to infection, inflammation or trauma (Gruys et al., 2005; Kim and Isaacson, 2015; Jain et al., 2011). Pro-inflammatory cytokines, such as Interferon Gamma (IFN-ɣ) have a critical role in the recognition and elimination of pathogens within the GIT, such as *Salmonella typhimurium* (Kak et al., 2018). Detection of pro-inflammatory cytokines in response to ETEC have also been observed in the small intestine of pigs, suggesting intestinal mucosal immune system activation (Gao et al., 2013). Interleukins, such as IL-6 and IL-17, are produced in response to environmental stress such as bacterial infections, which activate host defence mechanisms such as the synthesis of acute phase proteins (APPs) (Tanaka et al., 2014; Zhu et al., 2014; Cooper, 2009).

Acute-phase proteins, such as pig-major acute phase protein (pig-MAP) are released into the circulatory system of mammals during infection, and have been used to confirm inflammation and tissue injury as well as response to treatments in humans (Thompson et al., 1992) and pigs (Hulten et al., 2003). As a result, they could be more widely used as less invasive markers of inflammation within the host, compared to bacterial identification and analysis of cytokine production directly in the GIT. Alongside this, faecal markers of intestinal inflammation, such as calprotectin, can be advantageous in measuring levels of mucosal inflammation that are insufficient to cause an increase in some blood APPs, such as C-Reactive protein in humans (Gisbert and McNicholl, 2009). Calprotectin has been widely used as a reliable marker of inflammatory bowel disease in humans (Canani et al., 2008). In pigs, the use of calprotectin as an inflammatory marker has identified average levels in both healthy (Lallès and Fagerhol, 2005; Bogere et al., 2019) and infected pigs (Xiao et al., 2014; Barbosa et al., 2021). Using 16S rRNA sequencing to identify commonalities and differences in bacterial

composition of the GIT of pigs reared indoors or outdoors and subsequently provided control or pharmacological levels of ZnO, could provide interesting insight into bacteria that could subsequently be linked to the improved performance often seen. This could provide an insight into the bacterial composition of pigs that showed improved performance in Chapter 4. Alongside this, identifying differences in immune response through inflammatory markers within the GIT, and whether less invasive markers in blood and faeces show similar results, is of interest to identify health differences between these groups of pigs.

5.1.1 Aims

The first aim of the research presented in this chapter was to determine whether the health status and inflammatory response of pigs reared indoors or outdoors and provided either control or pharmacological levels of ZnO differed, without a deliberate pathogenic challenge. Then, whether the bacterial composition of the GIT of pigs reared indoors or outdoors and then given control or pharmacological levels of ZnO differed 14 days after weaning, while accounting for factors such as GIT location, that can naturally show differences in bacterial composition, as identified in Chapter 3.

5.1.2 Primary Hypothesis

1. If pigs were reared outdoors or provided pharmacological levels of ZnO, they will have reduced expression of cytokines and markers (in blood and faeces) associated with an inflammatory response and have increased bacterial richness, with a bacterial composition consisting of more beneficial bacteria and less potentially pathogenic bacteria, 14 days after weaning.

Secondary hypotheses

2. Rearing pigs outdoors before weaning or providing pharmacological levels of ZnO after weaning will reduce faecal scores and rectal temperatures after weaning.
3. Along the length of the GIT (proximal to distal), and radially from the mucosa to the lumen within each location, the bacterial composition will show reduced levels of facultative anaerobes, regardless of rearing environment and dietary treatment.

5.2 Materials and Method

5.2.1 Animals and management

The experimental design is described in detail within Chapter 2. Samples analysed were from pigs housed at the National Pig Centre, University of Leeds in 2019. In brief, pigs were reared in indoor commercial farrowing crates or in outdoor paddocks and arks. At weaning, ten pigs per litter were weaned into indoor facilities and split into two pens of five pigs per pen, balanced for body weight and sex. One pen received a control (~200 ppm ZnO) diet while the other received a matched diet with ~2500 ppm ZnO for 14 days after weaning (Table 2.5). After day 14, all pigs received the same commercial feed until 28 days after weaning (Table 2.6). Average pen faecal scores were recorded daily based on a 0 to 4 scale, with 4 being watery faeces (see Section 2.2.5). Blood samples, faecal samples and rectal temperatures were collected from six pigs per litter three days prior to weaning and then from the same pigs, which equalled three pigs per pen (n = 96), at days 7, 13 and 28 after weaning. Faecal samples and rectal temperatures were also collected four days after weaning, as described in Section 2.4.1. In addition, at day 14, one pig per pen, of previously sampled pigs, (n = 32) were randomly selected and humanely killed under Schedule 1 of the Animals (Scientific Procedures) Act 1986 and dissected for sample collection according to Section 2.4.2.

5.2.2 Statistical analysis of faecal scores and rectal temperatures

Faecal scores were analysed using a generalised linear mixed model in SPSS (v. 26), with rearing environment and dietary treatment included within the model. Interactions were tested and removed from the model if no significant interactions were observed; main effects were presented in these cases.

To determine differences in rectal temperature overtime between rearing environment and dietary treatment, a linear mixed model in SPSS (v. 26) was used. The linear mixed model used compound symmetry as the repeated covariance type as variances for all data were homogenous. The model included rearing environment and dietary treatment as main, fixed effects, with batch as a random effect. Interactions between rearing environment and dietary treatment were included within the model if a significant interaction was observed. If no interaction was observed, main effects were reported without the interaction in

the model. The effect of rearing environment or dietary treatment within each time point was determined using a univariate general linear model in SPSS (v. 26). For three days before weaning, only rearing environment was included as a fixed effect in the model. Then, all days after weaning included both pre-weaning rearing environment and post weaning dietary treatment in the model. Interactions were included within the model if they were significant.

5.2.3 Quantitative PCR

Mucosal scrapings from the ileum of all dissected pigs (n = 32) and the colon of indoor pigs (n = 16) were used for qPCR analysis. Total RNA was isolated from samples using the Direct-zol™ RNA Miniprep with Zymo-Spin™ ICC Columns (Cambridge Bioscience, UK), following the 'Tough-to-lyse' tissue samples protocol (see Section 2.5.3). Isolated RNA was converted to cDNA using the First Strand cDNA Synthesis Kit (Thermo Scientific, USA), as described in Section 2.5.4. Primers for all genes of interest (IFN-ɣ, IL-6, IL-17; Table 2.6) were optimised and primer efficiency determined as described in Section 2.5.5. Raw qPCR data was analysed using the qbase+ software, (v.3.2) (Biogazelle, Belgium). The cycle threshold (Ct) values generated by qPCR, of all genes, were converted into normalised relative quantities (NRQ), which are corrected for variation within qbase+. The NRQ values represent relative expression levels compared to housekeeper genes, these values are Log10-transformed in qBase+ for statistical analysis using a general linear model in SPSS (v.26), before being back-transformed to present in relevant results tables.

5.2.4 Faecal calprotectin and plasma pig-major acute phase protein analysis using ELISAs

Faecal concentrations of calprotectin were measured in samples collected at days 4, 7, 13, 14 and 28 after weaning using the MBS033848 Porcine Calprotectin ELISA kit (MyBiosource, USA) and following the manufacturer's procedure, as described in Section 2.5.7.1. Results obtained from samples collected at days 13 and 14 were combined for statistical analysis. Plasma concentrations of pig-MAP were measured in plasma samples collected three days prior to weaning and at days 7 and 14 after weaning using the PigMAP ELISA kit (Acuvet Biotec, Spain), following the manufacturer's procedure, as described in Section 2.5.7.2.

5.2.4.1 Statistical analysis of pig-major acute phase protein and calprotectin concentrations

Calprotectin and pig-MAP concentrations were analysed from the same pig through time using a linear mixed model in SPSS (v.26) (Section 2.6.7.3). The linear mixed model used compound symmetry and included rearing environment and dietary treatment as main, fixed effects, with batch as a random effect within the model. Interactions between rearing environment and dietary treatment were included within the model if significant. Differences in calprotectin and pig-MAP concentration within each time point were analysed using a univariate general linear model, with rearing environment and dietary treatment as fixed effects, and batch as a random effect. Interactions between rearing environment and dietary treatment were included within the model if significant.

5.2.5 Microbiome sampling and DNA extraction

Samples from the lumen and mucosa of the jejunum, ileum and colon as well as faeces were collected from pigs during dissections at day 14 as described in Section 2.4.2. Samples were stored at -80ºC until analysis could be complete. DNA was extracted using the PureLink™ Microbiome DNA Purification Kit (Thermo Fisher Scientific, USA), as further described in Section 5.2.2. A total of 190 samples were sent to Novogene (China) for amplification and 16S rRNA sequencing of the V4 hypervariable region on a NovaSeq PE250 platform.

5.2.5.1 Microbial analysis

Due to the quantity of data produced from the increased number of samples used compared to Chapter 3, all downstream analysis was complete on the High Performance Computers at the University of Leeds. Raw sequence reads were quality filtered to remove unwanted sequences (such as those representing Archaea) and processed using Mothur v1.40.3, following the MiSeq standard operation procedure (Schloss et al., 2009). Unique sequences were identified and aligned against the SILVA (v.132) database, and filtered as described in Section 2.5.2.1. The effect of rearing environment, dietary treatment, GIT location and sample type (mucosa or lumen) on alpha diversity were analysed in R Studio (v. 3.4.3) using a general linear model on unrarefied data. Beta diversity was plotted using a non-metric multidimensional scaling plot (NMDS) using the Bray-Curtis distance, followed by a PERMANOVA (adonis) test to identify significant

interactions and main effects, as described in Section 2.5.2.2. DeSeq2 analysis was carried out to determine differences in OTU count. For DeSeq2 analysis, data were split into four groups to allow for pairwise comparisons between all groups, these groups were: indoor-reared, control-fed pigs (indoor control); indoor-reared, ZnO-fed pigs (indoor ZnO); outdoor-reared, control-fed pigs (outdoor control); outdoor-reared, ZnO-fed pigs (outdoor ZnO). These four groups were also analysed for differences in the percentage relative abundance of phyla and genera within each GIT location, using a Kruskal-Wallis or one way ANOVA, corrected for multiple tests in SPSS (v.26), as described in Section 2.5.2.3.

5.3 Results

5.3.1 Faecal scores and rectal temperatures up to 28 days after weaning

Average, weekly faecal scores for the first four weeks after weaning are presented in Table 5.1. No significant differences were observed between rearing environment and dietary treatment within the first week after weaning. During the second and third week, rearing pigs outdoors and the provision of ZnO significantly reduced faecal scores. In the fourth week, there was a significant interaction between rearing environment and provision of ZnO, whereby indoor pigs receiving ZnO and outdoor, control-fed pigs had significantly lower faecal scores than indoor control-fed pigs. Outdoor pigs that subsequently received ZnO had faecal scores in between indoor control pigs and the other two groups. Although faecal scores were reduced, it is important to note that no scores recorded watery faeces, thus indicating diarrhoea was not present for any groups.

Table 5.1 Average pen faecal scores from weaning until day 28 days after weaning for pigs that were reared either indoors or outdoors before weaning and then provided a diet containing control (~200ppm) or pharmacological (~2500 ppm) levels of ZnO for 14 days after weaning.

	Indoor		Outdoor			Wald Chi-Square$_{df}$		p values		
Faecal Score (day)	Control	ZnO	Control	ZnO	SEM	Environment	Treatment	Environment	Treatment	Environment * Treatment
0-7	2.29	2.31	2.18	2.19	0.037	$2.698_{1,44}$	$0.028_{1,44}$	0.107	0.868	N/A
8-15	2.43	2.27	2.25	2.08	0.046	$4.979_{1,44}$	$3.948_{1,44}$	**0.026**	**0.047**	N/A
16-22	2.28	2.14	2.12	1.98	0.036	$6.507_{1,44}$	$4.754_{1,44}$	**0.011**	**0.029**	N/A
23-28	2.16^{a}	2.02^{b}	2.01^{b}	2.04^{ab}	0.018	$5.524_{1,43}$	$4.635_{1,43}$	**0.019**	**0.031**	**0.002**

Faecal scores were recorded on a scale of 0-4 with 0= no faeces in pen; 1= Firm faeces; 2= Soft faeces; 3= very soft faeces; 4= watery faeces.

There were no interactions between rearing environment and dietary treatment on rectal temperatures that were analysed from three days prior to weaning and then 4, 7, 13 and 28 days after weaning; thus, the interaction was removed from the statistical model. Analysis of rectal temperatures over time revealed a significant effect of rearing environment, with outdoor pigs increasing in temperature ($F_{df} = 11.31_{1,90}$; p = 0.001). Dietary treatment did not affect rectal temperature over time ($F_{df} = 0.28_{1,90}$; p = 0.596; Figure 5.1).

There were also no interactions between rearing environment and dietary treatment on rectal temperatures of pigs within each point, thus it was removed from the statistical model. Three days prior to weaning there was no effect of rearing environment on rectal temperatures ($F_{df} = 0.387_{1,91}$; p = 0.535; Figure 5.1). At four days after weaning, when all pigs were within the indoor facilities, pigs that had previously been reared outdoors tended to have higher rectal temperatures ($F_{df} = 3.024_{1,90}$; p = *0.085)*, and temperatures were increased in these pigs at day 7 and 13 ($F_{df} = 8.627_{1,89}$, p = 0.004; $F_{df} = 13.711_{1,85}$, p <0.001, respectively) . Dietary treatment did not affect temperature at day four or seven ($F_{df} = 0.091_{1,90,}$ p = 0.763; $F_{df} = <0.001_{1,89,}$ p = 0.994, respectively), but did affect rectal temperature at day 13, with ZnO increasing rectal temperatures of indoor-reared pigs ($F_{df} = 10.240_{1,85}$; p = 0.002) but not outdoor-reared pigs. By day 28

after weaning there were no differences in rectal temperature between rearing environment and dietary treatment groups (F_{df} = $0.545_{1,47}$, p = 0.464; F_{df} = $0.568_{1,47,}$ p = 0.455, respectively).

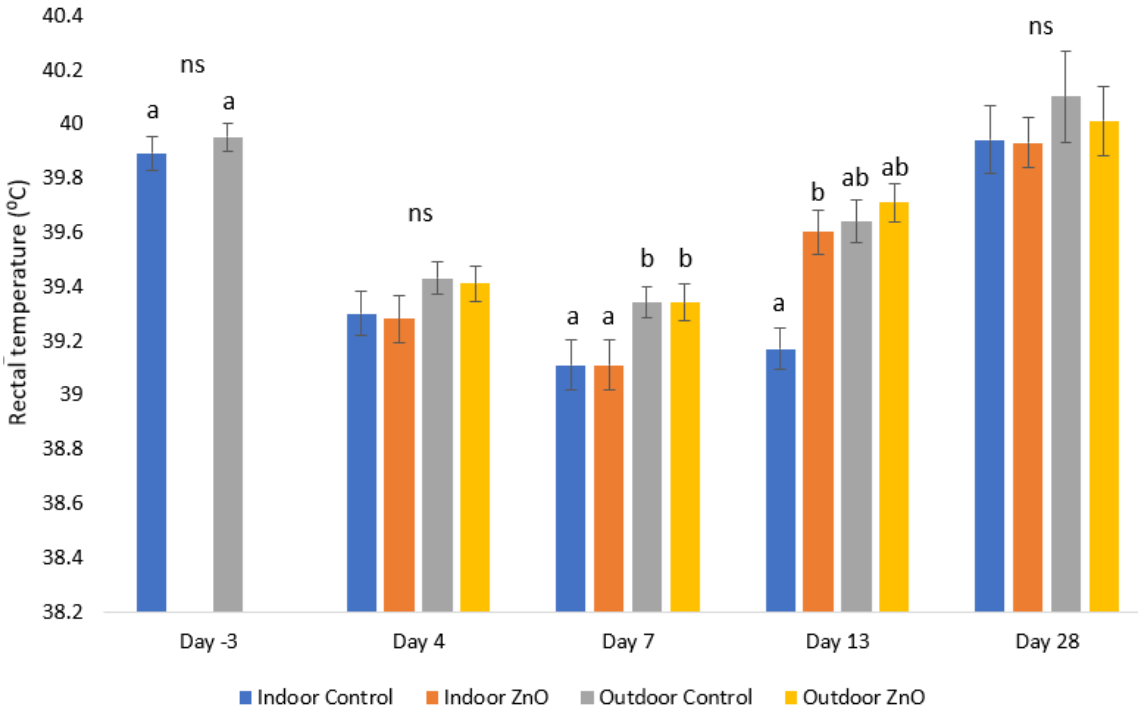

Figure 5.1 Rectal temperatures (°C) at three days before weaning and days 4, 7, 13 and 28 days after weaning, of pigs reared indoors or outdoors and then provided control (200ppm) or pharmacological (~2500 ppm) levels of ZnO for 14 days after weaning. Error bars showing standard error of the mean. Ns = not significant; different letters above bars within each time point indicate significant difference.

5.3.2 Inflammatory markers within the gastrointestinal tract, blood and faeces

5.3.3 Gene expression analysis using qPCR

The stability of the reference genes, β-actin and GAPDH, used for relative expression were identified by qBase+ as adequate (M= 0.571, CV = 0.199); therefore, the geometric mean of both reference genes were used as the normalisation factor for all genes of interest. Primer efficiency for IL-17A was not sufficient (90% - 110%) when using the pre-designed PrimePCR™ assay or primers designed specifically for this work (see Section 2.5.5) and therefore further analysis of this gene was not carried out. Hereafter, genes of interest refer only to IFN-ɣ and IL-6.

The normalised relative quantities (NRQ) of each gene of interest, relative to the housekeeper genes, showed no significant effect of rearing environment, dietary treatment an interaction between both within the ileum (Table 5.2). There was also no significant difference in normalised relative quantities of IL-6 and IFN- ɣ in the colon of pigs that were reared indoors and given either control or pharmacological levels of ZnO (Table 5.3).

Table 5.2 Normalised relative quantities of interferon gamma (IFN) and ilterleukin-6 (IL-6) mRNA in the ileum of pigs reared indoors or outdoors and subsequently provided of control (200ppm) or pharmacological (2500ppm) levels of ZnO.

df – degrees of freedom.

	INDOOR		OUTDOOR		F statistic$_{df}$		p value	
	Control	ZnO	Control	ZnO	Environment	Treatment	Environment	Treatment
IFN-ɣ	0.876	0.787	1.197	1.076	$1.213_{1,27}$	$0.137_{1,27}$	0.281	0.714
IL-6	1.030	1.151	1.567	1.750	$0.684_{1,27}$	$0.047_{1,27}$	0.415	0.829

Table 5.3 Normalised relative abundance of interferon gamma (IFN) and ilterleukin-6 (IL-6) mRNA in the colon of indoor reared pigs that received either control (200ppm) or pharmacological (2500ppm) levels of ZnO after weaning.

	Control	ZnO	F statistic$_{df}$	p value
IFN-ɣ	0.948	0.838	$0.931_{1,12}$	0.354
IL-6	0.762	0.351	$0.047_{1,27}$	0.372

df – degrees of freedom

5.3.4 Plasma concentration of pig-major acute phase protein

Neither rearing environment ($F_{df} = 2.811_{1,27}$; p = 0.105) or dietary treatment ($F_{df} = 0.007_{1,27}$; p = 0.935) had an effect on plasma concentrations of pig-MAP from three days prior to weaning to days 7 and 14 after weaning. Furthermore, there was no effect of rearing environment or dietary treatment on the concentrations of pig-MAP in plasma within each time point, as shown in Table 5.4.

Table 5.4 Plasma concentrations of pig-MAP (mg/ml) at three days before weaning as well as 7 and 14 days after weaning in response to pre-weaning rearing environment and post-weaning provision of control or pharmacological levels of ZnO.

	Indoor		Outdoor		F- statistic$_{df}$		P- value	
	Control	ZnO	Control	ZnO	Environment	Treatment	Environment	Treatment
Day – 3*	0.205		0.140		$2.394_{1,13}$	N/A	0.146	N/A
Day 7	0.124	0.122	0.087	0.085	$1.900_{1,27}$	$0.008_{1,27}$	0.179	0.928
Day 14	0.109	0.132	0.079	0.102	$1.293_{1,27}$	$0.762_{1,27}$	0.265	0.390

*Day -3 refers to three days prior to weaning; df = degrees of freedom

5.3.4.1 Faecal concentrations of calprotectin

There were no interactions between rearing environment and dietary treatment at any time point on faecal calprotectin concentrations, therefore results presented exclude the interaction from the model. Neither rearing environment ($F_{df} = 0.152_{1,69}$; p = 0.698) or dietary treatment ($F_{df} = 0.211_{1,69}$; p = 0.647) had a significant effect on calprotectin concentrations over time, from day 4 to day 28 after weaning. Furthermore, calprotectin concentrations within each time point did not differ between rearing environments and dietary treatments (Table 5.5).

Table 5.5 Faecal calprotectin (ng/ml of suspended faeces) levels at 4, 7, 13.5 (day 13 and 14 combined) and 28 days after weaning, for pigs reared indoors or outdoors before weaning and then provided a diet with control or pharmacological levels ZnO.

	Indoor		Outdoor		F- statistic		P- value	
	Control	ZnO	Control	ZnO	Environment	Treatment	Environment	Treatment
Day 4	53.14	53.14	53.14	53.14	$0.292_{1,22}$	$1.360_{1,22}$	0.592	0.256
Day 7	45.80	43.23	44.89	42.31	$0.059_{1,41}$	$0.483_{1,41}$	0.810	0.491
Day 13.5	42.46	38.23	43.62	45.25	$0.126_{1,43}$	$1.650_{1,43}$	0.724	0.206
Day 28	44.37	49.32	47.17	52.12	$0.370_{1,21}$	$1.185_{1,21}$	0.550	0.289

5.3.5 Bacterial sequencing data

After filtering of raw sequence reads from 16S rRNA sequencing, a total of 14,833,555 sequence reads from 190 samples were generated. The total number of OTUs was 36,382 from 190 samples. All the identified sequences that were present in at least one sample above 0.1% were able to be classified into 16 different phyla, with Firmicutes (63% ± 1.35), Bacteroidetes (18% ± 0.87) and Epsilonbacteraeota (8% ± 0.93) being the top three abundant phyla (Appendix C.1 to Appendix C.3). Epsilonbacteraeota is a newly reassigned phyla, from the class of *Protobacteria*, known as *Epsilonproteobacteria* and the order *Desulfurellales* (Waite et al., 2017).

5.3.6 Taxonomic classification and microbial diversity within the mucosa and lumen along the length of the gastrointestinal tract

Overall relative abundance of phyla within each GIT location was first examined, irrespective of sample type, environment and treatment. In the jejunum, Firmicutes (64% ± 3.60), Epsilonbacteraeota (15% ± 3.16) and Bacteroidetes (13% ± 1.56) were the three most abundant phyla. In the ileum, it was Firmicutes (64% ± 3.01), Bacteroidetes (13% ± 1.25) and Proteobacteria (12% ± 2.00). The colon was dominated by Firmicutes (61% ± 1.76), Bacteroidetes (24% ± 1.40) and Proteobacteria (6% ± 1.15) and finally in faeces, Firmicutes (67% ± 2.54), Bacteroidetes (24% ± 2.05) and Epsilonbacteraeota (3% ± 0.90) were the three dominating phyla. Comparing Proteobacteria, the decrease seen from the ileum to faeces was significant (12% ± 2.00 vs 2.99% ± 0.45, respectively; $p = 0.002$). Likewise, the decrease in Epsilonbacteraeota from the jejunum to faeces was statistically significant (15% ± 3.16 vs 3% ± 0.90, respectively; $p = 0.004$).

Alpha diversity was significantly different between GIT locations; specifically, species richness and evenness was lower in the jejunum and ileum compared to both colonic and faecal samples (Table 5.6). Beta diversity was significantly different between all GIT locations ($F_{df} = 4.100_{3,150}$; $p = 0.001$), except between colonic and faecal samples. This was supported by DeSeq2 analysis of OTU counts between each GIT location, although nine OTUs also significantly altered between the colon and faeces (Appendix D.4).

When looking at differences in diversity measures between the mucosa and lumen, overall differences were seen for Chao1, but not Shannon and Simpson

diversity indices (Table 5.6). Beta diversity also showed overall differences between all mucosa and luminal samples ($F_{df} = 3.534_{1,150}$; $p = 0.001$). To better understand these differences, more in depth comparisons were made between the mucosa and lumen within each GIT location rather than overall. Although overall differences were seen in alpha diversity when including all samples, these differences were not seen when comparing the mucosa and lumen specifically within each GIT location. Conversely, beta diversity did differ between the mucosa and lumen of the jejunum ($F_{df} = 2.224_{1,28}$; $p = 0.020$), ileum ($F_{df} = 2.328_{1,43}$; $p = 0.009$) and a trend in the colon ($F_{df} = 1.524_{1,50}$; $p = 0.084$). These results were supported by DeSeq2 analysis, whereby the majority of differences were observed between the mucosa and lumen of the jejunum (6 OTUs differed) and ileum (34 OTUs differed) rather than within the colon (3 OTUs differed) (Appendix E.5).

Table 5.6 Alpha Diversity indices for the bacterial community composition of the mucosa and lumen of the jejunum, ileum colon as well as faeces of pigs 14 days after weaning.

	GIT Location	Sample type		GIT Location		Sample type	
		Mucosa	Lumen	F- statistic$_{df}$	p value	F- statistic$_{df}$	p value
Simpson	Jejunum[a]	0.865	0.829	19.985$_{153,155}$	**<0.001**	0.577$_{153,154}$	0.449
	Ileum[a]	0.880	0.887				
	Colon[b]	0.951	0.946				
	Faeces[b]	0.955					
Shannon	Jejunum[a]	3.680	3.388	19.877$_{153,155}$	**<0.001**	1.182$_{153,154}$	0.279
	Ileum[a]	3.715	3.652				
	Colon[b]	4.287	4.272				
	Faeces[b]	4.431					
Chao1	Jejunum[a]	1697.1	1982.1	6.391$_{153,155}$	***0.002***	14.526$_{153,154}$	***<0.001***
	Ileum[a,b]	1609.3	2074.1				
	Colon[b,c]	2017.1	2307.5				
	Faeces[c]	2334.9					

df = degree of freedom show comparison of both statistical models
Numbers in bold show significant p-values; Different superscript letters next to GIT location, within each measure of diversity indicate differences between GIT locations (P<0.05), irrespective of sample type.

Given these differences in beta diversity measures between the mucosa and lumen of each GIT location, further classification of the phyla and genera within each of these microenvironments were investigated and showed significant differences in relative abundance at the phyla level, as shown in Figure 5.2. In the jejunum, Firmicutes were more abundant in the lumen compared to the mucosa ($p = 0.012$) whilst Epsilonbacteraeota were more abundant within the mucosa compared to the lumen ($p = 0.001$). In the Ileum, Firmicutes and Fusobacteria were more enriched in the lumen compared to the mucosa ($p = 0.003$; $p = 0.023$, respectively). The mucosa was more enriched with Proteobacteria, Epsilonbacteraeota, Actinobacteria and Cyanobacteria compared to the lumen ($p = 0.018$; $p = 0.001$; $p =0.017$; $p = 0.027$, respectively). In the colon, the lumen was more enriched with Firmicutes compared to the mucosa ($p = 0.001$) while the mucosa was again, more enriched with Epsilonbacteraeota compared to the lumen ($p < 0.0001$). Overall, bacteria within Firmicutes and Bacteroidetes were the predominant phyla within the lumen, while Proteobacteria, Epsilonbacteraeota (previously Proteobacteria) were more prominent in mucosal samples.

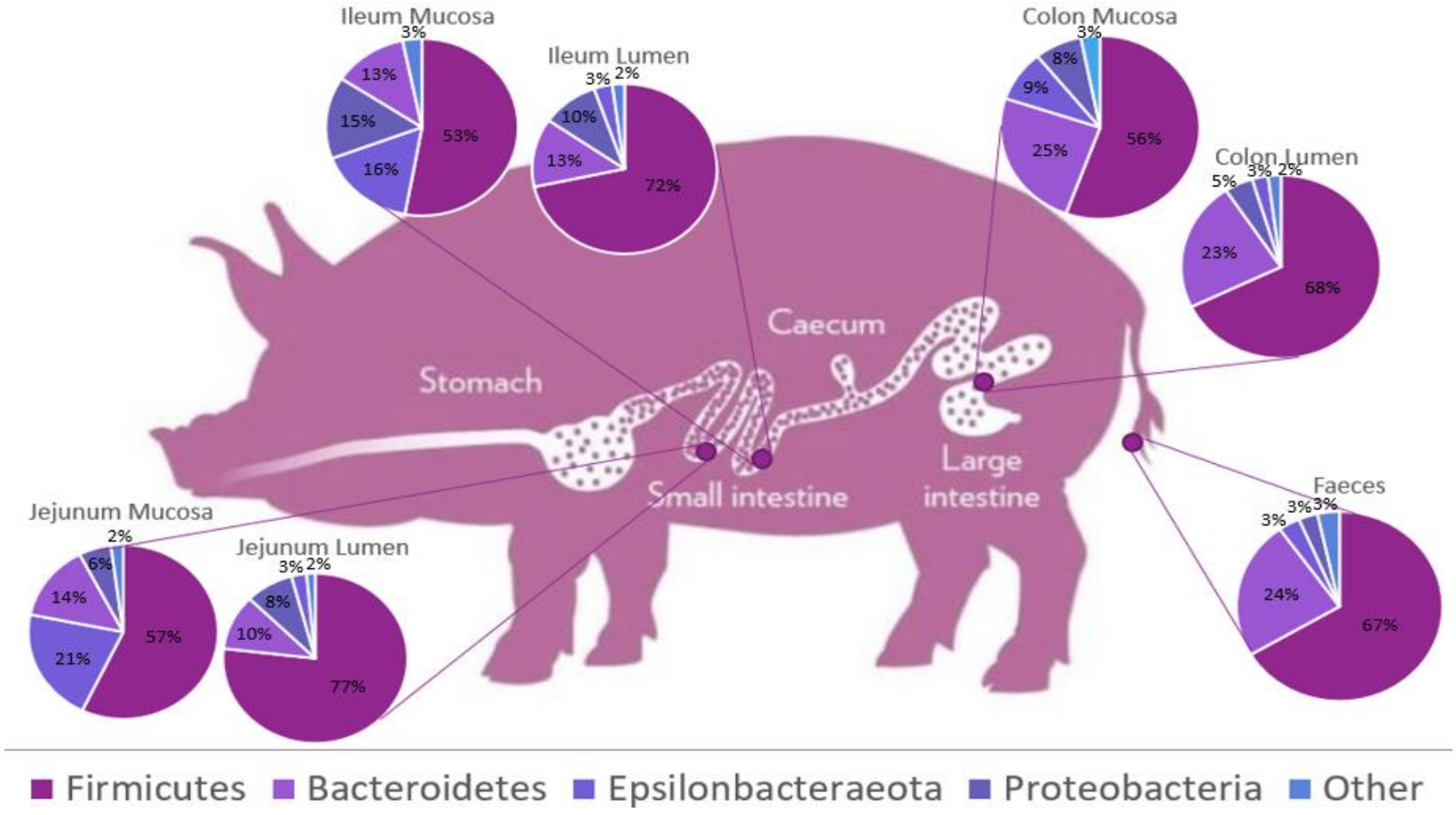

Figure 5.2 Community composition of the most dominant phyla (Firmicutes, Bacteroidetes, Epsilobacteraeota and Proteobacteria and all others in the mucosa and lumen of the ileum, caecum and colon as well as faeces of pigs 14 days after weaning.

5.3.7 Microbial diversity of pigs reared indoors or outdoors and provided control or pharmacological levels of zinc oxide after weaning

The phyla composition of indoor-reared, control-fed pigs was looked at first and showed higher abundance of Proteobacteria and Epsilonbacteraeota in the small intestine (jejunum and ileum) compared to the colon. Higher levels of Proteobacteria and Epsilonbacteraeota were also seen in the mucosa compared to the lumen of the small, but not large intestine. Across all groups of pigs, there were no differences in relative abundance, at the phyla level, within either the mucosa or lumen of the jejunum. Furthermore, there were consistently no differences observed between indoor control pigs and indoor ZnO-fed pigs or between indoor control pigs and outdoor control pigs (Appendix C.1).

Comparison of outdoor control pigs to outdoor ZnO-fed pigs showed an increase in Firmicutes from outdoor control (50%) to outdoor ZnO (78%; $p<0.001$) in the mucosa of the ileum (Figure 5.3; Appendix C.2). Firmicutes in the mucosal attached microbiome of the ileum were also different in relative abundance between indoor ZnO (43%) and outdoor ZnO (79%; $p = 0.001$), as were Bacteroidetes in the lumen and mucosa of the colon; increasing from 16% and 19%, respectively in indoor ZnO pigs to 32% and 35%, respectively in outdoor ZnO pigs ($p = 0.05$; $p < 0.001$;Figure 5.2; Appendix C.2). Furthermore, Epsilonbacteraeota decreased from indoor ZnO pigs (11%) to outdoor ZnO pigs (3%; $p = 0.041$; Figure 5.2).

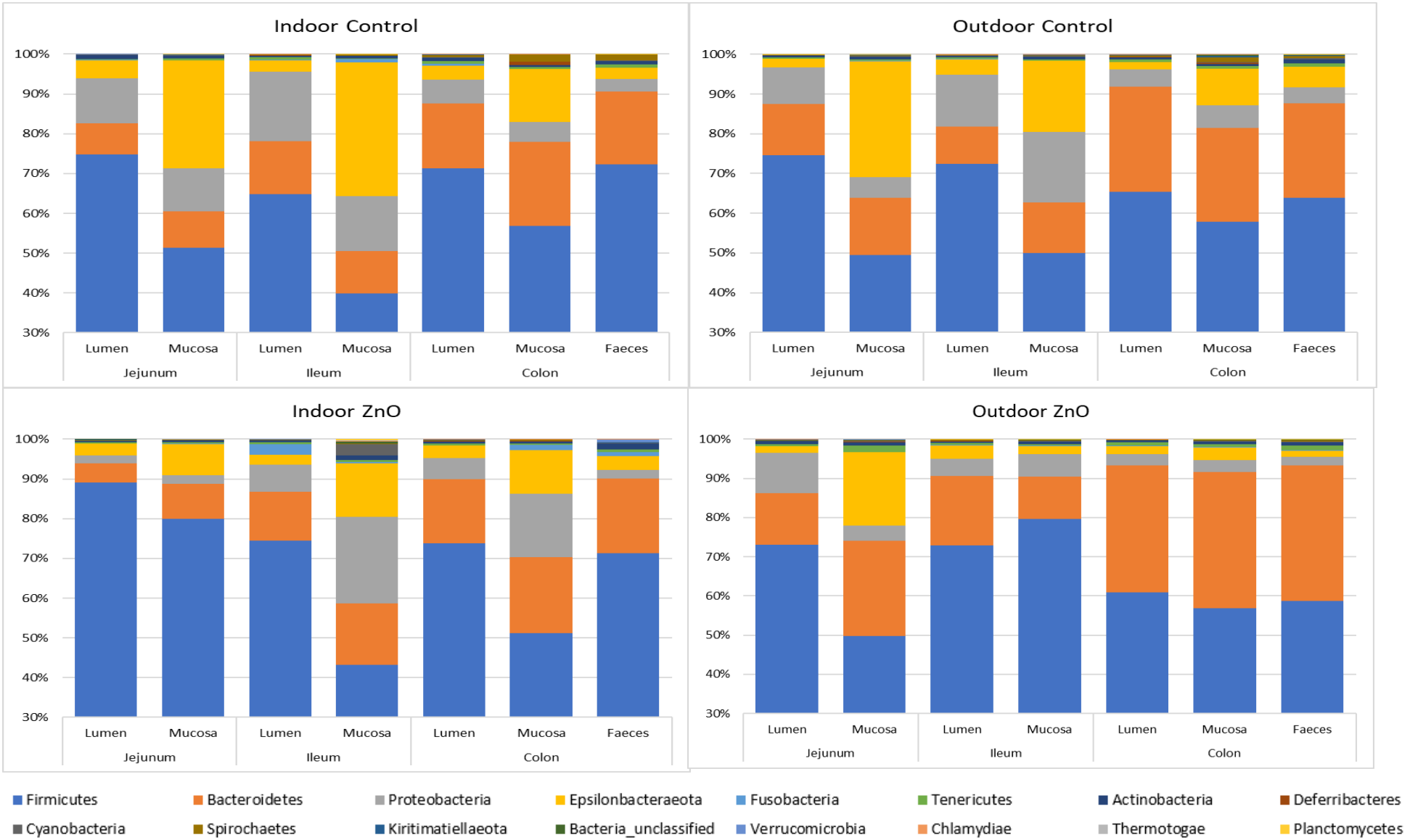

Figure 5.3 Relative abundance of phyla in the mucosa and lumen of the jejunum, ileum and colon and in faeces. Differences shown for indoor-reared, control fed; Indoor ZnO fed (~2500 ppm), outdoor-reared control fed and outdoor-reared ZnO (~2500 ppm) fed pigs. Note: relative abundance starts at 30% and includes all phyla that were present in at least one sample above 1%.

5.3.7.1 Alpha diversity

Differences in alpha diversity between rearing environments and dietary treatments were identified within the jejunum, ileum, colon and faeces. Sample type (luminal or mucosal) was kept within the statistical mode, but consistently no significant differences were observed and have therefore not been reported.

There were no significant interactions between rearing environment and dietary treatment for any alpha diversity measures within any GIT location and therefore the interaction was removed from the model and main effects are presented (Table 5.7). Alpha diversity, as measured by Simpson and Shannon diversity indices, significantly increased in the jejunum of outdoor, compared to indoor-reared pigs ($p=0.031$; $p=0.009$ for Simpson and Shannon, respectively). The provision of ZnO did not affect alpha diversity in any parts of the small intestine. Furthermore, the ileum was not affected by either rearing environment or dietary treatment (Table 5.7). In the colon, rearing environment tended to affect both Simpson ($p = 0.061$) and Shannon ($p = 0.073$) diversity measures, but did not significantly affect Chao1. Dietary treatment had no effect on any alpha diversity measures within the colon. In faeces, there were also no effects of dietary treatment, but rearing pigs outdoors significantly increased Shannon diversity ($p=0.024$) and tended to increase Simpson ($p=0.073$) and Chao1 (0.070) compared to pigs reared indoors.

Table 5.7 Alpha diversity indices of the bacterial community composition in the small intestine, colon and faeces of pigs reared indoors or outdoors and given a post-weaning diet containing control (200 ppm) or pharmacological levels (2500 ppm) of ZnO.

	GIT Location	INDOOR		OUTDOOR		Environment		Treatment	
		Control	ZnO	Control	ZnO	F-Statisitc$_{df}$	P-value	F-Statisitic$_{df}$	P-value
Simpson	Jejunum	0.860	0.753	0.863	0.920	$5.194_{28,29}$	**0.031**	$0.181_{28,29}$	0.674
	Ileum	0.911	0.857	0.884	0.891	$0.033_{43,44}$	0.857	$0.630_{43,44}$	0.432
	Colon	0.945	0.933	0.958	0.960	$3.672_{50,51}$	*0.061*	$0.190_{50,51}$	0.665
	Faeces	0.950	0.940	0.964	0.967	$3.529_{24,25}$	*0.073*	$0.101_{24,25}$	0.753
Shannon	Jejunum	3.379	3.016	3.635	4.181	$7.913_{28,29}$	**0.009**	$0.305_{28,29}$	0.586
	Ileum	3.811	3.620	3.616	3.696	$0.054_{43,44}$	0.817	$0.076_{43,44}$	0.784
	Colon	4.262	4.062	4.406	4.403	$3.357_{50,51}$	*0.073*	$0.514_{50,51}$	0.477
	Faeces	4.290	4.166	4.571	4.697	$5.793_{24,25}$	**0.024**	$0.002_{24,25}$	0.978
Chao1	Jejunum	1621.47	1770.45	1804.62	1956.48	$1.064_{28,29}$	0.311	$1.056_{28,29}$	0.313
	Ileum	1860.76	1982.46	1985.91	1799.71	$0.346_{43,44}$	0.560	$0.042_{43,44}$	0.839
	Colon	1228.68	1997.54	2185.59	2175.22	$0.029_{50,51}$	0.763	$1.000_{50,51}$	0.322
	Faeces	2279.61	2064.40	2417.37	2595.89	$3.612_{24,25}$	*0.070*	$0.005_{24,25}$	0.943

df = Degree of freedom of both models
Numbers in bold show significant p-values

5.3.7.2 Beta diversity

The relationships between the community composition of the pigs microbiome was investigated using an NMDS plot, which indicated differences in distribution of bacteria between indoor and outdoor-reared pigs, that was more evident in the jejunum and faeces (Appendix E.1 to Appendix E.4). However, dietary treatments consistently did not cluster separately within any GIT location. There were also no significant interactions between rearing environment and dietary treatment, leading to main effects being reported. There were significant differences in beta diversity of indoor and outdoor-reared pigs within the lumen and mucosa of the jejunum and colon as well as in faeces. In the ileum, only the mucosa attached microbiome differed in composition between rearing environments. In comparison, dietary treatment did not alter the bacterial composition within the GIT, but tended to alter composition of the faeces (Table 5.8).

Table 5.8 The effect of environment and dietary treatment on beta diversity measures within the mucosa and lumen of the jejunum, ileum, colon and in faeces at 14 days after weaning.

		Test statistic$_{df}$		p value	
		Environment	Treatment	Environment	Treatment
Jejunum	Mucosa	$2.336_{1,18}$	$1.456_{1,18}$	**0.008**	0.123
	Lumen	$2.666_{1,8}$	$0.844_{1,8}$	**0.023**	0.560
Ileum	Mucosa	$1.625_{1,17}$	$1.363_{1,17}$	**0.045**	0.148
	Lumen	$1.465_{1,24}$	$1.215_{1,24}$	0.165	0.274
Colon	Mucosa	$1.728_{1,26}$	$1.380_{1,26}$	**0.036**	0.127
	Lumen	$2.757_{1,22}$	$1.277_{1,22}$	**0.001**	0.202
Faeces		$2.281_{1,24}$	$1.539_{1,24}$	**0.003**	*0.079*

Df – Degrees of freedom

5.3.7.3 DeSeq2 Analysis

In the jejunal mucosa, six OTUs changed between indoor control and indoor ZnO, including the increase of two members of the *Lachnospiraceae* family (*Marvinbryantia* and *CHKCI001*) and two *Ruminococcaceae* (unclassified and *Ruminococcaceae UCG-002*) (Table 5.9). These exact four genera, alongside unclassified *Muribaculaceae* all also significantly increased in the jejunal mucosa of both groups of outdoor pigs (Table 5.9). These genera clearly showed greater

association to the mucosal attached microbiome as they were not replicated in the lumen, or indeed in any other GIT location (Table 5.10 and Table 5.11). This pattern between groups was also replicated in faecal samples, but with the increase of Unclassified *Prevotellaceae* in indoor ZnO and both outdoor groups when compared to indoor control pigs (Table 5.11).

Although the pattern of similarity between indoor control pigs to indoor ZnO and all outdoor pigs was not replicated in the ileum, similarities between indoor and outdoor ZnO fed pigs were observed. The OTUs associated with the phyla Bacteroidetes (*Salinirepens* and unlclassified *Bacteroidia*) as well unclassified *Alphaproteobacteria* significantly increased in the ileal mucosa of indoor control pigs compared to both indoor ZnO and outdoor ZnO pigs. The majority of changes within the ileum were observed between the mucosa of outdoor control and ZnO pigs. In the lumen, outdoor-reared pigs receiving ZnO consistently had lower levels of the OTU associated with *Lactobacillus* than all other groups of pigs.

Fewer changes were observed within the colon and faeces compared to the jejunum and ileum (Table 5.11). Significant differences were only observed between indoor ZnO and outdoor ZnO pigs in the mucosa of the colon, indicating an environmental effect on these pigs. Unlike in chapter 3, there were no differences in OTUs within the colon of indoor control and ZnO fed pigs, which was also partly true for outdoor control and ZnO fed pigs, except for the decrease of *Bradymondales* in the lumen of outdoor ZnO pigs. The majority of changes were observed between environments, with increases in Ruminococcaceae UCG-014 in all outdoor, compared to indoor pigs (Table 5.11).

Table 5.9 DeSeq2 analysis of the operational taxonomic units (OTU) in the mucosa and lumen of the jejunum of pigs reared either indoors or outdoors and provided control (200ppm) or pharmacological (2500ppm) levels of ZnO after weaning. P values adjusted for multiple testing with Benjamin-Hochberg correction. Fold changes represent Log2 fold increases and decreases.

OTU Number	Genera	Fold Change	p value
JEJUNUM MUCOSA			
OTUs Changed from Indoor Control to Indoor ZnO pigs			
OTU480	*Marvinbryantia*	18.69	<0.001
OTU535	*CHKCI001*	17.60	<0.001
OTU684	*Ruminococcaceae, unclassified*	16.47	0.002
OTU412	*Ruminococcaceae UCG-002*	16.44	0.003
OTU498	*Muribaculaceae, unclassified*	17.58	0.003
OTU450	*Muribaculaceae ge*	-16.64	0.029
OTUs Changed from Outdoor Control to Outdoor ZnO pigs			
OTU526	*Alistipes*	25.97	<0.001
OTU244	*Butyricicoccus*	-5.66	0.003
OTUs Changed from Indoor Control to Outdoor Control pigs			
OTU412	*Ruminococcaceae UCG-002*	21.20	<0.001
OTU684	*Ruminococcaceae, unclassified*	20.39	<0.001
OTU480	*Marvinbryantia*	20.73	<0.001
OTU535	*CHKCI001*	20.43	<0.001
OTU498	*Muribaculaceae, unclassified*	20.46	<0.001
OTUs Changed from Indoor ZnO to Outdoor ZnO pigs			
OTU450	*Muribaculaceae ge*	23.74	<0.001
OTUs Changed from Indoor Control to Outdoor ZnO			
OTU412	*Ruminococcaceae UCG-002*	21.04	<0.001
OTU535	*CHKCI001*	20.31	<0.001
OTU480	*Marvinbryantia*	20.16	<0.001
OTU526	*Alistipes*	22.18	<0.001
OTU684	*Ruminococcaceae, unclassified*	18.51	<0.001
OTU498	*Muribaculaceae, unclassified*	19.37	<0.001
JEJUNUM LUMEN			
OTUs Changed from Indoor Control to Indoor ZnO pigs			
OTU138	*Dialister*	-23.45	<0.001
OTU147	*Prevotella 6*	-24.43	<0.001
OTU414	*Ruminococcaceae UCG-014*	-17.76	0.018
OTUs Changed from Outdoor Control to Outdoor ZnO pigs			
OTU147	*Prevotella 6*	14.84	0.004
OTUs Changed from Indoor Control to Outdoor Control pigs			
OTU147	*Prevotella 6*	-25.26	<0.001
OTU071	*Enterococcus*	-7.35	0.033
OTUs Changed from Indoor ZnO to Outdoor ZnO pigs			
OTU138	*Dialister*	20.32	<0.001
OTU414	*Ruminococcaceae UCG-014*	22.25	<0.001
No changes from Indoor Control to Outdoor ZnO			

Table 5.10 DeSeq2 analysis of the operational taxonomic units (OTU) in the mucosa and lumen of the ileum of pigs reared either indoors or outdoors and provided control (200ppm) or pharmacological (2500ppm) levels of ZnO after weaning. P values adjusted for multiple testing with Benjamin-Hochberg correction. Fold changes represent Log2 fold changes.

OTU Number	Genera	Fold Change	p value
ILEUM MUCOSA			
OTUs Changed from Indoor Control to Indoor ZnO pigs			
OTU218	*Bacteria, unclassified*	23.96	<0.001
OTU802	*Salinirepens*	20.87	<0.001
OTU830	*Bacteroidia, unclassified*	20.57	<0.001
OTU712	*Alphaproteobacteria, unclassified*	20.84	<0.001
OTUs Changed from Outdoor Control to Outdoor ZnO pigs			
OTU277	*Megasphaera*	22.12	<0.001
OTU712	*Alphaproteobacteria, unclassified*	32.91	<0.001
OTU294	*Sphingobium*	23.15	<0.001
OTU802	*Salinirepens*	29.71	<0.001
OTU830	*Bacteroidia, unclassified*	29.60	<0.001
OTU218	*Bacteria, unclassified*	26.66	<0.001
OTU306	*Sphingobium*	23.08	<0.001
OTU235	*Ruminiclostridium 5*	-25.86	<0.001
OTU216	*Helicobacter*	-22.86	<0.001
OTUs Changed from Indoor Control to Outdoor Control pigs			
OTU277	*Megasphaera*	-22.00	<0.001
OTU294	*Sphingobium*	-16.04	0.004
OTU306	*Sphingobium*	-17.52	0.004
OTUs Changed from Indoor ZnO to Outdoor ZnO pigs			
OTU216	*Helicobacter*	-23.52	<0.001
OTU235	*Ruminiclostridium 5*	-22.68	<0.001
OTUs Changed from Indoor Control to Outdoor ZnO pigs			
OTU218	*Bacteria, unclassified*	22.88	<0.001
OTU712	*Alphaproteobacteria, unclassified*	19.47	<0.001
OTU830	*Bacteroidia, unclassified*	17.69	<0.001
OTU802	*Salinirepens*	17.11	<0.001
OTU216	*Helicobacter*	-18.61	<0.001
OTU235	*Ruminiclostridium 5*	-18.07	<0.001
ILEUM LUMEN			
OTUs Changed from Indoor Control to Indoor ZnO pigs			
OTU259	*Muribaculaceae, unclassified*	-19.09	<0.001
OTUs Changed from Outdoor Control to Outdoor ZnO pigs			
OTU935	*Lactobacillus*	-18.62	<0.001
No changes from Indoor Control to Outdoor Control pigs			
OTUs Changed from Indoor ZnO to Outdoor ZnO pigs			
OTU259	*Muribaculaceae, unclassified*	23.30	<0.001
OTU935	*Lactobacillus*	-21.89	<0.001
OTUs Changed from Indoor Control to Outdoor ZnO pigs			
OTU935	*Lactobacillus*	-24.63	<0.001

Table 5.11 DeSeq2 analysis of the operational taxonomic units (OTU) in the mucosa and lumen of the colon and in faeces of pigs reared either indoors or outdoors and provided control (200ppm) or pharmacological (2500ppm) levels of ZnO after weaning. P values adjusted for multiple testing with Benjamin-Hochberg correction. Fold changes represent Log2 fold changes.

OTU Number	Genera	Fold Change	p value
COLON MUCOSA			
No changes from Indoor Control to Indoor ZnO pigs			
No changes from Outdoor Control to Outdoor ZnO pigs			
No changes from Indoor Control to Outdoor Control pigs			
OTUs Changed from Indoor ZnO to Outdoor ZnO pigs			
OTU105	*Muribaculaceae, unclassified*	5.38	0.025
OTU123	*Sutterella*	-3.41	0.025
No changes from Indoor Control to Outdoor ZnO pigs			
COLON LUMEN			
No changes from Indoor Control to Indoor ZnO pigs			
OTUs Changed from Outdoor Control to Outdoor ZnO pigs			
OTU399	*Bradymonadales, ge*	-22.51	<0.001
OTUs Changed from Indoor Control to Outdoor Control pigs			
OTU424	*Ruminococcaceae UCG-014*	20.67	0.022
OTUs Changed from Indoor ZnO to Outdoor ZnO pigs			
OTU424	*Ruminococcaceae, UCG-014*	*31.10*	<0.001
OTU173	*Dubosiella*	*25.57*	<0.001
OTU670	*Enterorhabdus*	*22.51*	<0.001
OTU335	*Lachnospiraceae, unclassified*	*20.97*	<0.001
OTU399	*Bradymonadales ge*	*-16.66*	<0.001
OTUs Changes from Indoor Control to Outdoor ZnO pigs			
OTU424	Ruminococcaceae UCG-014	20.62	0.024
OUT399	Bradymonadales ge	-21.55	<0.001
FAECES			
OTUs Changed from Indoor Control to Indoor ZnO pigs			
OTU345	*Prevotellaceae, unclassified*	17.89	<0.001
No changes from Outdoor Control to Outdoor ZnO pigs			
OTUs Changed from Indoor Control to Outdoor Control pigs			
OTU345	*Prevotellaceae, unclassified*	19.93	<0.001
OTU250	*Blautia*	5.17	0.034
No changes from Indoor ZnO to Outdoor ZnO pigs			
OTUs Changed from Indoor Control to OutdoorZnO pigs			
OTU345	*Prevotellaceae, unclassified*	22.56	<0.001

5.3.7.4 Taxonomic classification of bacteria found within pigs reared indoors or outdoors and provided control or pharmacological levels of zinc oxide after weaning

Compared to DeSeq2, which looks at the counts of each OTU and how these vary between groups, relative percentage abundance takes into consideration the percentage abundance of each genera within the bacterial community and how this alters. Analysis of the top 20 genera present in at least one sample above 0.1% within each GIT environment gave a total of 41 genera (Figure 5.4). As seen with phyla, there were no significant differences in relative abundance of genera in the lumen of the jejunum or ileum between pigs reared indoors or outdoors and provided control or pharmacological levels of ZnO (Figure 5.4). In the mucosa of the jejunum, a similar pattern of relative abundance of *Campylobacter* was observed to that of OTU counts with DeSeq2. Specifically, *Campylobacter* was more abundant in indoor control pigs (4%) compared to indoor ZnO pigs (0.8%; $p = 0.045$), outdoor control pigs (0.4%; $p = 0.013$) and outdoor ZnO pigs (0.6%; $p = 0.028$). While *Lactobacillus* showed an increase from indoor control (21.6%) to indoor ZnO (53.0%; $p = 0.004$), its relative abundance decreased from indoor control to both outdoor groups (16.0%, $p = 0.001$; 9.4%, $p < 0.001$, for outdoor control and ZnO pigs, respectively).

As indicated in Figure 5.4, the relative abundance of *Clostridium Sensu Stricto* within the ileal mucosa of outdoor ZnO pigs (28.8%) was significantly higher than outdoor control (5.3%; $p = 0.011$) as well as both indoor-reared pigs (4.1%, $p = 0.005$; 2.7%, $p = 0.002$ for control and ZnO pigs, respectively). *Terrisporobacter* was also higher in outdoor ZnO pigs (5.0%) compared to both indoor control (1.4%; $p = 0.028$) and indoor ZnO pigs (0.7%; $p = 0.005$).

The lumen of the colon of outdoor ZnO pigs was less enriched with *Lactobacillus* compared to indoor control pigs (8.1% vs 27.5%, respectively $p = 0.05$). In conjunction, *Prevotella-2* significantly increased from indoor control (0.6%) to outdoor ZnO in the lumen of the colon (1.9%; $p = 0.048$). There was a decrease in abundance of *Helicobacter* in outdoor ZnO pigs (2.4%) compared to indoor ZnO (6.9%; $p = 0.039$) in the mucosa of the colon. Meanwhile, increases in unclassified *Lachnospiraceae,* unclassified *Prevotellaceae* and unclassified *Ruminococcaceae* were all seen from indoor ZnO to outdoor ZnO pigs. For *Lachnospiraceae* the increase was from 2.2% to 4.3% ($p = 0.021$),

Prevotellaceae was from 1.3% to 7.6% (p = 0.009) and *Ruminococcaceae* from 0.7% to 1.8% (p = 0.035).

In partial support of the OTU count data presented in Table 5.11, the faeces of outdoor ZnO pigs were more enriched with *Prevotellaceae* compared to indoor control pigs (10.6% vs 1.0%, respectively; p = 0.027). While the demise of *Megasphaera* was seen in outdoor ZnO pigs (0.8%) compared to indoor control (4.5%; p = 0.022). In addition, *Parabacteroides* also increased from indoor control (0.3%) to outdoor ZnO pigs (1.9%, p = 0.022).

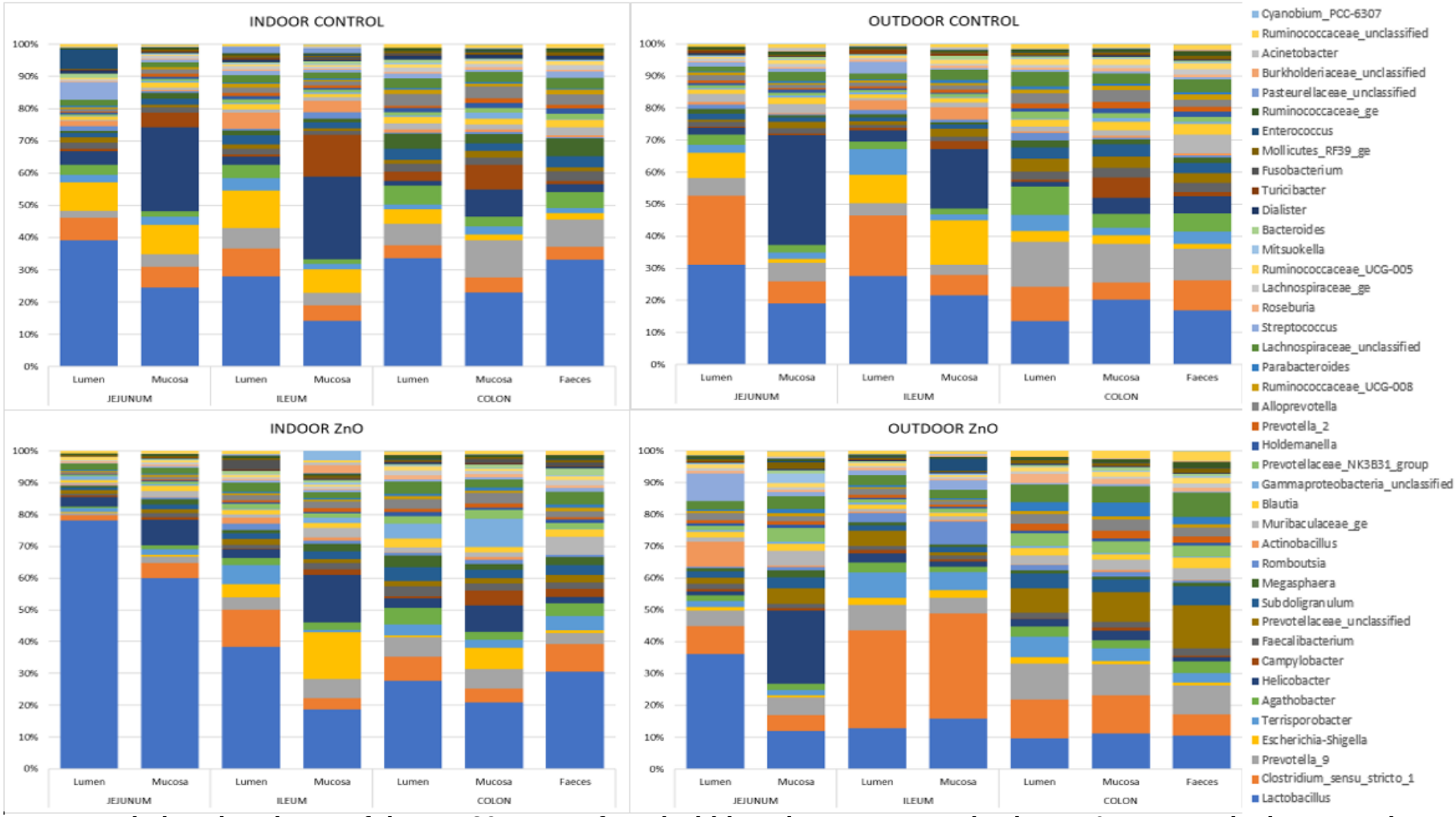

Figure 5.4 Relative abundance of the top 20 genera found within at least one sample above 1%, across the lumen and mucosa of the small (jejunum/ileum) and large (colon) intestines as well as in the faeces of pigs reared either indoors or outdoors and then provided a diet containing control (200ppm) or pharmacological levels (2500ppm) of ZnO after weaning.

5.4 Discussion

Part of the primary hypothesis of this research stated that rearing of pigs outdoors or providing pharmacological levels of ZnO would reduce expression of cytokines and markers associated with inflammation. Results presented in this chapter dispute this hypothesis, as there were no significant differences between all groups of pigs on trial. Given that there were no differences in any inflammatory markers and all levels were within the expected range for healthy pigs, it could be concluded that no pigs within the study were effected by a virulent infection and were all considered healthy, at the time that pigs were sampled (Section 5.3.1 to 5.3.4). Given there were significant improvements in performance of pigs reared outdoors or provided pharmacological levels of ZnO after weaning (Chapter 4), these improvements cannot be associated with benefitting pigs in response to a virulent infection. Performance data in Chapter 4 identified outdoor-reared pigs were consistently heavier from weaning until slaughter, with improved average daily gain (ADG) for the first month after weaning (Table 4.2). In addition, the provision of pharmacological levels of ZnO provided improved weight and ADG when it was included in the diet, until day 14 (Table 4.2). To understand the differences in GIT microbiome of these pigs, 16S rRNA sequencing was used to determine differences in alpha and beta diversity, as well as determining differences in counts of individual OTUs that cause overall shifts in beta diversity. Furthermore, classification of OTUs at the genera and phyla level allow for overall changes in the relative abundance of genera and phyla to be investigated. This analysis compares how the proportion of a community (i.e. a certain genera) changes relative to the rest of the community. In comparison, DeSeq2 analysis of individual OTU counts between groups indicate OTUs that can be responsible for changes within the community composition. Both of these methods are important to look at to give ideas of overall composition and relative changes, as well as individual OTUs within a genera that provide more specific detail. Before determining the effect of rearing environment and dietary treatment on the GIT microbiome composition, the effect of GIT location, including differences between the mucosa and lumen, were identified as location within the GIT is known to influence the microbiome (Chapter 3) (Holman et al., 2017; Quan et al., 2018; Yang et al., 2016; Zhao et al., 2015).

The major phyla across all GIT locations were Firmicutes, Bacteroidetes, Proteobacteria and Epsilonbacteraeota, which is a newly classified phyla, previously within Proteobacteria. Alpha diversity, measured by Shannon, Simpson and Chao1, was lower in the jejunum and ileum compared to the colon and faeces, while beta diversity also showed differences between GIT environments. Unlike in Chapter 3, results presented in this chapter showed differences between the mucosa and lumen of all GIT locations, when looking at beta diversity, with an increase of oxygen-tolerant bacteria such as Proteobacteria and Epsilonbacteraeota within the mucosa-attached microbiome compared to the lumen (Figure 5.2). Remarkably, similar shifts in some bacteria were observed in the small intestine when comparing indoor control pigs to both outdoor groups of pigs and indoor ZnO pigs, which will be discussed later in Section 5.4.3.

5.4.1 Identifying the overall disease status of the pigs used and differences between rearing environment or dietary treatment

Previous research has identified one of the benefits of providing pharmacological levels of ZnO to pigs, after weaning, is the reduced incidence of diarrhoea (Stensland et al., 2015; Heo et al., 2013). Therefore, the first aim of this research was to determine the overall health of pigs used and whether any differences were observed between rearing environment and treatment groups.

5.4.1.1 Faecal scores did not show presence of diarrhoea

Rearing pigs outdoors and the provision of ZnO showed reduced faecal scores after weaning, accepting one of the secondary hypotheses of this research (Hypothesis 2) and supporting previous findings (Lei and Kim, 2018; Cho et al., 2015). While reductions were observed for these pigs, it must be noted that there were no faecal scores relating to diarrhoea during this time. Therefore indoor, control-fed pigs, along with all other pigs, were not showing clinical signs of virulent pathogenic infection. This was further supported by the rectal temperatures of pigs recorded before and after weaning.

5.4.1.2 Rectal temperatures differed with environment and dietary treatment

During the pre-weaning stage, rectal temperatures of piglets were in accordance with previous findings, and were not different between rearing environments, showing no obvious signs of infection (Soerensen and Pedersen, 2015). Rectal temperatures recorded after weaning also supported the absence of clinical signs of infection and were all within the expected range for healthy pigs (MSD, 2015). Recorded temperatures did not go above 40.5ºC, which has previously been associated with the onset of observable infection (Martínez-Avilés et al., 2017). However, in contrast to expectations, pigs reared outdoors had consistently higher rectal temperatures during the first two weeks after weaning and the provision of ZnO also significantly increased rectal temperatures during this time, thus rejecting part of Hypothesis 2 of this research. Although this increase is not associated with observable infection, it is still surprising. Increased rectal temperatures have previously been associated with a decrease in feed intake (Quiniou et al., 2001), which contradicts the results seen in the data presented here, and in feed intake results presented in Chapter 4 during this time (Table 4.2).

One explanation for the increase seen in outdoor pigs rectal temperature during the post-weaning stage, could be the removal of their ability to wallow in mud, which is a behaviour often used in outdoor pigs to self-regulate their body temperature (Bracke, 2011). By moving these pigs indoors after weaning, they lost their ability to exhibit this behaviour, thus potentially resulting in an increase in temperature compared to indoor pigs- who may have adapted to the indoor ambient temperature from birth. Nonetheless, this would not explain the increase in body temperature of pigs fed pharmacological levels of ZnO compared to control pigs. Although it is hard to explain why these differences were observed, it is important to note that the temperatures did not exceed those expected in the presence of infection.

5.4.1.3 Neither rearing environment or pharmacological levels of zinc oxide had an effect on expression of inflammatory markers in the gastrointestinal tract of pigs 14 days after weaning

The results of the qPCR analysis reported in this chapter, suggest that the expression levels of IL-6 and IFN-ɣ, compared to reference genes (β-actin and GAPDH) 14 days after weaning, were not different across rearing environments or dietary treatment, partially rejecting the primary hypothesis of this work. Given there were also no significant differences observed in symptoms of virulent infections, this is perhaps unsurprising. Previous work carried out at the University of Leeds has identified an effect of both rearing environment and pharmacological levels of ZnO on the innate immune response, including the reduction of IL-8 in ZnO fed pigs, although other markers such as tumour necrosis factor α (TNF- α) were not reported to differ. These results were obtained after a deliberate challenge with pathogenic ETEC, which deliberately elicited an immune response. When a deliberate challenge has not been used, decreases in the expression of IFN-ɣ and IL-6 have been seen at day 7 but not 14 after weaning, in response to ZnO (Hu et al., 2013b). Furthermore, Liu et al. (2014) found no differences in expression of IFN-ɣ in response to ZnO in the colon from weaning to three weeks after weaning, without a deliberate pathogenic challenge. Clearly, the effect of ZnO on immune markers can vary, and is likely due to the differences in health of the pigs used. A deliberate exposure to pathogenic bacteria could be required to increase the likelihood of differences being observed. Alternatively, sampling pigs sooner after weaning, when a natural immune response is more likely, may provide significant differences.

5.4.1.4 Neither rearing environment or pharmacological levels of zinc oxide had an effect on inflammatory markers in plasma and faeces

Although differences in markers associated with inflammation within the ileum and colon were not identified, faecal and plasma samples collected at multiple time points closer to weaning could have indicated an inflammatory response occurred earlier than day 14. Before identifying differences at other time points, comparisons were made at day 14, to support the qPCR work. As differences in IL-6 and IFN-ɣ mRNA expression were not observed, it was not surprising that differences were also not seen in concentrations of pig-MAP and calprotectin at the same time point, further disputing the primary hypothesis of this research.

Although no differences were observed with these markers, the similarity of results between both techniques does support the use of these less invasive markers when an inflammatory response is not observed. To successfully identify the use of these markers during a disease incidence, future research should include a deliberate pathogenic infection, such as ETEC, to elicit an immune and inflammatory response.

The results of plasma pig-MAP and faecal calprotectin concentrations reported in this chapter also suggest that rearing environment and dietary treatment did not affect inflammatory markers closer to weaning. This consolidates the assumption that no infectious pathogens were present at sufficient levels to cause ill-health during this research. Concentration of pig-MAP in the data presented within this chapter ranged from 0.08 to 0.21 mg/ml plasma, which was within the expected range provided by the kit manufacturer. Although these values are lower than the reported values for high health herds by Piñeiro et al. (2009) (0.83 mg/ml), they are closer to these levels than reported values of pigs with poor health (1.68 mg/ml) (Piñeiro et al., 2009; Pomorska-Mól et al., 2013). This further emphasises the high health of pigs used for the research presented in this chapter.

Average recovery of calprotectin per sample ranged between 38 – 53 ng/ml of suspended faeces, which was also within the expected range suggested by the manufacturer of this kit. The concentration obtained is also similar to the amount recovered from healthy pigs at 28 days of age (weaning) by Bogere et al. (2019), when using the same extraction kit. Slinger et al. (2019) identified that levels of calprotectin dropped from day 14 to 28 after weaning, although overall concentrations were lower than those described within this chapter, which could be due to the use of a different ELISA kit or, again, in overall health status of the pigs sampled. Interpreting the analysis on inflammatory markers and faecal scores allows the assumption that infectious virulent pathogens, were not present at levels to cause the onset of a response within the present study. As a result of this, any differences in the bacterial composition of pigs reared indoors or outdoors and provided pharmacological levels of ZnO are not in response to a pathogenic infection.

5.4.2 Identifying differences longitudinally and radially within the gastrointestinal tract of all sampled pigs

As previously discussed in Chapter 3, the activity and role of the GIT changes along the length of the small and large intestines, resulting in different microenvironments (Hornbuckle and Tennant, 1997; Maslowski and Mackay, 2011; Kogut and Arsenault, 2016). As different bacterial compositions are naturally expected to reside within the different microenvironments longitudinally along the GIT, they should be considered when determining the effect of external factors, such as rearing environment and dietary treatment on the bacterial composition of the GIT in pigs. Alpha diversity can be used to study the complexity of species diversity within samples and looks at species richness and evenness. Results reported in this chapter support previous work, with increased alpha diversity in the colon and faeces compared to the small intestine (Yang et al., 2016; Quan et al., 2018). Furthermore, the differences in beta diversity between all locations, except colon and faeces support previous work and was not surprising given the differences observed in composition.

The dominant phyla identified in Section 5.3.6 support results of Chapter 3 and previous work, identifying Firmicutes, Bacteroidetes and Epsilonbacteraeota (a new, reassigned phyla, from the class of *Protobacteria)* as most dominant across all samples (Waite et al., 2017; Holman et al., 2017). Although results presented in this chapter show overall lower percentage abundance of these phyla compared to Chapter 3, they are more in line with expected values (Quan et al., 2018; Guevarra et al., 2018). Along the length of the GIT, significant variation in Bacteroidetes, Proteobacteria and Epsilonbacteraeota were found between locations of the small intestine and the colon/faeces, similar to previous findings in pigs (Looft et al., 2014a; Quan et al., 2018; Zhang et al., 2018). Results presented in this chapter support the expectation of bacterial composition changing along the length of the GIT as a result of differing microenvironments within each GIT location, validating preliminary data presented in Chapter 3, accepting part of hypothesis 3 of this chapter, and supporting previous research (Holman et al., 2017; Zhao et al., 2015; Xiao et al., 2018). Specifically, increases of anaerobic Bacteroidetes were seen within the large intestine, while significant decreases of facultative anaerobes such as Proteobacteria, or Epsilonbacteraeota, were seen from the small intestine to the colon and faeces,

as seen previously in both pigs (Zhang et al., 2018; Crespo-Piazuelo et al., 2018; Zhao et al., 2015) and humans (Donaldson et al., 2016).

Different microenvironments can also be expected when comparing the mucosa and lumen within each GIT location as proximity to the mucosal layer exposes bacteria to host-derived oxygen, thus promoting the growth of oxygen tolerant bacteria, such as Proteobacteria (Marteyn et al., 2011; Kelly et al., 2017). Although when comparing all luminal and mucosal samples, there was higher alpha diversity (as measured by Chao1) in the lumen, these results were not replicated when looking at differences within each GIT location. It should also be noted that Chao1 values presented in this chapter were higher than those seen in Chapter 3, but more in line with previous work and expected values (Guevarra et al., 2018; Quan et al., 2018). In terms of beta diversity, the distribution of mucosa-associated bacteria differed from the luminal bacterial composition of all GIT locations in the current research, unlike in Chapter 3 (Appendix D.5). Differences may have been observed as a result of different storage conditions of samples, with improved preservation of some bacterial species seen at -80ºC, which was used in the current research (Bahl et al., 2012). In mucosa, oxygen diffusion from the epithelial capillary network creates an oxygen-rich environment, promoting the colonisation of oxygen tolerant bacteria, such as Proteobacteria and the newly classified Epsilonbacteraeota, which was observed (Albenberg et al., 2014). At the genus level, enrichment of *Campylobacter*, *Helicobacter* and *Pseudomonas* in the mucosa compared to the lumen has also previously been reported (Zhang et al., 2018; Kelly et al., 2017). The mucin-colonizing ability reported for *Campylobacter* and *Helicobacter* support their colonisation of the outer mucus layer (Naughton et al., 2013). Although overgrowth of these genera can disturb the intestinal barrier function, Zhang et al. (2018) concluded that their commensal presence within the mucus layer could stimulate the immunoprotecting function of the gut barrier. As each GIT environment is clearly functionally and microbially diverse, the effect of both rearing environment and dietary treatment were determined within each GIT microenvironment, as stated in Section 5.3.1.

5.4.3 The remarkable similarity of bacterial shifts between rearing environments and provision of pharmacological levels of zinc oxide

A remarkable finding of the research presented in this chapter is the similar shift in bacterial composition within the jejunal mucosa between all outdoor pigs and indoor-reared pigs provided pharmacological levels of ZnO, when compared to indoor, control-fed pigs (Table 5.9). Specifically, the increase of *Marvinbryantia* and *CHKI001* from the family *Lachnospiraceae,* two members of the *Ruminococcaceae* family (unclassified and *Ruminococcaceae UCG-002*) as well as unclassified *Muribaclaceae*. Given the performance data reported in Chapter 4 showed outdoor pigs, and indoor pigs receiving pharmacological levels of ZnO were significantly heavier than indoor control pigs at the point where the samples were collected for 16S sequencing, this could suggest a link between the improved performance and these specific genera. However, it was not possible to determine exact correlations for the purpose of this work. Furthermore, DeSeq2 does not allow for interactions between factors such as rearing environment and dietary treatment to be determined, hence analysis was conducted comparing all four groups. This is a limitation as it is unclear whether the pattern observed with these genera would be considered an interaction, statistically. Nonetheless, **increases in OTUs associated with** *Marvinbryantia* from faecal samples have been associated with high residual feed intake in pigs (Kubasova et al., 2018; Yang et al., 2018). This could be linked to the increased ADFI seen for both outdoor-reared pigs and pigs provided ZnO. This could suggest a move towards a more beneficial microbiome of these pigs, supporting the primary hypothesis of this research. Similar patterns between these four groups of pigs were also seen when looking at the relative percentage abundance of genera, rather than OTU counts. *Campylobacter* was increased in indoor control pigs compared to all outdoor reared pigs and indoor pigs receiving ZnO. The reduction of *Campylobacter* in response to ZnO has previously been reported (Yu et al., 2017). As previously mentioned, the overgrowth of this genera can result in disruption to the intestinal barrier function and subsequent onset of infection, although given the health of the pigs used within this study was high, it is unlikely that there was an overgrowth of *Campylobacter* in any of the pigs (Zhang et al., 2018).

Although no differences were seen in health status of the pigs used within the current research, an increase in bacteria from the Lachnospiraceae family has previously been associated with the microbiomes of healthy pigs compared to those with diarrhoea (Dou et al., 2017). This potentially suggests these genera could also be of benefit to the pigs if they had been exposed to a virulent pathogen. It could therefore be of benefit to include a deliberate infectious challenge to pigs in future work, to identify whether these genera are still influenced. Although the commonality of these bacteria are of interest, identifying causal links between them and subsequent performance would be essential to further this work as correlational analysis was not possible within the timeframe of the work presented here. Although correlational analysis can be beneficial, it cannot provide exact causal links. In human work, the use of germ free mammals, such as mice, has been pivotal in identifying causal links between changes in the human microbiome and diseases such as Parkinson's (Sampson et al., 2016). By transplanting jejunal microbes such as the genera identified here, into germ free animals, causal links could be established. Subsequent research could then focus on identifying how these beneficial bacterial could be provided to pigs to improve their performance after weaning, as a potential alternative to pharmacological levels of ZnO. One method to achieve this would be through the use of probiotics. Probiotics are live microbial feed supplements that can be defined as 'a preparation or a product containing viable, defined microorganisms in sufficient numbers, which alter the microbiota in a compartment of the host, and by that exert beneficial health effects on the host' (Schrezenmeir and de Vrese, 2001). This means that if causal links were established between the bacteria and performance benefits to the host, they could be developed into a probiotic to work within the jejunum of the pig.

5.4.4 Pharmacological levels of zinc oxide had minimal effect on alpha and beta diversity but did alter composition of the small intestine

The provision of pharmacological levels of ZnO did not alter measures of alpha diversity within any GIT location, contrary to expectations and disputing aspects of the primary hypothesis of this study. The effect of ZnO on intestinal diversity in previous research has been varied, with some reporting increases in alpha diversity as a result of ZnO or antibiotic-treatment, in the small intestine of indoor-reared pigs (Yu et al., 2017; Wei et al., 2020), while others have seen reduced

diversity (Shen et al., 2014; Namkung et al., 2006). More agreement is shown in the reduced diversity in the colon in response to ZnO in the diet (Yu et al., 2017; Rattigan et al., 2020). Measures of beta diversity were not affected by the provision of ZnO in all GIT locations within the data presented in this chapter. This disputes results presented in Chapter 3 of this thesis, however, is in more agreeance with previous published studies (Yu et al., 2017). The differences across the two chapters of this research could be as a result of different health status of the pigs used (unknown health of pigs used in Chapter 3) or an increased sample size and improved storage technique in the latter work (-80ºC). Particularly as long-term storage of bacterial samples at -20ºC, as used in Chapter 3, can alter the abundance of bacteria upon analysis (Bahl et al., 2012).

Although overall beta diversity was not affected by the provision of ZnO, there were still some individual OTUs that changed in abundance as a result of ZnO. Overall measures of beta diversity do not take into account the total number of reads obtained for a sample or reflect the absolute number of microbes present (Weiss et al., 2017). As a result, differences may not be evident in overall beta diversity, while absolute count of individual OTUs can still show as being significantly different between groups. Although individual OTUs make up a small aspect of the entire bacterial population, significant differences in their abundance between groups should still be investigated as their change in abundance may still be directly linked to benefits seen, such as with performance.

The changes seen in OTUs in response to ZnO are more in accordance with Zn availability, as the majority of changes in OTUs were observed within the mucosa of the small intestine of both indoor and outdoor reared pigs. This is expected given the higher availability of Zn as free Zn^{2+} ions within the proximal parts of the intestine, as a result of increasing pH in the large intestine that reduces the solubility of Zn (Starke et al., 2014). The changes seen partially support the primary hypothesis as increases in potentially beneficial bacteria are seen with ZnO. As previously discussed in Section 5.4.3, the changes seen from indoor control pigs to indoor and outdoor pigs receiving ZnO was similar; with increases in bacteria that have been reported to be associated with improved feed intake (Kubasova et al., 2018). Further patterns were revealed in the ileum when providing pharmacological levels of ZnO, as four of the five genera that changed from control to ZnO fed pigs reared indoors, were also seen between outdoor

control and ZnO pigs. Although the genera that changed have not been widely reported in the literature in pigs or humans.

Zinc oxide has previously been effective at reducing counts of the *Helicobacter* genus, including species such as *Helicobacter pylori* (Yu et al., 2017). *Helicobacter pylori* is responsible for causing inflammation of the stomach (gastritis) and parts of the small intestine in both humans (Blaser and Atherton, 2004) and pigs (Sharma et al., 2010; Mann et al., 2014). In data presented within this chapter, *Helicobacter* was reduced in the ileal mucosa of outdoor pigs receiving ZnO compared to outdoor control pigs, supporting Yu et al. (2017). In addition, outdoor-reared pigs provided ZnO also had reduced counts of the OTU associated with *Helicobacter* compared to indoor pigs given ZnO. Although it has previously been concluded that the commensal presence of *Campylobacter* could stimulate the immunoprotecting function of the gut barrier, overgrowth can disturb the intestinal barrier function so it is difficult to draw conclusions on the exact benefit or disadvantage of this bacteria (Zhang et al., 2018). In accordance with previous work, OTUs associated with *Lactobacillus* decreased in outdoor pigs receiving ZnO compared to control pigs. This effect of ZnO has been widely reported for indoor-reared pigs (Højberg et al., 2005; Starke et al., 2014). Although perhaps surprisingly, no differences were observed in this genera between indoor pigs receiving control or pharmacological levels of ZnO.

5.4.5 Pre-weaning rearing environment effected bacterial diversity of pigs 14 days after weaning

The exposure of outdoor-reared pigs to soil-borne microorganisms and plant-derived compounds (carbohydrates, fibres) from soil, pasture and straw can be logically assumed to increase microbial diversity, and alter the abundance of particular genera associated with fibre and carbohydrate fermentation within the GIT, compared to pigs reared in a less diverse environment, indoors. A more diverse microbiome can be expected to be of more benefit to the host as it can reduce the opportunity for infectious agents to colonise, thus reducing the likelihood of disease (Keesing et al., 2010). Previously, pigs exposed to soil from outdoor environments have shown significant increases in species richness and evenness in the large intestine and faeces, two weeks after weaning, compared to indoor-reared pigs (Pluske et al., 2007; Vo et al., 2017). Results presented in Table 5.2 support these previous findings, with Shannon and Simpson diversities

showing a tendency to increase in the colon and Shannon diversity significantly increasing in faeces. Furthermore, both Simpson and Shannon diversity were significantly higher for outdoor-reared pigs in the jejunum, indicating increased richness and evenness of bacterial species and supporting the primary hypothesis of this study. Although Chao1 values presented within this chapter are higher than the values shown in Chapter 3, they are more in line with the expected values within the GIT (Guevarra et al., 2018; Quan et al., 2018). Values may also be higher due to an increase in the number of samples used, meaning a higher number of species are more likely to have been recovered. Differences between the microbial communities from indoor and outdoor pigs, as determined by beta diversity, showed environment effected all GIT environments apart from the lumen of the ileum.

5.4.5.1 Different pre-weaning rearing environments resulted in different bacterial composition within the gastrointestinal tract of pigs

The exposure of outdoor-reared pigs to plant-derived compounds such as carbohydrates and fibres as well as bacteria commonly found in soil, such as *Bacteroidetes* (Thomas et al., 2011), is the likely cause of the greatest increase of *Muribaculaceae* in all GIT locations of outdoor-reared pigs compared to those indoors. The *Muribaculaceae* family, previously known as S24-7, has been associated with the degradation of complex carbohydrates, which outdoor pigs would likely have increased exposure too (Lagkouvardos et al., 2019; Ormerod et al., 2016). Although surprisingly, Prevotella 6 decreased in the jejunum of outdoor reared pigs compared to those indoors. Prevotella is known to be dominant in the GIT microbiota of mammals and humans that consume a diet rich in plant polysaccharides and fibre, as it is linked to fermentation of plant-derived non-start polysaccharides to SCFAs (Ivarsson et al., 2014). Therefore the increased exposure to plant polysaccharides of outdoor pigs would be expected to increase this genera in their GIT.

Although *Prevotella* reduced in the jejunum, an increased relative abundance of *Prevotellaceae,* was seen in the colon of outdoor reared pigs. The increase of these bacteria in the colon can be of particular benefit as *Prevotella* species can produce acetate in the gut, providing a source of energy for butyrate-producing bacteria such as *Ruminococcaceae* (Looft et al., 2014b). *Ruminococcaceae* also increased in the colon of outdoor-reared pigs; this family are known to be present

within soil, and are capable of degrading complex polysaccharides and fibres to produce SCFAs, such as butyrate (Thomas et al., 2011). Not only has an increase in butyrate been reported to reduce inflammation within the GIT (Looft et al., 2014a), it is also an energy source for colonocytes (Thomas et al., 2011). In Chapter 3, the increase in *Prevotellaceae* and *Ruminococcaceae* was linked with age and a more stable GIT composition. This could suggest that the GIT of outdoor-reared pigs matures faster than pigs reared indoors, which was also a conclusion made by Vo et al. (2017). Given the known presence of *Ruminococcaceae* in soil, their increase in the GIT of outdoor-reared pigs could be as a result of increased exposure to these genera within the soil. It could be beneficial in future research to include 16S rRNA sequencing of the soil microbiome to identify similarities between the composition of soil and the GIT of outdoor-reared pigs.

5.5 Conclusion

Rearing pigs outdoors before weaning or providing pharmacological levels of ZnO after weaning showed no differences in the inflammatory response of pigs, rejecting aspects of the primary hypothesis. In addition, faecal scores, rectal temperatures and markers of inflammation within the GIT, blood and faeces of sampled pigs within this study indicate that pigs were healthy and showed no sign of a virulent infection. Dietary ZnO had minimal effects on the bacterial diversity of pigs 14 days after weaning. However, of the few differences observed in OUT counts, the majority were within the small intestine. In comparison, rearing pigs outdoors increased some alpha diversity measures in the jejunum and faeces of pigs and affected beta diversity, accepting aspects of the primary hypothesis of this study. Remarkably, when looking at individual OTU counts between indoor- and outdoor-reared pigs given either control or pharmacological levels of ZnO, similar patterns of changes were observed within the small intestine, highlighting four genera that significantly increased from indoor control pigs, to indoor ZnO-fed pigs and all outdoor-reared pigs. These findings warrant further investigation to determine correlations and potential causal links between the genera that are more abundant in pigs that also had improved performance, as seen in Chapter 4. If causal links can be identified then the use of these bacteria in probiotics for pig diets could be an area of future development as an a alternative strategy to improve pig performance after weaning.

Chapter 6
General Discussion

Pharmacological levels of zinc (~2500 ppm) in the form of zinc oxide have been widely used in the diet of pigs for 14 days after weaning, showing improvements in growth rate, as seen in Chapter 4 and in previous research (Stensland et al., 2015; Heo et al., 2013). These levels of ZnO have also been shown to reduce the incidence of post-weaning diarrhoea (PWD) (Heo et al., 2010). Post-weaning diarrhoea can pose a significant economic loss to the pig industry; therefore, ways to reduce it are crucial areas for continued investigation (Zhou et al., 2016; Klose et al., 2010). Pharmacological levels of ZnO can impact the host in a variety of ways, including reports of increased villous height: crypt depth ratio within the gastrointestinal tract (GIT) and reduced expression of genes involved in the innate immune response, as determined through *in vitro* work (Zhu et al., 2017; Sargeant et al., 2010). In addition, the development of next generation 16S rRNA sequencing technologies, has increased understanding of the changes within the GIT microbiome after the provision of ZnO (Yu et al., 2017; Starke et al., 2014; Vahjen et al., 2015) .

Concerns relating to pharmacological levels of Zn being excreted into pig slurry and subsequently being spread onto agricultural lands; increasing soil and groundwater concentrations have led to the upcoming ban of these levels of ZnO across the EU in 2022 (Monteiro et al., 2010; Directorate, 2017). These concerns are alongside fears that high levels of Zn are reported to potentially contribute to the acquisition and spread of antibiotic resistant genes in bacteria, through horizontal gene transfer (Yazdankhah et al., 2014; Ciesinski et al., 2018). Although alternatives to ZnO are often dietary alternatives, additional factors such as management techniques of pigs are also an area that warrant further investigation. The UK pig industry is unique, as 40% of the sow breeding herd are managed in outdoor production facilities (AHDB, 2021c). Although rearing piglets outdoors comes with challenges, previous work has identified beneficial effects on pig welfare and pre- and post-weaning performance that could outweigh the benefits of providing ZnO after weaning (Cox and Cooper, 2001; Miller et al., 2009; Gentry et al., 2002). The exposure of outdoor pigs to an increased abundance of bacteria within the soil, alongside complex carbohydrates in their external environment are the potential cause of an increase

in bacterial diversity and acceleration of gut maturation reported after weaning (Vo et al., 2017; Pluske et al., 2007).

The comparison of indoor and outdoor produced pigs during the pre-weaning stage, in addition to the provision of pharmacological levels of ZnO after weaning, is an area that has not been extensively investigated. Studies that have been conducted have often included a deliberate pathogenic challenge, the use of antimicrobial growth promotors (AGPs) and/or not always assessed lifetime performance benefits (Miller et al., 2009). Therefore, one of the aims of this research were to identify the lifetime performance benefits of rearing pigs in either indoor commercial farrowing pens or outdoor paddocks and arks, and then provided control (~200 ppm) or pharmacological levels of ZnO (~2500 ppm) for 14 days after weaning. In addition, this work aimed to develop a method of analysing 16S rRNA sequencing data to identify influencing factors, including pharmacological levels of ZnO, on the GIT microbiome, in the absence of a deliberate pathogenic challenge. Then, to use this method of analysis to investigate the effect of rearing environment and subsequent provision of ZnO on the GIT microbiome, when no obvious pathogenic challenge was present.

6.1 Outdoor rearing provided heavier pigs and a similar shift in bacterial composition within the gastrointestinal tract compared to zinc oxide

The primary hypothesis of this research stated that rearing pigs outdoors or providing pharmacological levels of ZnO would show improved lifetime performance, a more diverse bacterial composition in the pigs GIT and reduced GIT inflammation. Findings in this thesis showed that rearing piglets outdoors, with the ability to free-roam individual paddocks, provided lifetime performance benefits, enabling a higher percentage of outdoor-reared pigs to be sent to slaughter at day 116, compared to pigs that had been reared indoors (Chapter 4). Although ZnO improved ADG and ADFI for the first two weeks after weaning, this improvement was short-lived and differences were not maintained past its inclusion in the diet, partially rejecting the overall hypothesis of this research. Furthermore, the provision of ZnO showed more of a benefit to indoor-reared pigs, compared to those outdoors. Alongside the performance benefits seen, the differences in bacterial composition of outdoor reared pigs and pigs given ZnO is of significant interest. Although ZnO did not increase bacterial richness and evenness within the GIT, outdoor rearing of pigs did, and both outdoor-reared

pigs and pigs receiving ZnO showed similar changes in their bacterial composition compared to indoor-reared, control-fed pigs, supporting the primary hypothesis of this research. Specifically, in the mucosa of the jejunum, the shift in composition of pigs reared outdoors was near identical to the shifts seen when providing ZnO to indoor pigs, when compared to indoor, control-fed pigs. This shift in bacteria indicate a potential benefit of these genera, which included members of the *Lachnospiraceae* family (*Marvinbryantia* and *CHKCI001*) and *Ruminococcaceae* family. *Marvinbryantia* has previously been associated with improved feed intake of pigs but direct causal links between the inclusion of these bacteria and subsequent improved performance is essential. Future work should include correlational analysis and methods to determine causal links, through the use of gnotobiotic animals or faecal microbial transplants.

6.1.1 Identifying causal links between bacterial composition of the gastrointestinal tract of outdoor reared pigs or pigs receiving zinc oxide

Although the work presented in this thesis shows, for the first time, the similarity in microbial shifts within the small intestine of outdoor-reared pigs and indoor pigs receiving ZnO, it does not provide a causal link between the microbiome and subsequent performance improvements. Correlational analysis between microbial composition and performance of pigs in this study was not conducted due to time restraints to complete the analysis; however, this form of analysis would be useful to determine potential links between the common bacteria that changed in the GIT of these pigs. A review conducted by Nowland et al. (2021) showed bacteria that has previously been correlated with performance of young pigs, which showed that bacteria from both the *Lachnospiraceae* and *Ruminococcaceae* families were correlated with increased abundance in piglets not displaying PWD (Mach et al., 2015; Dou et al., 2017). Although correlational research can be advantageous in determining possible links between these factors, a major disadvantage is that it cannot definitely determine causal links (Curtis et al., 2016).

The use of gnotobiotic animal models in previous research to determine causal links between bacterial composition changes and their associated impact on, for example, disease, has been pivotal (Fritz et al., 2013). Therefore, to determine whether the effect of both rearing environment and dietary provision of ZnO on

the GIT microbiome is the direct cause of performance benefits seen in Chapter 4, the use of germ-free models and/or microbial transplants should be an area of further research. The use of germ-free mice as models for humans have previously identified causal links between the gut microbiota and Parkinson's disease, through microbial transplants (Sampson et al., 2016). Furthermore, the use of faecal transplantation into mice has also been essential in establishing a causal link between the GIT microbiota, host susceptibility to *Clostridium difficile* and subsequent identification of infection resistance, when *Clostridium scindens* was present (Buffie et al., 2015). Given that pigs, as well as mice, are used as human models due to similarities in their GIT structure, the transplant of GIT microbiota from pigs reared outdoors, or provided ZnO into mice could be an avenue of further research (Zhang et al., 2013). Specifically, the transplant of GIT contents, particularly the inclusion of the common bacteria that altered in outdoor pigs and indoor pigs that received ZnO supplementation, could be of benefit to identify potential associations with performance. This could prove or disprove a causal link between the GIT microbiome and subsequent growth performance in pigs.

The use of germ-free piglets have also been used as a human model with success in identifying a donor-like microbial community with limited individual variation (Pang et al., 2007). Germ-free pigs could therefore also be considered for this work, although mice would likely allow for increased replication and validation of results, initially. The use of pigs for faecal transplants, without being germ-free has also shown positive results in identifying causal effects of the microbiome of different pig breeds on GIT morphology, digestion and barrier function, which could be of interest in determining the overall effects of environment and ZnO in pigs (Diao et al., 2018). Future research in this area could provide mechanistic understanding as to whether the microbiome causes differences in immune response, which would be of particular interest after the provision of an artificial bacterial infection, such as the use of ETEC K88 in Sargeant et al. (2010) work.

If causal links can be identified between the common bacteria that changed from indoor control-fed pigs to all other groups, and the improved performance of these pigs, then commercial application would need to be considered. One avenue for stimulating establishment of beneficial GIT microbes is through the provision of

probiotics in animal feed. Probiotics are live microbial feed supplements that have been defined as ‘a preparation or a product containing viable, defined microorganisms in sufficient numbers, which alter the microbiota in a compartment of the host, and by that exert beneficial health effects on the host’ (Schrezenmeir and de Vrese, 2001). The most common bacterial probiotics for pigs include *Lactobacillus* spp., *Enterococcus* spp., *Pediococcus* spp., and *Bacillus* spp., which typically target the caecum and colon (Chaucheyras-Durand and Durand, 2010). Previous research has shown the benefits of using lactic-acid producing bacteria, such as *Lactobacillus*, as alternatives to antibiotics, with positive responses to growth performance parameters and in some studies, reduced PWD (Shu et al., 2001; Taras et al., 2006). However inconsistencies are often seen in the benefit of these probiotics, which has been attributed to differing doses and types of strain used (Heo et al., 2013).

It is important for any probiotic to be able to survive in the gastric acidic environment with bile salts within the GIT, which would need to be considered in development of probiotics to include or promote growth of *Lachnospiraceae* and *Ruminococcaceae* bacteria. However, if causal links were determined between these bacteria and subsequent performance benefits, and probiotics could be developed to ensure growth of these bacteria within the small intestine of pigs, this could provide a feasible alternative to the provision of ZnO, without suggesting all pigs should be reared outdoors. This could be particularly beneficial as it is not feasible to expect that all UK farmers could rear pigs outdoors due to the increased space that would be required. Furthermore, the cost to farmers that currently rear their piglets indoors would need to be considered. A more realistic approach would be to identify the beneficial aspects of outdoor production, such as bacterial shifts in the GIT from soil, or whether there are other elements of outdoor pig production that result in the improved performance seen after weaning.

6.1.2 Investigating other beneficial effects of outdoor rearing

Although previous research has reported that exposure to soil can increase the rate of maturation of the GIT microbiome in pigs after weaning, which was also indicated in the research presented in Chapters 5 (Vo et al., 2017), understanding the maturation of the GIT through the use of immunohistochemistry and further histological analysis could be beneficial. For example, staining of GIT samples

for markers of gut maturation, such as intestinal fatty acid binding protein could be included in future work, from multiple time points before and after weaning. In lambs, the staining of ileal tissue samples has identified a distribution pattern along the crypts of epithelial cells overtime, which has also been reported in human tissue samples (Reisinger et al., 2014). Faster maturation of the GIT of pigs reared outdoors, alongside shifts towards a beneficial microbiome seen in the current research, could further explain the improved performance of pigs after weaning.

Differences in pasture types could impact performance benefits that were seen within the current research, presented in Chapter 4. Previous research has identified that different soil types can compromise health and welfare of sows and their piglets, such as an increase in piglet mortality when reared on poached soils, given their favourable conditions for pathogens (Pietrosemoli and Tang, 2020). Although the paddocks used in the current experiment were beneficial to pig performance during the summer months, it could be valuable to replicate the study during winter. Alongside this work, understanding climatic differences, groundwater level and soil composition, could be beneficial as these may all influence the GIT development of pigs and their subsequent performance. Further studies, under similar conditions to those used in the present study, could also involve the analysis of soil and vegetation composition, including assessment of bacterial composition and levels of organic acids, vitamins and carbohydrates often found in soil (Vranova et al., 2013). This could determine whether it is the soil microbiome that directly affects the pigs GIT microbiome or whether there are additional aspects of soil and pasture that can be linked to improved performance. Any differences identified within such research could then be compared to the provision of creep feed to indoor-reared piglets.

Although it is speculated that greater environmental complexity and exposure to nutrients and bacteria within the soil and vegetation results in the improved performance of outdoor reared pigs, other factors leading to improved welfare, such as decreased stocking density and improved air quality (less shared airspace) may also play a role. Although decreased stocking density in outdoor production is beneficial for pig welfare, the logistics of rearing all UK pigs outdoors is unrealistic due to the land-use change and cost to move production systems, for farmers already set-up to rear piglets indoors. A decreased stocking density

results from an increase in space per litter compared to intensive indoor systems. Therefore, to increase the number of pigs reared outdoors would require substantially more land, potentially taking land away from crop growth, making the shift in production unrealistic (Guy et al., 2012). Changing use of land to increase outdoor pig farming would also not be in line with the UK government roadmap for increasing habitats for nature recovery and new areas of woodland (DEFRA, 2020). Nonetheless, identifying whether it is the exposure to the environment itself, or other factors such as stocking density or improved air quality, could be conducted through the use of a barren outdoor environment and a typical pasture paddock.

6.2 The effect of zinc oxide on the gastrointestinal microbiome and health of pigs

The use of pharmacological levels of ZnO in the diet of pigs for 14 days after weaning has been used for many years. However, the benefits of ZnO on pig performance and health have been varied (Stensland et al., 2015; Broom et al., 2003; Heo et al., 2010). The research presented in this thesis shows the beneficial effect of pharmacological levels of ZnO on ADG, ADFI and weight for the first two weeks after weaning. However, the improvements seen were short lived and no performance benefits were seen once ZnO was removed from the diet at day 14, disputing the overall hypothesis of this research (Chapter 4). As demonstrated in Chapter 3, the effect of ZnO on the microbiome of pigs is predominantly seen 14 days after weaning, with minimal prolonged effects, similar to performance data. This is why subsequent analysis of the microbiome was focussed on day 14 in Chapter 5, whereby some differences were again seen in response to ZnO.

The experiments conducted and presented in Chapters 3, 4 and 5 involved pigs that were not deliberately challenged with a bacterial pathogen, such as ETEC. Samples used for research in Chapter 3 were collected prior to the start of the current PhD, so health status of pigs is unknown, besides no deliberate pathogenic challenge. However, for the work in Chapters 4 and 5, it is evident in faecal scores and inflammatory markers that the overall health of pigs used within that research was high, with no obvious disease challenge. Although differences in the bacterial composition were identified between rearing environments and dietary treatment, differences in markers of inflammation, both within the GIT as

well as plasma and faecal samples were not observed (Chapter 5). This is likely as a result of the pigs used across the trials not being exposed to a deliberate challenge to their immune system and suggests that improved performance was not dependant on pigs being infected. Previous work conducted at the University of Leeds on the impact of ZnO has used an artificial challenge of ETEC K88, which is a strain that causes PWD in pigs, and identified significant decreases in expression of immune response genes associated with inflammation, including chemokines such as CXCL6 and CXCL1 with ZnO (Sargeant et al., 2010). This could suggest that the use of an artificial challenge could be beneficial when trying to identify differences in host immune response to rearing environment and ZnO. Due to Home Office building requirements, a disease challenge was not feasible for the work completed for this thesis, but should be considered again for future work when looking at immune responses.

6.3 Confirmation of alternative factors that should be considered when completing 16S rRNA sequencing

The bacterial composition of the GIT can be significantly affected by environmental factors such as diet and immediate surroundings as well as host factors such as genetics, sex, age and disease status (Chen et al., 2018; De Filippo et al., 2010; Goodrich et al., 2014). Results presented in Chapter 3 identified bacterial succession from weaning to 28 days after weaning, which has been described in detail previously in both humans (Penders et al., 2006; Leser and Mølbak, 2009) and pigs (Frese et al., 2015; De Rodas et al., 2018). In support of previous work, significant differences in diversity were observed between the small and large intestine in Chapters 3 and 5, emphasising the inadequacy of faecal samples in defining the microbiome of the small intestine and the proximal large intestine. However, differences in the composition of the ileum and colon between experiments were seen. Storage techniques can alter the composition of bacteria within samples, as discussed in Section 5.4.2. This may also have resulted in differences in the effect of sample type (luminal/mucosal) between the experiments. Experiment 1, presented in Chapter 3, showed no differences between the sample types, while results of experiment 2, presented in Chapter 5, did, which was more in line with expected results (Marteyn et al., 2011; Kelly et al., 2017). The second experiment used a snap freezing method to instantly

freeze bacteria and samples were subsequently stored at -80ºC compared to -20ºC for experiment 1.

Host genetics can also impact bacterial composition, which has been reported in humans (Goodrich et al., 2014) and pigs (Chen et al., 2018). Both experiments included the artificial insemination of sows with single sire semen, to reduce genetic differences as much as possible, within each experiment. However, sow and boar genetics were different between both experiments presented in Chapters 3 and 5; this could potentially explain some of the compositional changes observed in the microbiome. Although it is acknowledged that host genetics can affect the microbiome of pigs, analysis of results presented in Chapter 5 did not factor in the effect of batch as this was not a primary area of interest, but does warrant further investigation due to the increase in genetic differences, particularly between batches. In addition, the effect of sex was not considered across results presented in Chapters 3 and 5, but is also known to potentially cause differences in the microbiome (Xiao et al., 2016).

A disadvantage of 16S rRNA sequencing on platforms such as the Illumina ones used across the work presented in this thesis, is the inability to accurately identify bacteria below the genus level of classification. Upcoming technology, termed third generation sequencing technology, includes the use of Oxford Nanopore technology (Winand et al., 2020). The use of Oxford Nanopore technology allows for much longer sequence reads, going from the typical 300 base pairs in Illumina sequencing, to full length reads, classifying down to the species level of bacteria (Winand et al., 2020). This could provide a better insight into species of bacteria that are changing, given species can be highly varied within a genus. However, as this technology is still in development, it also has limitations, including higher read error rates with ~40% of species being misclassified, compared to over 99% being correctly classified at the genus level (Winand et al., 2020). Nonetheless, this is a technology that should be further investigated to determine changes at the species level within the pig GIT in the future.

6.4 Considering further aspects of outdoor pig production compared to pharmacological levels of zinc oxide

One of the main concerns leading to the upcoming ban of pharmacological levels of ZnO in post-wean diets results from approximately 80% of Zn being excreted

in faeces, increasing Zn leaching in soil (Buff et al., 2005). Zinc leaching in soil can become toxic to microorganisms and plants and is therefore a leading environmental concern (Gräber et al., 2005). Zinc contaminated agricultural soil (400 mg kg^{-1}) has shown a decrease in bacterial diversity and caused stunted crop growth for pea seed and barley grain, leading to a yield two tonnes per hectare less compared to soils without Zn toxicity in the UK (57 mg kg^{-1})(Moffett et al., 2003). Furthermore, additional concerns relating to the acquisition and spread of antibiotic, and in some cases heavy metal, resistant genes in bacteria through horizontal gene transfer, support the requirement for ZnO alternatives to be sought (Yazdankhah et al., 2014; Ciesinski et al., 2018).

Given the performance benefits reported in Chapter 4, rearing of pigs outdoors could be considered an alternative to providing pharmacological levels of ZnO after weaning, potentially enabling reduced levels of ZnO to be used. Although reducing the level of ZnO within the diet of pigs after weaning could reduce the potential for Zn leaching, outdoor production also has concerns relating to sustainable production that mean it is unrealistic to expect all UK farmers to move to outdoor rearing of piglets. Outdoor pigs require more space per litter than intensive indoor systems and require land that could otherwise be used for growing grains or pasture (Guy et al., 2012). However, other factors are reduced, such as the overall cost of outdoor rearing per sow compared to standard farrowing crates used on indoor units due to reduced building and maintenance cost. Although this reduced cost is only applicable in comparing production systems that are already set up for indoor or outdoor-rearing, the cost to move production from an indoor system, to an outdoor one, would incur significant costs that individual farmers would need to consider. Costs are often a significant factor in determining the viability and sustainability of a commercial farm, alongside overall environmental sustainability of the industry. Therefore, there must be a balance between economic, environmental and societal sustainability.

Besides rearing pigs outdoors, identifying ways to expose indoor pigs to the beneficial aspects of outdoor rearing, such as *Lachnospiraceae* and *Ruminococcaceae* bacteria through probiotic use if causal links were established, could reduce the requirement for pharmacological levels of ZnO, while mitigating the need for increased land for outdoor pig production. This could potentially reduce the level of Zn used and, based on performance data presented in

Chapter 4, could enable a higher percentage of pigs to be sent to slaughter at an earlier age. This would only be feasible if causal links can be determined between these bacteria and the performance seen, otherwise other factors that differ between indoor- and outdoor rearing systems would also need to be investigated. Faster growth and reduced time to slaughter has been suggested to reduce the relative environmental impact per 1 kg of live pig weight (Ottosen et al., 2021). Although consumers favour pork products from pigs reared outdoors due to increased welfare, a 2019 survey revealed that cost was the driving factor for pork products, meaning more indoor produced pork is consumed and should remain a driving factor when determining best practice for the pre-weaning stage of production (AHDB, 2019).

6.5 Conclusions

Finding alternative ways to improve the health and performance of pigs after weaning is imperative for the UK industry, as the ban on pharmacological levels of ZnO is approaching. Dietary alternatives to ZnO are readily researched, but the effect of different management strategies on pig performance also warrants further investigation in identifying new ways to improve performance and health of pigs after weaning.

The provision of pharmacological levels of ZnO showed significant improvements in performance for the first 14 days after weaning, but did not show lifetime benefits. It was also during this time that the majority of changes in bacterial composition within the pig GIT were seen, although increases in bacterial richness were not observed, as expected. A key finding of this research was that rearing of piglets in an outdoor environment showed lifetime performance benefits, allowing a higher percentage of outdoor pigs to be sent to slaughter at day 116, accepting part of the primary hypothesis of this research and showing significant commercial benefit of outdoor rearing. A further key finding of this research was the similar effect on the bacterial composition of the small intestine of pigs reared outdoors or provided ZnO. Changes in the bacterial composition showed similar shifts in genera in the small intestine of outdoor-reared and ZnO-fed pig, with the addition of changes in the colon associated with the breakdown of plant poly-saccharides, that outdoor pigs would have had greater exposure too. No differences were seen in markers of the immune and inflammatory response within the GIT, blood or faeces, which indicates no obvious disease

challenge was present in these pigs. Further research should consider the use of correlational analysis and potentially gnotobiotic animals and/or microbial transplants to identify causal links between the microbiome and improved performance of these pigs. Although the performance benefits and shifts in bacterial composition identified in the research presented in this thesis show the benefit of rearing pigs outdoors, given the land-use change and cost associated with rearing all UK pigs outdoors, it is perhaps not feasible to expect this to be a suitable replacement to pharmacological levels of ZnO. Instead, future work should now focus on identifying the specific aspects of outdoor rearing that cause these improvements. This could include identifying potential causal links between bacterial composition and performance of outdoor-reared pigs, which could allow for new probiotics to be developed to promote growth of these bacteria within the GIT, acting as a possible alternative to pharmacological levels of ZnO.

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Appendix A

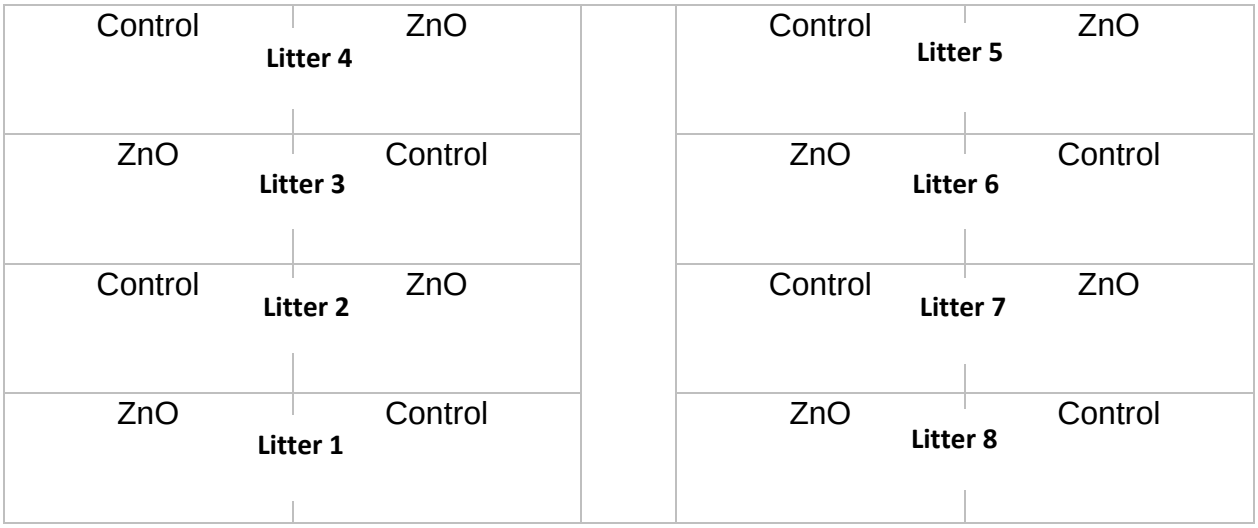

Figure A. 1 Pen layout for the sampling room in Trial 1. Showing litter number, split between pens and their allocated treatment.

BATCH 1- Performance

Litter 7	Litter 8		PERFORMANCE ROOM	Litter 3	Litter 4
OUT- ZnO				IN-ZnO	
Litter 5	Litter 6			Litter 1	Litter 2
Litter 3	Litter 4			Litter 7	Litter 8
IN-CONTROL				OUT-CONTROL	
Litter 1	Litter 2			Litter 5	Litter 6

BATCH 1- Sampling

Litter 3	Litter 4		SAMPLING ROOM	Litter 7	Litter 8
IN-CONTROL				OUT- ZnO	
Litter 1	Litter 2			Litter 5	Litter 6
Litter 7	Litter 8			Litter 3	Litter 4
OUT-CONTROL				IN-ZnO	
Litter 5	Litter 6			Litter 1	Litter 2

*litter 6 in performance room was an indoor litter, not outdoor (not sufficient numbers)
*Both pens of litter 1 in performance room were separately taken off trial prior to D29 and excluded from subsequent analysis

BATCH 2- Sampling

Litter 15	Litter 16		SAMPLING ROOM	Litter 11	Litter 12
OUT-CONTROL				IN-CONTROL	
Litter 13	Litter 14			Litter 9	Litter 10
Litter 11	Litter 12			Litter 15	Litter 16
IN-ZnO				OUT- ZnO	
Litter 9	Litter 10			Litter 13	Litter 14

*Litter 9, ZnO and litter 10, Control were taken off trial prior to D29 and excluded for subsequent analysis.

Figure A. 2 Pen layout for 'sampling' and 'performance' rooms in batch 1 and 2 in Trial 2. Showing litter number, split between pens and their allocated treatment.

Appendix B

Table B. 1 DeSeq2 analysis results for OTUs classified to the genera level that significantly changed between the ileum and caecum at day 28, irrespective of dietary treatment. P values are adjusted for multiple testing using the Benjamin-Hochberg correction. Fold changes represent Log2 fold changes.

OTU Number	Phyla	Genera	Base mean	Fold Change	P value
Increased from ileum to caecum					
OTU 12	Bacteroidetes	Muribaculaceae unclassified	32.52	2.45	0.002
OTU 23	Firmicutes	Ruminococcaceae ge	19.94	3.13	0.002
OTU 16	Bacteroidetes	Muribaculaceae ge	20.02	2.29	0.003
OTU 11	Firmicutes	Veillonellaceae unclassified	136.72	2.12	0.004
OTU 01	Firmicutes	Megasphaera	2495.96	1.69	0.005
OTU 69	Firmicutes	Family XIII UCG-001	25.23	2.73	0.005
OTU 59	Epsilonbacteraeota	Campylobacter	13.67	2.93	0.016
OTU 07	Firmicutes	Mitsuokella	948.51	1.70	0.020
OTU 06	Firmicutes	Phascolarctobacterium	734.38	1.48	0.025
OTU 65	Firmicutes	Fournierella	8.63	2.28	0.027
OTU 38	Firmicutes	Allisonella	59.43	1.69	0.030
OTU 26	Bacteroidetes	Alloprevotella	18.54	2.37	0.036
OTU 20	Bacteroidetes	Prevotellaceae UCG-003	15.11	2.15	0.044
Decreased from ileum to caecum					
OTU 19	Firmicutes	Terrisporobacter	449.39	-9.46	<0.001
OTU 83	Firmicutes	Lactococcus	40.30	-8.20	<0.001
OTU 18	Firmicutes	Clostridium sensustricto1	639.93	-6.54	<0.001
OTU 25	Proteobacteria	Pasteurellaceae unclassified	68.21	-6.85	<0.001
OTU 39	Firmicutes	Clostridiaceae 1 unclassified	88.60	-6.16	<0.001
OTU 55	Firmicutes	Intestinibacter	50.73	-7.14	<0.001
OTU 08	Firmicutes	Lactobacillus	1172.14	-6.29	<0.001
OTU 02	Firmicutes	Streptococcus	10282.3	-5.86	<0.001
OTU 68	Proteobacteria	Actinobacillus	12.70	-7.56	<0.001
OTU 31	Firmicutes	Lactobacillales unclassified	114.82	-4.89	<0.001
OTU 35	Firmicutes	Veillonella	6.92	-6.20	<0.001
OTU147	Firmicutes	Tepidimicrobium	8.89	-7.45	<0.001
OTU 53	Firmicutes	Peptostreptococcaceae unclassified	24.47	-4.80	<0.001
OTU 79	Actinobacteria	Micrococcales unclassified	6.31	-5.89	<0.001
OTU 49	Proteobacteria	Enterobacteriaceae unclassified	6.85	-4.46	<0.001
OTU102	Actinobacteria	Sanguibacter	5.74	-5.14	<0.001
OTU 51	Firmicutes	Streptococcaceae unclassified	42.45	-4.56	<0.001
OTU 36	Firmicutes	Romboutsia	39.93	-3.99	0.013
OTU 66	Firmicutes	RuminococcaceaeUCG-005	3.56	-2.19	0.022
OTU119	Firmicutes	Parvimonas	2.81	-6.28	0.025
OTU108	Firmicutes	Mogibacterium	0.95	-3.07	0.038
OTU120	Firmicutes	Ruminiclostridium 5	1.49	-3.60	0.039
OTU 77	Firmicutes	Turicibacter	14.93	-3.74	0.044

Table B. 2 DeSeq2 analysis results for OTUs classified to the genera level that significantly changed between the ileum and colon at day 28, irrespective of dietary treatment. P values are adjusted for multiple testing using the Benjamin-Hochberg correction. Fold changes represent Log2 fold changes.

OTU Number	Phyla	Genera	Base mean	Fold Change	P value
Increased from ileum to colon					
OTU 16	Bacteroidetes	Muribaculaceae ge	20.02	2.86	<0.001
OTU 12	Bacteroidetes	Muribaculaceae unclassified	32.52	2.69	<0.001
OTU 11	Firmicutes	Veillonellaceae unclassified	136.72	2.08	0.005
OTU 04	Bacteria, unclassified	Bacteria unclassified	171.31	1.29	0.005
OTU 05	Firmicutes	Ruminococcaceae unclassified	185.56	1.91	0.006
OTU 20	Bacteroidetes	Prevotellaceae UCG-003	15.11	2.77	0.006
OTU 23	Firmicutes	Ruminococcaceae ge	19.94	2.79	0.006
OTU 37	Firmicutes	Ruminococcaceae UCG-002	8.25	2.89	0.006
OTU 69	Firmicutes	Family XIII UCG-001	25.23	2.61	0.007
OTU 65	Firmicutes	Fournierella	8.63	2.40	0.016
OTU 01	Firmicutes	Megasphaera	2495.96	1.33	0.034
OTU 06	Firmicutes	Phascolarctobacterium	734.38	1.38	0.038
OTU 13	Bacteroidetes	Bacteroidales unclassified	11.22	2.05	0.039
OTU 40	Bacteroidetes	Prevotellaceae unclassified	6.66	2.57	0.039
Decreased from ileum to colon					
OTU 19	Firmicutes	Terrisporobacter	449.39	-9.39	<0.001
OTU 83	Firmicutes	Lactococcus	40.30	-9.73	<0.001
OTU 18	Firmicutes	Clostridium sensu stricto 1	639.93	-7.41	<0.001
OTU 39	Firmicutes	Clostridiaceae 1 unclassified	88.60	-7.23	<0.001
OTU 25	Proteobacteria	Pasteurellaceae unclassified	68.21	-7.38	<0.001
OTU 08	Firmicutes	Lactobacillus	1172.14	-7.06	<0.001
OTU 02	Firmicutes	Streptococcus	10282.35	-7.27	<0.001
OTU 55	Firmicutes	Intestinibacter	50.73	-7.38	<0.001
OTU 31	Firmicutes	Lactobacillales unclassified	114.82	-6.43	<0.001
OTU 51	Firmicutes	Streptococcaceae unclassified	42.45	-6.83	<0.001
OTU 68	Proteobacteria	Actinobacillus	12.70	-7.66	<0.001
OTU102	Actinobacteria	Sanguibacter	5.74	-7.42	<0.001
OTU 53	Firmicutes	Peptostreptococcaceae unclassified	24.47	-5.97	<0.001
OTU 79	Actinobacteria	Micrococcales unclassified	6.31	-7.34	<0.001
OTU 35	Firmicutes	Veillonella	6.92	-6.71	<0.001
OTU147	Firmicutes	Tepidimicrobium	8.89	-8.12	<0.001

OTU 49	Proteobacteria	Enterobacteriaceae unclassified	6.85	-4.75	<0.001
OTU 36	Firmicutes	Romboutsia	39.93	-5.56	<0.001
OTU 77	Firmicutes	Turicibacter	14.93	-6.02	<0.001
OTU 95	Firmicutes	Bacilli unclassified	4.50	-4.51	0.007
OTU120	Firmicutes	Ruminiclostridium 5	1.49	-4.51	0.007
OTU108	Firmicutes	Mogibacterium	0.95	-3.67	0.010
OTU119	Firmicutes	Parvimonas	2.81	-6.76	0.012
OTU 90	Firmicutes	Blautia	1.51	-3.04	0.033
OTU 10	Firmicutes	Firmicutes unclassified	117.41	-1.57	0.039
OTU155	Firmicutes	Anaerostipes	0.70	-3.56	0.039

Table B. 3 DeSeq2 analysis results for OTUs classified to the genera level that significantly changed between day 0 and day 14 within the ileum, caecum and colon, irrespective of dietary treatment. P values are adjusted for multiple testing using the Benjamin-Hochberg correction. Fold changes represent Log2 fold changes.

OTU Number	Phyla	Genera	Base mean	Fold Change	P value
Ileum					
Increased from day 0 to day 14					
OTU 28	Firmicutes	Negativibacillus	40.56	8.61	<0.001
OTU 09	Firmicutes	Anaerovibrio	362.28	6.66	<0.001
OTU 34	Firmicutes	Dialister	40.97	7.52	<0.001
OTU 83	Firmicutes	Lactococcus	32.49	4.69	0.004
OTU 102	Actinobacteria	Sanguibacter	15.41	6.99	0.007
OTU 18	Firmicutes	Clostridium sensu stricto 1	270.26	3.60	0.014
OTU 30	Firmicutes	Faecalibacterium	7.88	5.60	0.019
OTU 32	Firmicutes	Ruminococcaceae UCG-014	6.25	5.40	0.019
OTU 14	Firmicutes	Acidaminococcus	48.15	4.42	0.027
OTU 20	Bacteroidetes	Prevotellaceae UCG-003	13.47	4.77	0.027
OTU 17	Firmicutes	Subdoligranulum	18.51	4.58	0.029
OTU 122	Firmicutes	Paenibacillus	10.39	7.59	0.033
OTU 71	Firmicutes	Ruminococcus 2	2.94	5.73	0.040
OTU 29	Bacteroidetes	Prevotella 9	5.37	4.38	0.046
OTU 108	Firmicutes	Mogibacterium	3.90	5.36	0.046
OTU 90	Firmicutes	Blautia	2.80	5.32	0.047
OTU 154	Actinobacteria	Oerskovia	4.28	6.31	0.047
Decreased from day 0 to day 14					
OTU 11	Firmicutes	Veillonellaceae unclassified	70.10	-4.74	0.001
OTU 35	Firmicutes	Veillonella	642.76	-6.02	0.001
OTU 49	Proteobacteria	Enterobacteriaceae unclassified	23.02	-3.60	0.014
OTU 89	Fusobacteria	Fusobacteriaceae unclassified	3.38	-5.78	0.033
OTU 10	Firmicutes	Firmicutes unclassified	46.55	-3.03	0.047
CAECUM					
Increased from day 0 to day 14					
OTU 17	Firmicutes	Subdoligranulum	87.61	4.58	<0.001
OTU 30	Firmicutes	Faecalibacterium	22.85	5.83	<0.001
OTU 18	Firmicutes	Clostridium sensu stricto 1	28.33	6.33	0.002
OTU 38	Firmicutes	Allisonella	73.20	4.53	0.006
OTU 54	Actinobacteria	Enterorhabdus	13.56	5.53	0.006
OTU 52	Actinobacteria	Olsenella	12.76	5.27	0.007
OTU 07	Firmicutes	Mitsuokella	937.84	4.23	0.013
OTU 27	Firmicutes	Solobacterium	39.21	4.02	0.013
OTU 34	Firmicutes	Dialister	195.78	6.72	0.013
OTU 71	Firmicutes	Ruminococcus 2	7.39	5.32	0.013
OTU 65	Firmicutes	Fournierella	8.12	4.56	0.017
OTU 33	Firmicutes	Holdemanella	33.17	2.73	0.019
OTU 96	Firmicutes	Ruminococcaceae UCG-008	3.54	5.01	0.021
OTU 62	Firmicutes	Ruminococcus 1	9.64	4.23	0.034
OTU 28	Firmicutes	Negativibacillus	131.41	2.42	0.046
Decreased from day 0 to day 14					
OTU 43	Synergistetes	Pyramidobacter	272.37	-10.33	<0.001
OTU 35	Firmicutes	Veillonella	8.19	-6.38	0.002

OTU 15	Firmicutes	uncultured	49.77	-3.14	0.005
OTU 93	Kiritimatiellaeota	WCHB1-41 ge	2.56	-5.14	0.013
OTU 88	Fusobacteria	Fusobacterium	3.06	-5.49	0.015
OTU 123	Firmicutes	Hydrogenoanaerobacterium	3.18	-5.10	0.016
COLON					
Increased from day 0 to day 14					
OTU 34	Firmicutes	Dialister	349.27	24.34	<0.001
OTU 17	Firmicutes	Subdoligranulum	51.96	4.24	<0.001
OTU 30	Firmicutes	Faecalibacterium	35.16	8.68	<0.001
OTU 38	Firmicutes	Allisonella	75.39	5.85	<0.001
OTU 28	Firmicutes	Negativibacillus	194.55	4.02	<0.001
OTU 54	Actinobacteria	Enterorhabdus	11.77	6.17	<0.001
OTU 65	Firmicutes	Fournierella	9.86	5.75	0.001
OTU 52	Actinobacteria	Olsenella	11.72	5.63	0.001
OTU 27	Firmicutes	Solobacterium	16.54	4.29	0.001
OTU 07	Firmicutes	Mitsuokella	1227.35	4.99	0.003
OTU 32	Firmicutes	Ruminococcaceae UCG-014	18.79	2.86	0.005
OTU 22	Firmicutes	Lachnospiraceae unclassified	28.99	2.12	0.008
OTU 18	Firmicutes	Clostridium sensu stricto 1	13.10	4.13	0.010
OTU 96	Firmicutes	Ruminococcaceae UCG-008	2.70	5.56	0.011
OTU 69	Firmicutes	Family XIII UCG-001	34.34	3.06	0.016
OTU 90	Firmicutes	Blautia	2.69	5.58	0.016
OTU 21	Firmicutes	Clostridiales unclassified	102.68	2.37	0.018
OTU 14	Firmicutes	Acidaminococcus	693.66	4.76	0.023
OTU 33	Firmicutes	Holdemanella	24.50	2.49	0.023
OTU 67	Actinobacteria	Collinsella	4.74	3.76	0.037
Decreased from day 0 to day 14					
OTU 43	Synergistetes	Pyramidobacter	72.76	-11.59	<0.001
OTU 35	Firmicutes	Veillonella	6.02	-5.96	<0.001
OTU 88	Fusobacteria	Fusobacterium	1.59	-5.82	0.011
OTU 36	Firmicutes	Romboutsia	2.49	-4.46	0.021

Table B. 4 DeSeq2 analysis results for OTUs classified to the genera level that significantly changed between the ileum and caecum at day 0. P-values are adjusted for multiple testing using the Benjamin-Hochberg correction. Fold changes represent Log2 fold changes.

OTU Number	Phyla	Genera	Base mean	Fold Change	P value
Increased from ileum to caecum					
OTU 20	Bacteroidetes	Prevotellaceae UCG-003	77.90	6.84	<0.001
OTU 43	Synergistetes	Pyramidobacter	1253.84	9.58	<0.001
OTU 23	Firmicutes	Ruminococcaceae ge	31.00	6.62	<0.001
OTU 15	Firmicutes	uncultured	198.12	5.02	<0.001
OTU 92	Firmicutes	Anaerofilum	8.99	7.47	<0.001
OTU123	Firmicutes	Hydrogenoanaero-bacterium	25.13	8.21	<0.001
OTU 28	Firmicutes	Negativibacillus	33.18	8.01	<0.001
OTU 05	Firmicutes	Ruminococcaceae unclassified	723.48	5.07	<0.001
OTU 82	Firmicutes	Ruminiclostridium 9	7.05	6.31	<0.001
OTU 56	Bacteroidetes	Prevotella 2	12.74	6.46	<0.001
OTU 93	Kiritimatiellaeota	WCHB1-41 ge	12.67	5.01	<0.001
OTU 29	Bacteroidetes	Prevotella 9	9.90	4.78	<0.001
OTU 22	Firmicutes	Lachnospiraceae unclassified	14.63	4.20	0.001
OTU127	Bacteroidetes	Butyricimonas	4.94	6.90	0.001
OTU 75	Firmicutes	Family XIII AD3011 group	11.92	6.00	0.001
OTU 87	Proteobacteria	Anaerobiospirillum	5.31	6.87	0.001
OTU 06	Firmicutes	Phascolarctobacterium	1884.81	4.31	0.001
OTU 57	Proteobacteria	Desulfovibrio	16.07	4.15	0.001
OTU 44	Firmicutes	Erysipelotrichaceae unclassified	29.69	4.44	0.002
OTU 16	Bacteroidetes	Muribaculaceae ge	107.92	4.40	0.002
OTU 26	Bacteroidetes	Alloprevotella	31.41	4.99	0.003
OTU106	Bacteroidetes	Bacteroides	6.24	4.64	0.004
OTU137	Proteobacteria	Bilophila	3.93	6.33	0.004
OTU 04	Bacteria, unclassified	Bacteria unclassified	316.72	3.58	0.006
OTU 03	Tenericutes	Mollicutes RF39 ge	48.35	4.20	0.007
OTU 60	Cyanobacteria	Gastranaerophilales ge	3.93	4.58	0.007
OTU 85	Bacteroidetes	Parabacteroides	6.30	4.13	0.008
OTU 24	Firmicutes	Catenibacterium	12.77	4.82	0.010
OTU 98	Firmicutes	Ruminococcaceae NK4A214 group	2.01	4.55	0.011
OTU 46	Firmicutes	Candidatus Soleaferrea	60.97	3.50	0.013
OTU 12	Bacteroidetes	Muribaculaceae unclassified	123.63	3.91	0.014
OTU 13	Bacteroidetes	Bacteroidales unclassified	186.29	4.06	0.014

OTU104	Firmicutes	Erysipelotrichaceae ge	2.24	4.43	0.016
OTU 37	Firmicutes	Ruminococcaceae UCG-002	17.54	3.94	0.016
OTU 32	Firmicutes	Ruminococcaceae UCG-014	4.01	4.38	0.018
OTU 64	Bacteroidetes	Rikenellaceae RC9 gut group	15.70	3.70	0.018
OTU149	Planctomycetes	p-1088-a5 gut group	3.91	4.78	0.020
OTU148	Bacteroidetes	Bacteroidales ge	2.12	4.17	0.026
OTU 69	Firmicutes	Family XIII UCG-001	9.24	5.24	0.032
OTU 73	Firmicutes	Ruminococcaceae UCG-003	2.73	5.51	0.032
OTU 47	Firmicutes	Erysipelotrichaceae UCG-006	5.92	5.14	0.032
OTU111	Bacteroidetes	Tannerellaceae unclassified	2.99	3.97	0.033
OTU 63	Firmicutes	Oscillospira	10.10	3.80	0.033
OTU157	Bacteroidetes	dgA-11 gut group	1.29	4.21	0.041
OTU 45	Firmicutes	Christensenellaceae R-7 group	29.00	3.19	0.045
OTU103	Firmicutes	Family XIII unclassified	2.23	3.81	0.045
OTU 14	Firmicutes	Acidaminococcus	17.21	4.32	0.047
Decreased from Ileum to Caecum					
OTU 35	Firmicutes	Veillonella	1147.11	-5.43	<0.001
OTU 39	Firmicutes	Clostridiaceae 1 unclassified	25.20	-4.37	0.001
OTU 18	Firmicutes	Clostridium sensu stricto 1	8.19	-6.06	0.001
OTU 68	Proteobacteria	Actinobacillus	16.45	-5.30	0.001
OTU 36	Firmicutes	Romboutsia	60.03	-3.70	0.001
OTU 49	Proteobacteria	Enterobacteriaceae unclassified	31.25	-3.79	0.002
OTU 53	Firmicutes	Peptostreptococcaceae unclassified	22.74	-4.58	0.003
OTU 25	Proteobacteria	Pasteurellaceae unclassified	36.19	-2.68	0.018
OTU150	Actinobacteria	Actinomyces	3.01	-4.54	0.047

Table B. 5 DeSeq2 analysis results for OTUs classified to the genera level that significantly changed between the ileum and colon at day 0. P values are adjusted for multiple testing using the Benjamin-Hochberg correction. Fold changes represent Log2 fold changes.

OTU Number	Phyla	Genera	Base mean	Fold Change	P value
Increased from ileum to colon					
OTU123	Firmicutes	Hydrogenoanaerobacterium	25.13	8.91	<0.001
OTU 20	Bacteroidetes	Prevotellaceae UCG-003	77.90	6.04	<0.001
OTU 23	Firmicutes	Ruminococcaceae ge	31.00	6.20	<0.001
OTU 43	Synergistetes	Pyramidobacter	1253.84	8.31	<0.001
OTU 15	Firmicutes	uncultured	198.12	4.66	<0.001
OTU 28	Firmicutes	Negativibacillus	33.18	7.59	<0.001
OTU 92	Firmicutes	Anaerofilum	8.99	6.59	<0.001
OTU 29	Bacteroidetes	Prevotella 9	9.90	5.19	<0.001
OTU 05	Firmicutes	Ruminococcaceae unclassified	723.48	4.66	<0.001
OTU 56	Bacteroidetes	Prevotella 2	12.74	6.39	<0.001
OTU 82	Firmicutes	Ruminiclostridium 9	7.05	5.57	0.001
OTU 93	Kiritimatiellaeota	WCHB1-41 ge	12.67	4.24	0.003
OTU 75	Firmicutes	Family XIII AD3011 group	11.92	5.25	0.005
OTU 06	Firmicutes	Phascolarctobacterium	1884.81	3.86	0.005
OTU106	Bacteroidetes	Bacteroides	6.24	4.53	0.006
OTU 57	Proteobacteria	Desulfovibrio	16.07	3.63	0.007
OTU 22	Firmicutes	Lachnospiraceae unclassified	14.63	3.36	0.007
OTU 26	Bacteroidetes	Alloprevotella	31.41	4.49	0.009
OTU 16	Bacteroidetes	Muribaculaceae ge	107.92	3.74	0.011
OTU 87	Proteobacteria	Anaerobiospirillum	5.31	5.49	0.011
OTU 44	Firmicutes	Erysipelotrichaceae unclassified	29.69	3.68	0.012
OTU148	Bacteroidetes	Bacteroidales ge	2.12	4.45	0.020
OTU 13	Bacteroidetes	Bacteroidales unclassified	186.29	3.89	0.020
OTU 32	Firmicutes	Ruminococcaceae UCG-014	4.01	4.36	0.020
OTU 37	Firmicutes	Ruminococcaceae UCG-002	17.54	3.86	0.020
OTU 64	Bacteroidetes	Rikenellaceae RC9 gut group	15.70	3.72	0.020
OTU127	Bacteroidetes	Butyricimonas	4.94	4.96	0.020
OTU137	Proteobacteria	Bilophila	3.93	5.28	0.020
OTU 60	Cyanobacteria	Gastranaerophilales ge	3.93	3.96	0.020
OTU 24	Firmicutes	Catenibacterium	12.77	4.21	0.025
OTU 85	Bacteroidetes	Parabacteroides	6.30	3.51	0.025
OTU 12	Bacteroidetes	Muribaculaceae unclassified	123.63	3.50	0.029
OTU 03	Tenericutes	Mollicutes RF39 ge	48.35	3.27	0.039
OTU 73	Firmicutes	Ruminococcaceae UCG-003	2.73	5.31	0.040
OTU 99	Firmicutes	Coprococcus 3	2.16	3.88	0.040
OTU 98	Firmicutes	Ruminococcaceae NK4A214 group	2.01	3.71	0.040
OTU 45	Firmicutes	Christensenellaceae R-7 group	29.00	3.20	0.046

Decreased from ileum to colon					
OTU 39	Firmicutes	Clostridiaceae 1 unclassified	25.20	-5.16	<0.001
OTU 35	Firmicutes	Veillonella	1147.11	-5.27	<0.001
OTU 36	Firmicutes	Romboutsia	60.03	-3.85	0.001
OTU 53	Firmicutes	Peptostreptococcaceae unclassified	22.74	-5.01	0.001
OTU 18	Firmicutes	Clostridium sensu stricto 1	8.19	-5.04	0.003
OTU 49	Proteobacteria	Enterobacteriaceae unclassified	31.25	-3.62	0.003
OTU 68	Proteobacteria	Actinobacillus	16.45	-3.42	0.018
OTU 25	Proteobacteria	Pasteurellaceae unclassified	36.19	-2.57	0.024
OTU 77	Firmicutes	Turicibacter	2.31	-5.33	0.040

Table B. 6 DeSeq2 analysis results for OTUs classified to the genera level that significantly changed between the ileum and caecum at day 14, irrespective of dietary treatment. P values are adjusted for multiple testing using the Benjamin-Hochberg correction. Fold changes represent Log2 fold changes.

OTU Number	Phyla	Genera	Base mean	Fold Change	P value
Decreased from ileum to caecum					
OTU 19	Firmicutes	Terrisporobacter	208.11	-8.00	<0.001
OTU 18	Firmicutes	Clostridium sensu stricto 1	316.07	-6.33	<0.001
OTU 35	Firmicutes	Veillonella	61.02	-7.95	<0.001
OTU 36	Firmicutes	Romboutsia	44.68	-7.64	<0.001
OTU 53	Firmicutes	Peptostreptococcaceae unclassified	17.65	-6.06	<0.001
OTU 25	Proteobacteria	Pasteurellaceae unclassified	164.18	-7.45	<0.001
OTU 39	Firmicutes	Clostridiaceae 1 unclassified	55.81	-5.79	<0.001
OTU 02	Firmicutes	Streptococcus	970.43	-4.83	<0.001
OTU 31	Firmicutes	Lactobacillales unclassified	38.43	-4.34	<0.001
OTU 55	Firmicutes	Intestinibacter	27.21	-7.20	<0.001
OTU 51	Firmicutes	Streptococcaceae unclassified	26.29	-5.23	<0.001
OTU 72	Epsilonbacteraeota	Helicobacter	6.30	-4.70	<0.001
OTU 83	Firmicutes	Lactococcus	21.85	-4.49	<0.001
OTU 68	Proteobacteria	Actinobacillus	18.98	-7.14	<0.001
Otu122	Firmicutes	Paenibacillus	7.30	-6.95	<0.001
OTU 79	Actinobacteria	Micrococcales unclassified	5.96	-4.03	<0.001
OTU 08	Firmicutes	Lactobacillus	428.26	-2.73	0.004
OTU 119	Firmicutes	Parvimonas	17.53	-6.25	0.004
OTU 147	Firmicutes	Tepidimicrobium	5.02	-6.52	0.007
OTU 102	Actinobacteria	Sanguibacter	4.54	-3.62	0.009
OTU 95	Firmicutes	Bacilli unclassified	2.00	-3.59	0.009
OTU 159	Firmicutes	Jeotgalibaca	1.21	-4.42	0.010
OTU 154	Actinobacteria	Oerskovia	1.24	-4.23	0.028
Increased from ileum to caecum					
OTU 12	Bacteroidetes	Muribaculaceae unclassified	58.55	2.83	<0.001
OTU 16	Bacteroidetes	Muribaculaceae ge	49.93	2.78	<0.001
OTU 23	Firmicutes	Ruminococcaceae ge	38.23	2.59	<0.001
OTU 54	Actinobacteria	Enterorhabdus	15.17	3.55	0.002
OTU 17	Firmicutes	Subdoligranulum	84.35	2.36	0.002
OTU 04	Bacteria_unclassified	Bacteria unclassified	159.61	1.96	0.003
OTU 52	Actinobacteria	Olsenella	8.91	2.03	0.028
OTU 41	Bacteroidetes	Bacteroidia unclassified	7.27	2.00	0.034
OTU 69	Firmicutes	Family XIII UCG-001	25.64	1.95	0.044

Table B. 7 DeSeq2 analysis results for OTUs classified to the genera level that significantly changed between the ileum and colon at day 14, irrespective of dietary treatment. P values are adjusted for multiple testing using the Benjamin-Hochberg correction. Fold changes represent Log2 fold changes

OTU Number	Phyla	Genera	Base mean	Fold Change	P value
Increased from ileum to colon					
OTU 12	Bacteroidetes	Muribaculaceae unclassified	58.55	3.10	<0.001
OTU 16	Bacteroidetes	Muribaculaceae ge	49.93	3.14	<0.001
OTU 54	Actinobacteria	Enterorhabdus	15.17	3.58	0.002
OTU 17	Firmicutes	Subdoligranulum	84.35	2.16	0.006
OTU 69	Firmicutes	Family XIII UCG-001	25.64	2.52	0.006
OTU 06	Firmicutes	Phascolarctobacterium	845.17	1.56	0.007
OTU 05	Firmicutes	Ruminococcaceae unclassified	284.26	1.51	0.010
OTU 23	Firmicutes	Ruminococcaceae ge	38.23	1.87	0.016
OTU 92	Firmicutes	Anaerofilum	3.90	3.29	0.016
OTU 04	Bacteria, unclassified	Bacteria unclassified	159.61	1.50	0.035
OTU 41	Bacteroidetes	Bacteroidia unclassified	7.27	1.99	0.036
OTU 52	Actinobacteria	Olsenella	8.91	1.95	0.036
OTU 29	Bacteroidetes	Prevotella 9	14.05	1.66	0.045
Decreased from ileum to colon					
OTU 18	Firmicutes	Clostridium sensu stricto 1	316.07	-7.60	<0.001
OTU 19	Firmicutes	Terrisporobacter	208.11	-7.97	<0.001
OTU 36	Firmicutes	Romboutsia	44.68	-9.59	<0.001
OTU 53	Firmicutes	Peptostreptococcaceae unclassified	17.65	-7.51	<0.001
OTU 25	Proteobacteria	Pasteurellaceae unclassified	164.18	-8.95	<0.001
OTU 31	Firmicutes	Lactobacillales unclassified	38.43	-5.54	<0.001
OTU 35	Firmicutes	Veillonella	61.02	-8.91	<0.001
OTU 39	Firmicutes	Clostridiaceae 1 unclassified	55.81	-6.61	<0.001
OTU 02	Firmicutes	Streptococcus	970.43	-5.27	<0.001
OTU 83	Firmicutes	Lactococcus	21.85	-6.68	<0.001
OTU 55	Firmicutes	Intestinibacter	27.21	-8.23	<0.001
OTU 51	Firmicutes	Streptococcaceae unclassified	26.29	-6.47	<0.001
OTU 72	Epsilonbacteraeota	Helicobacter	6.30	-5.66	<0.001
OTU 08	Firmicutes	Lactobacillus	428.26	-4.21	<0.001
OTU 68	Proteobacteria	Actinobacillus	18.98	-8.22	<0.001
OTU 79	Actinobacteria	Micrococcales unclassified	5.96	-4.94	<0.001
OTU 122	Firmicutes	Paenibacillus	7.30	-7.22	<0.001
OTU 119	Firmicutes	Parvimonas	17.53	-8.63	<0.001
OTU 159	Firmicutes	Jeotgalibaca	1.21	-4.90	0.005
OTU 102	Actinobacteria	Sanguibacter	4.54	-3.79	0.006

OTU 154	Actinobacteria	Oerskovia	1.24	-5.12	0.006
OTU 95	Firmicutes	Bacilli unclassified	2.00	-3.77	0.006
OTU 43	Synergistetes	Pyramidobacter	2.75	-5.75	0.011
OTU 147	Firmicutes	Tepidimicrobium	5.02	-6.15	0.012
OTU 166	Firmicutes	Carnobacterium	1.53	-5.16	0.020
OTU 153	Firmicutes	Carnobacteriaceae unclassified	1.16	-4.58	0.028
OTU 112	Proteobacteria	Burkholderiaceae unclassified	1.62	-3.38	0.034

Appendix C

Table C. 1 Percentage relative abundance of phyla present within at least one sample above 0.1% in the lumen and mucosa of the jejunum of pigs reared indoors or outdoors and subsequently provided control (~200 ppm) or pharmacological (~2500 ppm) levels of ZnO for 14 days after weaning. P marked with * is one way ANOVA, rest are Krushkal-wallis. Pairwise differences marked with different superscript.

	JEJUNUM LUMEN				JEJUNUM MUCOSAL					
	Indoor		Outdoor		Indoor		Outdoor		P – values	
	Con	ZnO	Con	ZnO	Con	ZnO	Con	ZnO	Lumen	Mucosa
Firmicutes	74.81	89.10	74.61	73.09	51.18	79.83	49.40	49.75	0.300	**0.048**
Bacteroidetes	7.86	4.76	12.86	13.16	9.20	8.73	14.48	24.25	0.266	0.093
Proteobacteria	11.18	1.96	9.13	10.21	10.69	2.28	5.13	3.89	0.268	0.442
Epsilonbacteraeota	4.51	3.12	2.17	1.68	27.08	7.83	29.25	18.70	0.761	0.411
Fusobacteria	0.14	0.00	0.01	0.01	0.01	0.05	0.00	0.08	0.284	0.642
Tenericutes	0.20	0.30	0.35	0.57	0.47	0.36	0.36	1.58	0.106	0.695
Actinobacteria	1.09	0.24	0.49	0.78	0.65	0.48	0.73	0.98	0.151	0.316*
Deferribacteres	0.02	0.07	0.02	0.04	0.07	0.04	0.04	0.08	0.939	0.498*
Cyanobacteria	0.08	0.02	0.12	0.02	0.12	0.06	0.16	0.23	0.222	0.277
Spirochaetes	0.04	0.10	0.09	0.16	0.13	0.08	0.10	0.18	0.208	0.462*
Kiritimatiellaeota	0.00	0.20	0.01	0.15	0.03	0.01	0.14	0.06	0.154	0.817
Bacteria, unclassified	0.04	0.10	0.04	0.07	0.05	0.06	0.12	0.13	0.821	0.427
Verrucomicrobia	0.01	0.00	0.00	0.00	0.05	0.01	0.00	0.00	0.449	0.474
Chlamydiae	0.00	0.00	0.02	0.00	0.00	0.00	0.00	0.00	0.521	0.657
Thermotogae	0.00	0.00	0.00	0.00	0.01	0.01	0.01	0.03	0.626	0.976
Planctomycetes	0.00	0.00	0.04	0.01	0.04	0.00	0.01	0.01	0.387	0.557

P-values marked with * are based on one way ANOVA, all other values are from non-parametric Kruskal Wallis test.
Significant P values are shown in bold, and significant pairwise differences are marked with different superscripts.

Table C. 2 Percentage relative abundance of phyla present within at least one sample above 0.1% in the lumen and mucosa of the ileum of pigs reared indoors or outdoors and subsequently provided control (~200 ppm) or pharmacological (~2500 ppm) levels of ZnO for 14 days after weaning. P marked with * is one way ANOVA, rest are Krushkal-wallis. Pairwise differences marked with different superscript.

	ILEUM LUMEN				ILEUM MUCOSAL					
	Indoor		Outdoor		Indoor		Outdoor		P – values	
	Con	ZnO	Con	ZnO	Con	ZnO	Con	ZnO	Lumen	Mucosa
Firmicutes	64.74	74.48	72.33	72.92	39.90^{a}	43.17^{a}	49.89^{a}	79.66^{b}	0.822*	**<0.001***
Bacteroidetes	13.37	12.33	9.49	17.74	10.53	15.40	12.76	10.65	0.446*	0.670*
Proteobacteria	17.51	6.68	13.05	4.30	13.77	21.77	17.82	5.85	0.506	0.288*
Epsilonbacteraeota	2.77	2.58	3.88	3.46	33.68^{a}	13.37^{ab}	17.80^{ab}	2.05^{b}	0.930	**0.029**
Fusobacteria	0.25	2.70	0.03	0.01	0.72	0.37	0.12	0.05	0.425	0.445
Tenericutes	0.55	0.46	0.36	0.51	0.29	0.51	0.35	0.42	0.598*	0.655*
Actinobacteria	0.42	0.46	0.37	0.49	0.52	1.13	0.55	0.64	0.888	0.364
Deferribacteres	0.05	0.04	0.07	0.06	0.06	0.13	0.10	0.05	0.465	0.527
Cyanobacteria	0.07	0.03	0.04	0.06	0.07	2.70	0.05	0.18	0.404	0.196
Spirochaetes	0.18	0.13	0.18	0.29	0.21	0.21	0.30	0.15	0.113	0.143
Kiritimatiellaeota	0.00	0.03	0.03	0.04	0.07	0.03	0.05	0.02	0.570	0.322*
Bacteria, unclassified	0.02	0.04	0.04	0.05	0.06	0.55	0.07	0.16	0.189	0.831
Verrucomicrobia	0.00	0.01	0.02	0.00	0.01	0.01	0.01	0.00	0.982	0.172
Chlamydiae	0.04	0.00	0.05	0.01	0.00	0.01	0.06	0.00	0.197	0.526
Thermotogae	0.00	0.00	0.00	0.00	0.01	0.32	0.00	0.03	0.168	0.859
Planctomycetes	0.00	0.00	0.00	0.01	0.01	0.18	0.00	0.01	0.256	0.437

P-values marked with * are based on one way ANOVA, all other values are from non-parametric Kruskal Wallis test.
Significant P values are shown in bold, and pairwise differences are marked with different superscripts.

Table C. 3 Percentage relative abundance of phyla present within at least one sample above 0.1% in the lumen and mucosa of the ileum of pigs reared indoors or outdoors and subsequently provided control (~200 ppm) or pharmacological (~2500 ppm) levels of ZnO for 14 days after weaning. P marked with * is one way ANOVA, rest are Krushkal-wallis. Pairwise differences marked with different superscript.

	COLON LUMEN				COLON MUCOSA				FAECES				P - values		
	Indoor		Outdoor		Indoor		Outdoor		Indoor		Outdoor		Colon		
	Con	ZnO	Con	ZnO	Con	ZnO	Con	ZnO	Con	ZnO	Con	ZnO	Lumen	Mucosa	Faeces
Firmicutes	71.32	73.69	65.41	60.84	56.73	51.21	57.73	56.76	72.21	71.27	63.91	58.74	*0.197	*0.680	0.217
Bacteroidetes	16.18^{ab}	16.12^{a}	26.49^{ab}	32.39^{b}	21.26^{ab}	19.12^{a}	23.73^{ab}	34.89^{b}	18.33	18.74	23.67	34.58	***0.033**	***<0.001**	**0.013**
Proteobacteria	6.04	5.34	4.27	2.97	4.92	15.90	5.73	3.05	3.22	2.19	4.15	2.14	0.787	0.138	0.311
Epsilonbacteraeota	3.54	3.14	1.78	2.06	13.22^{a}	11.01^{a}	9.12^{ab}	3.23^{b}	2.77	3.57	5.16	1.55	0.686	***0.027**	0.922
Fusobacteria	0.48	0.01	0.06	0.05	0.04	1.20	0.00	0.10	0.04	0.93	0.00	0.05	0.937	0.315	0.666
Tenericutes	0.59	0.54	0.65	0.83	0.56	0.35	0.66	0.69	0.82	0.71	0.79	1.23	0.831	0.117	0.944
Actinobacteria	0.81	0.60	0.61	0.56	0.44	0.55	0.53	0.61	0.78	1.47	1.00	0.90	*0.632	0.417	0.418
Deferribacteres	0.09	0.03	0.02	0.04	0.78	0.18	0.28	0.06	0.06	0.03	0.04	0.05	0.788	0.091	0.680
Cyanobacteria	0.07	0.13	0.11	0.03	0.11	0.05	0.16	0.10	0.06	0.05	0.26	0.16	0.209	0.062	0.191
Spirochaetes	0.56	0.13	0.37	0.15	1.73	0.21	1.16	0.25	1.51	0.14	0.57	0.30	0.086	0.146	0.280
Kiritimatiellaeota	0.11	0.09	0.06	0.01	0.03	0.02	0.44	0.04	0.02	0.01	0.06	0.02	0.778	0.808	0.843
Bacteria, unclassified	0.08	0.14	0.09	0.03	0.08	0.04	0.26	0.14	0.08	0.03	0.25	0.18	0.565	0.334	0.188
Verrucomicrobia	0.06	0.01	0.01	0.00	0.00	0.04	0.01	0.00	0.00	0.79	0.00	0.00	0.666	0.949	0.888
Chlamydiae	0.03	0.00	0.03	0.01	0.05	0.07	0.13	0.04	0.02	0.01	0.06	0.00	0.742	0.866	0.625
Thermotogae	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.01	0.00	0.00	0.00	0.01	0.285	0.261	0..382
Planctomycetes	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.01	0.01	0.00	0.02	0.02	0.908	0.408	0.392

P-values marked with * are based on one way ANOVA, all other values are from non-parametric Kruskal Wallis test.
Significant P values are shown in bold, and pairwise differences are marked with different superscripts.

Appendix D

Table D. 1 DeSeq2 analysis results for OTUs classified to the genera level that significantly changed between the jejunum and all other locations at day 14, irrespective of environment, diet or sample type. P values are adjusted for multiple testing using the Benjamin-Hochberg correction. Fold changes represent Log2 fold changes.

	Phylum	Genus	Base Mean	LogFold Change	P value
Increased from Jejunum to Ileum					
OTU 0095	Proteobacteria	*Pasteurellaceae unclassified*	120.74	6.98	<0.001
OTU 0062	Firmicutes	*Streptococcus*	252.47	2.69	<0.001
OTU 0200	Bacteroidetes	*Alistipes*	64.76	7.21	<0.001
OTU 0036	Firmicutes	*Lactobacillus*	845.33	3.99	<0.001
OTU 0018	Firmicutes	*Romboutsia*	1150.59	1.67	0.005
OTU 0064	Fusobacteria	*Fusobacterium*	63.88	5.59	0.005
OTU 1152	Firmicutes	*Intestinimonas*	3.93	5.17	0.005
OTU 0007	Firmicutes	*Terrisporobacter*	2797.81	1.35	0.009
OTU 0016	Firmicutes	*Clostridium sensu stricto 1*	1296.25	1.76	0.010
OTU 0129	Proteobacteria	*Enterobacteriaceae unclassified*	154.64	2.22	0.010
OTU 0783	Proteobacteria	*Pasteurellaceae unclassified*	3.86	5.42	0.011
OTU 1271	Firmicutes	*Peptostreptococcaceae unclassified*	1.39	3.38	0.014
OTU 0173	Firmicutes	*Dubosiella*	37.63	9.57	0.016
OTU 0620	Firmicutes	*Terrisporobacter*	5.25	1.65	0.019
OTU 0098	Proteobacteria	*Burkholderiaceae unclassified*	18.37	2.75	0.020
OTU 1312	Proteobacteria	*Pasteurellaceae unclassified*	1.76	3.07	0.020
OTU 0908	Firmicutes	*Peptostreptococcaceae unclassified*	3.29	2.44	0.026
OTU 0572	Firmicutes	*Clostridium sensu stricto 6*	6.05	3.19	0.030
OTU 0287	Spirochaetes	*Treponema 2*	4.26	4.24	0.032
OTU 0951	Firmicutes	*Ruminococcaceae UCG-014*	5.31	5.67	0.041
Decreased from jejunum to ileum					
OTU 0152	Bacteroidetes	*Bacteroidia unclassified*	49.72	-2.03	0.002
OTU 0045	Bacteroidetes	*Prevotellaceae UCG-003*	220.84	-0.68	0.004
OTU 0434	Firmicutes	*Peptococcus*	13.33	-2.09	0.031
OTU 0525	Firmicutes	*Family XIII unclassified*	7.43	-1.54	0.034
Increased from Jejunum to Colon					

OTU 0095	Proteobacteria	*Pasteurellaceae unclassified*	120.74	6.98	<0.001
OTU 0062	Firmicutes	*Streptococcus*	252.47	2.69	<0.001
OTU 0200	Bacteroidetes	*Alistipes*	64.76	7.21	<0.001
OTU 0036	Firmicutes	*Lactobacillus*	845.33	3.99	<0.001
OTU 0018	Firmicutes	*Romboutsia*	1150.59	1.67	0.005
OTU 0064	Fusobacteria	*Fusobacterium*	63.88	5.59	0.005
OTU 1152	Firmicutes	*Intestinimonas*	3.93	5.17	0.005
OTU 0007	Firmicutes	*Terrisporobacter*	2797.81	1.35	0.009
OTU 0016	Firmicutes	*Clostridium sensu stricto 1*	1296.25	1.76	0.010
OTU 0129	Proteobacteria	*Enterobacteriaceae unclassified*	154.64	2.22	0.010
OTU 0783	Proteobacteria	*Pasteurellaceae unclassified*	3.86	5.42	0.011
OTU 1271	Firmicutes	*Peptostreptococcaceae unclassified*	1.39	3.38	0.014
OTU 0173	Firmicutes	*Dubosiella*	37.63	9.57	0.016
OTU 0620	Firmicutes	*Terrisporobacter*	5.25	1.65	0.019
OTU 0098	Proteobacteria	*Burkholderiaceae unclassified*	18.37	2.75	0.020
OTU 1312	Proteobacteria	*Pasteurellaceae unclassified*	1.76	3.07	0.020
OTU 0908	Firmicutes	*Peptostreptococcaceae unclassified*	3.29	2.44	0.026
OTU 0572	Firmicutes	*Clostridium sensu stricto 6*	6.05	3.19	0.030
OTU 0287	Spirochaetes	*Treponema 2*	4.26	4.24	0.032
OTU 0951	Firmicutes	*Ruminococcaceae UCG-014*	5.31	5.67	0.041
Decreased from jejunum to Colon					
OTU 0152	Bacteroidetes	*Bacteroidia unclassified*	49.72	-2.03	0.002
OTU 0045	Bacteroidetes	*Prevotellaceae UCG-003*	220.84	-0.68	0.004
OTU 0434	Firmicutes	*Peptococcus*	13.33	-2.09	0.031
OTU 0525	Firmicutes	*Family XIII unclassified*	7.43	-1.54	0.034
Decreased from jejunum to faeces					
OTU 0104	Proteobacteria	*Acinetobacter*	169.28	-3.27	<0.001
OTU 0124	Proteobacteria	*Burkholderia-Caballeronia-Paraburkholderia*	122.85	-3.31	<0.001
OTU 0251	Proteobacteria	*Massilia*	43.05	-3.31	<0.001
OTU 0208	NA	*NA*	22.66	-5.05	<0.001
OTU 0470	Actinobacteria	*Rothia*	11.35	-2.81	<0.001
OTU 0009	Epsilonbacteraeota	*Helicobacter*	4087.09	-3.68	<0.001
OTU 0034	Firmicutes	*Streptococcus*	528.03	-2.80	<0.001

OTU 0438	Actinobacteria	*Corynebacterium 1*	11.17	-3.07	<0.001
OTU 0187	Proteobacteria	*Acinetobacter*	96.33	-3.35	<0.001
OTU 0392	Proteobacteria	*Pseudomonas*	20.29	-3.01	<0.001
OTU 0025	NA	*NA*	148.23	-3.38	<0.001
OTU 0710	Actinobacteria	*Actinomyces*	4.96	-2.75	<0.001
OTU 0153	Proteobacteria	*Pseudomonas*	96.58	-2.46	<0.001
OTU 0010	Epsilonbacteraeota	*Helicobacter*	2982.05	-3.42	<0.001
OTU 0005	Firmicutes	*Lactobacillus*	3606.55	-1.47	<0.001
OTU 0619	Proteobacteria	*Delftia*	9.84	-3.39	0.001
OTU 0780	Firmicutes	*Streptococcus*	4.08	-3.00	0.001
OTU 0514	Proteobacteria	*Pseudomonas*	15.50	-3.51	0.002
OTU 0483	Proteobacteria	*Phyllobacterium*	13.26	-2.58	0.002
OTU 0001	Firmicutes	*Lactobacillus*	10734.0	-2.04	0.002
OTU 0930	Firmicutes	*Streptococcus*	2.99	-2.96	0.003
OTU 1287	Proteobacteria	*Achromobacter*	1.63	-3.92	0.004
OTU 0808	Proteobacteria	*Enterobacteriaceae unclassified*	5.87	-3.05	0.005
OTU 1080	Firmicutes	*Bacillales unclassified*	1.95	-3.70	0.006
OTU 0019	Proteobacteria	*Actinobacillus*	1190.86	-2.32	0.008
OTU 0198	Kiritimatiellaeota	*WCHB1-41 ge*	34.63	-4.22	0.008
OTU 0006	Proteobacteria	*Escherichia-Shigella*	6264.20	-2.54	0.009
OTU 0471	Firmicutes	*Streptococcus*	11.57	-2.03	0.009
OTU 0640	Proteobacteria	*Moraxella*	5.07	-2.54	0.009
OTU 1049	Firmicutes	*Lactobacillales unclassified*	1.71	-2.92	0.009
OTU 0206	Proteobacteria	*Vulcaniibacterium*	56.69	-2.99	0.016
OTU 0402	Proteobacteria	*Cupriavidus*	17.78	-3.72	0.016
OTU 0419	Firmicutes	*Faecalitalea*	8.06	-5.99	0.025
OTU 0536	Firmicutes	*Gemella*	8.39	-2.25	0.025
OTU 0556	Actinobacteria	*Bifidobacterium*	5.66	-2.69	0.026
OTU 0544	Proteobacteria	*Halomonas*	7.99	-2.47	0.026
OTU 1217	Proteobacteria	*Rhizobiaceae unclassified*	1.47	-2.43	0.029
OTU 0403	Firmicutes	*Lactobacillus*	12.01	-2.27	0.037
OTU 0052	Bacteroidetes	*Bacteroides*	167.90	-3.62	0.042
OTU 0887	Proteobacteria	*Enhydrobacter*	3.32	-2.32	0.048

Table D. 2 DeSeq2 analysis results for OTUs classified to the genera level that significantly changed between the ileum and the colon at day 14, irrespective of environment, diet or sample type. P values are adjusted for multiple testing using the Benjamin-Hochberg correction. Fold changes represent Log2 fold changes.

	Phylum	Genus	Base Mean	logFold Change	P value
Increased from Ileum to colon					
OTU 0135	Bacteroidetes	*Prevotellaceae UCG-001*	62.53	1.19	0.010
OTU 0032	Bacteroidetes	*Parabacteroides*	425.59	1.34	0.015
OTU 0045	Bacteroidetes	*Prevotellaceae UCG-003*	220.84	0.50	0.017
OTU 0051	Bacteroidetes	*Muribaculaceae ge*	198.93	1.27	0.023
OTU 0014	Firmicutes	*Subdoligranulum*	1166.31	0.75	0.023
OTU 0028	Epsilonbacteraeota	*Helicobacter*	653.26	1.42	0.033
OTU 0068	Firmicutes	*Roseburia*	182.74	0.80	0.050
Decreased from Ileum to colon					
OTU 0062	Firmicutes	*Streptococcus*	252.47	-2.65	<0.001
OTU 0129	Proteobacteria	*Enterobacteriaceae unclassified*	154.64	-3.37	<0.001
OTU 0251	Proteobacteria	*Massilia*	43.05	-2.48	<0.001
OTU 0036	Firmicutes	*Lactobacillus*	845.33	-3.64	<0.001
OTU 0071	Firmicutes	*Enterococcus*	47.45	-2.07	<0.001
OTU 0019	Proteobacteria	*Actinobacillus*	1190.86	-2.56	<0.001
OTU 0095	Proteobacteria	*Pasteurellaceae unclassified*	120.74	-4.04	<0.001
OTU 0098	Proteobacteria	*Burkholderiaceae unclassified*	18.37	-3.55	<0.001
OTU 0002	Firmicutes	*Clostridium sensu stricto 1*	7394.69	-1.70	<0.001
OTU 0104	Proteobacteria	*Acinetobacter*	169.28	-2.09	<0.001
OTU 0124	Proteobacteria	*Burkholderia-Caballeronia-Paraburkholderia*	122.85	-2.02	<0.001
OTU 1024	Bacteroidetes	*Muribaculaceae ge*	2.96	-4.46	<0.001
OTU 0170	Cyanobacteria	*Cyanobium PCC-6307*	9.31	-3.28	<0.001
OTU 0331	Firmicutes	*Streptococcus*	21.69	-1.98	<0.001
OTU 0946	Proteobacteria	*Burkholderiaceae unclassified*	2.79	-2.78	<0.001
OTU 1312	Proteobacteria	*Pasteurellaceae unclassified*	1.76	-3.39	<0.001
OTU 0034	Firmicutes	*Streptococcus*	528.03	-1.73	<0.001
OTU 0213	Proteobacteria	*Stenotrophomonas*	45.25	-2.49	<0.001

OTU 0187	Proteobacteria	*Acinetobacter*	96.33	-2.38	<0.001
OTU 0808	Proteobacteria	*Enterobacteriaceae unclassified*	5.87	-2.62	<0.001
OTU 0010	Epsilonbacteraeota	*Helicobacter*	2982.05	-2.55	0.001
OTU 0544	Proteobacteria	*Halomonas*	7.99	-2.52	0.001
OTU 1152	Firmicutes	*Intestinimonas*	3.93	-4.88	0.001
OTU 0294	Proteobacteria	*Sphingobium*	3.69	-5.63	0.001
OTU 1368	Firmicutes	*Streptococcus*	1.64	-3.92	0.001
OTU 0585	Firmicutes	*Clostridium sensu stricto 1*	6.44	-1.98	0.002
OTU 0306	Proteobacteria	*Sphingobium*	3.23	-5.36	0.002
OTU 0470	Actinobacteria	*Rothia*	11.35	-1.47	0.003
OTU 0018	Firmicutes	*Romboutsia*	1150.59	-1.45	0.003
OTU 0569	Firmicutes	*Ruminococcaceae UCG-014*	10.55	-2.63	0.003
OTU 0177	Cyanobacteria	*Cyanobium PCC-6307*	6.49	-3.36	0.004
OTU 0823	Proteobacteria	*Pasteurellaceae unclassified*	4.05	-2.52	0.004
OTU 0248	Firmicutes	*Lactobacillus*	33.22	-1.49	0.004
OTU 0865	Firmicutes	*Lachnospiraceae NK4A136 group*	3.14	-4.48	0.004
OTU 0514	Proteobacteria	*Pseudomonas*	15.50	-2.51	0.005
OTU 0389	Actinobacteria	*Flaviflexus*	3.78	-4.23	0.008
OTU 0471	Firmicutes	*Streptococcus*	11.57	-1.64	0.008
OTU 0483	Proteobacteria	*Phyllobacterium*	13.26	-1.81	0.008
OTU 1426	Actinobacteria	*Trueperella*	1.59	-3.15	0.008
OTU 0060	Firmicutes	*Turicibacter*	244.35	-1.47	0.009
OTU 0402	Proteobacteria	*Cupriavidus*	17.78	-3.04	0.009
OTU 0153	Proteobacteria	*Pseudomonas*	96.58	-1.51	0.009
OTU 0202	Firmicutes	*Lactobacillus*	57.92	-1.59	0.010
OTU 1179	Firmicutes	*Clostridiales unclassified*	1.61	-2.14	0.010
OTU 0108	Firmicutes	*Veillonella*	12.17	-2.12	0.012
OTU 0844	Firmicutes	*Clostridium sensu stricto 1*	3.05	-1.42	0.013
OTU 0016	Firmicutes	*Clostridium sensu stricto 1*	1296.25	-1.44	0.013
OTU 0392	Proteobacteria	*Pseudomonas*	20.29	-1.73	0.013
OTU 1619	Firmicutes	*Lactobacillus*	1.38	-3.38	0.013
OTU 0395	Firmicutes	*Clostridium sensu stricto 1*	12.55	-1.32	0.013
OTU 0784	Firmicutes	*Clostridium sensu stricto 8*	3.30	-4.57	0.013

OTU 0206	Proteobacteria	*Vulcaniibacterium*	56.69	-2.34	0.013
OTU 0760	Firmicutes	*Lactobacillus*	3.80	-1.75	0.014
OTU 0281	Firmicutes	*Ruminococcaceae UCG-014*	32.67	-1.23	0.021
OTU 0783	Proteobacteria	*Pasteurellaceae unclassified*	3.86	-4.18	0.024
OTU 0901	Proteobacteria	*Brevundimonas*	2.76	-2.04	0.024
OTU 0820	Proteobacteria	*Ralstonia*	4.16	-2.84	0.028
OTU 0731	Firmicutes	*Lactobacillus*	4.71	-1.40	0.029
OTU 0908	Firmicutes	*Peptostreptococcaceae unclassified*	3.29	-1.95	0.029
OTU 1493	Firmicutes	*Globicatella*	1.10	-2.55	0.029
OTU 1271	Firmicutes	*Peptostreptococcaceae unclassified*	1.39	-2.54	0.029
OTU 1407	Proteobacteria	*Enterobacteriaceae unclassified*	1.25	-2.26	0.029
OTU 0572	Firmicutes	*Clostridium sensu stricto 6*	6.05	-2.63	0.030
OTU 0674	Bacteroidetes	*Alloprevotella*	1.78	-4.26	0.030
OTU 1545	Firmicutes	*Lactobacillus*	0.87	-2.35	0.030
OTU 0619	Proteobacteria	*Delftia*	9.84	-1.91	0.031
OTU 0997	Firmicutes	*Lactobacillus*	2.30	-2.73	0.032
OTU 0005	Firmicutes	*Lactobacillus*	3606.55	-0.83	0.033
OTU 0667	Firmicutes	*Lactobacillus*	5.87	-1.96	0.033
OTU 0854	Firmicutes	*Lactobacillus*	3.15	-1.58	0.033
OTU 0880	Bacteroidetes	*Muribaculaceae ge*	1.42	-3.69	0.037
OTU 0979	Proteobacteria	*Neisseriaceae unclassified*	3.17	-2.26	0.038
OTU 0899	Firmicutes	*Lactobacillus*	4.77	-2.93	0.038
OTU 0221	Proteobacteria	*Hydrogenophilus*	15.61	-2.04	0.039
OTU 0007	Firmicutes	*Terrisporobacter*	2797.81	-0.97	0.039
OTU 1093	Proteobacteria	*Duganella*	3.66	-3.14	0.039
OTU 0218	Bacteria_unclassified	*Bacteria unclassified*	2.50	-5.60	0.041
OTU 0955	Proteobacteria	*Psychrobacter*	2.90	-2.15	0.043
OTU 1916	Firmicutes	*Lactobacillus*	0.54	-2.74	0.043
OTU 1189	Proteobacteria	*Stenotrophomonas*	1.45	-3.21	0.046
OTU 0265	Firmicutes	*Clostridiaceae 1 unclassified*	25.79	-1.37	0.048
OTU 0009	Epsilonbacteraeota	*Helicobacter*	4087.09	-1.55	0.048

Table D. 3 DeSeq2 analysis results for OTUs classified to the genera level that significantly changed between the ileum and faeces at day 14, irrespective of environment, diet or sample type. P values are adjusted for multiple testing using the Benjamin-Hochberg correction. Fold changes represent Log2 fold changes.

	Phylum	Genus	Base Mean	logFold Change	P value
Increased from ileum to faeces					
OTU 0020	Bacteroidetes	Muribaculaceae ge	695.06	1.62	<0.001
OTU 0118	Actinobacteria	Eggerthellaceae unclassified	83.60	1.10	<0.001
OTU 0152	Bacteroidetes	Bacteroidia unclassified	49.72	1.97	0.002
OTU 0045	Bacteroidetes	Prevotellaceae UCG-003	220.84	0.65	0.004
OTU 0776	Bacteroidetes	Muribaculaceae ge	2.55	2.22	0.007
OTU 0106	Bacteroidetes	Rikenellaceae RC9 gut group	87.05	0.85	0.023
OTU 0525	Firmicutes	Family XIII unclassified	7.43	1.55	0.025
OTU 0134	Firmicutes	Family XIII AD3011 group	66.02	0.77	0.041
OTU 0675	Actinobacteria	Slackia	4.23	1.15	0.041
OTU 0049	Firmicutes	Dorea	213.64	0.56	0.049
Decreased from ileum to faeces					
OTU 0062	Firmicutes	Streptococcus	252.47	-3.09	<0.001
OTU 0018	Firmicutes	Romboutsia	1150.59	-2.95	<0.001
OTU 0030	NA	NA	199.83	-7.99	<0.001
OTU 0019	Proteobacteria	Actinobacillus	1190.86	-3.70	<0.001
OTU 0129	Proteobacteria	Enterobacteriaceae unclassified	154.64	-3.89	<0.001
OTU 0006	Proteobacteria	Escherichia-Shigella	6264.20	-4.02	<0.001
OTU 0187	Proteobacteria	Acinetobacter	96.33	-3.75	<0.001
OTU 0036	Firmicutes	Lactobacillus	845.33	-4.43	<0.001
OTU 0808	Proteobacteria	Enterobacteriaceae unclassified	5.87	-4.06	<0.001
OTU 0016	Firmicutes	Clostridium sensu stricto 1	1296.25	-2.62	<0.001
OTU 0251	Proteobacteria	Massilia	43.05	-2.67	<0.001
OTU 0095	Proteobacteria	Pasteurellaceae unclassified	120.74	-4.89	<0.001
OTU 0104	Proteobacteria	Acinetobacter	169.28	-2.57	<0.001

OTU 0124	Proteobacteria	Burkholderia-Caballeronia-Paraburkholderia	122.85	-2.57	<0.001
OTU 0331	Firmicutes	Streptococcus	21.69	-2.69	<0.001
OTU 0002	Firmicutes	Clostridium sensu stricto 1	7394.69	-2.01	<0.001
OTU 0071	Firmicutes	Enterococcus	47.45	-2.18	<0.001
OTU 0123	Proteobacteria	Sutterella	75.69	-1.65	<0.001
OTU 0098	Proteobacteria	Burkholderiaceae unclassified	18.37	-3.85	<0.001
OTU 0619	Proteobacteria	Delftia	9.84	-3.41	<0.001
OTU 0011	Epsilonbacteraeota	Campylobacter	1543.90	-2.47	<0.001
OTU 0052	Bacteroidetes	Bacteroides	167.90	-4.80	<0.001
OTU 0060	Firmicutes	Turicibacter	244.35	-2.11	<0.001
OTU 0281	Firmicutes	Ruminococcaceae UCG-014	32.67	-1.92	<0.001
OTU 0853	Proteobacteria	Enterobacteriaceae unclassified	5.45	-3.77	0.001
OTU 1024	Bacteroidetes	Muribaculaceae ge	2.96	-5.03	0.001
OTU 0585	Firmicutes	Clostridium sensu stricto 1	6.44	-2.48	0.001
OTU 0208	NA	NA	22.66	-3.33	0.001
OTU 0514	Proteobacteria	Pseudomonas	15.50	-3.35	0.001
OTU 0081	Firmicutes	Lactococcus	270.80	-3.09	0.001
OTU 0200	Bacteroidetes	Alistipes	64.76	-5.49	0.001
OTU 0544	Proteobacteria	Halomonas	7.99	-2.86	0.001
OTU 0470	Actinobacteria	Rothia	11.35	-1.81	0.001
OTU 0153	Proteobacteria	Pseudomonas	96.58	-2.02	0.002
OTU 0640	Proteobacteria	Moraxella	5.07	-2.66	0.002
OTU 0823	Proteobacteria	Pasteurellaceae unclassified	4.05	-3.20	0.002
OTU 1140	Firmicutes	Clostridiales unclassified	2.08	-3.47	0.002
OTU 0170	Cyanobacteria	Cyanobium PCC-6307	9.31	-3.43	0.002
OTU 1287	Proteobacteria	Achromobacter	1.63	-3.78	0.002
OTU 0483	Proteobacteria	Phyllobacterium	13.26	-2.34	0.002
OTU 0177	Cyanobacteria	Cyanobium PCC-6307	6.49	-4.12	0.002

OTU 0847	Proteobacteria	Burkholderiaceae unclassified	6.34	-4.81	0.002
OTU 0009	Epsilonbacteraeota	Helicobacter	4087.09	-2.32	0.004
OTU 0034	Firmicutes	Streptococcus	528.03	-1.75	0.004
OTU 0108	Firmicutes	Veillonella	12.17	-2.72	0.004
OTU 0979	Proteobacteria	Neisseriaceae unclassified	3.17	-3.35	0.005
OTU 0007	Firmicutes	Terrisporobacter	2797.81	-1.36	0.007
OTU 0402	Proteobacteria	Cupriavidus	17.78	-3.66	0.007
OTU 0025	NA	NA	148.23	-2.40	0.008
OTU 1093	Proteobacteria	Duganella	3.66	-4.43	0.009
OTU 0294	Proteobacteria	Sphingobium	3.69	-5.70	0.009
OTU 0844	Firmicutes	Clostridium sensu stricto 1	3.05	-1.73	0.009
OTU 0667	Firmicutes	Lactobacillus	5.87	-2.65	0.009
OTU 0741	Firmicutes	Peptostreptococcaceae unclassified	4.41	-1.98	0.009
OTU 1312	Proteobacteria	Pasteurellaceae unclassified	1.76	-3.21	0.009
OTU 1404	Firmicutes	Peptostreptococcaceae unclassified	1.05	-3.01	0.009
OTU 0452	Firmicutes	Ruminiclostridium 9	22.03	-5.58	0.010
OTU 1107	Firmicutes	Peptostreptococcaceae unclassified	1.88	-2.47	0.010
OTU 0132	Epsilonbacteraeota	Campylobacter	74.39	-2.40	0.010
OTU 0206	Proteobacteria	Vulcaniibacterium	56.69	-2.78	0.011
OTU 0010	Epsilonbacteraeota	Helicobacter	2982.05	-2.43	0.012
OTU 1121	Proteobacteria	Gammaproteobacteria unclassified	1.94	-4.41	0.012
OTU 0269	Proteobacteria	uncultured	4.18	-3.17	0.012
OTU 0306	Proteobacteria	Sphingobium	3.23	-5.53	0.012
OTU 0248	Firmicutes	Lactobacillus	33.22	-1.59	0.014
OTU 0202	Firmicutes	Lactobacillus	57.92	-1.79	0.015
OTU 0783	Proteobacteria	Pasteurellaceae unclassified	3.86	-5.20	0.015
OTU 1305	Firmicutes	Ruminococcaceae UCG-014	2.13	-4.47	0.015

OTU 0887	Proteobacteria	Enhydrobacter	3.32	-2.36	0.016
OTU 1187	Firmicutes	Clostridium sensu stricto 1	1.71	-2.96	0.016
OTU 0265	Firmicutes	Clostridiaceae 1 unclassified	25.79	-1.81	0.017
OTU 0463	Bacteroidetes	Muribaculaceae ge	21.99	-6.80	0.023
OTU 0005	Firmicutes	Lactobacillus	3606.55	-1.01	0.023
OTU 0943	Firmicutes	Lactobacillus	2.59	-3.08	0.024
OTU 0556	Actinobacteria	Bifidobacterium	5.66	-2.44	0.027
OTU 1760	Proteobacteria	Escherichia-Shigella	0.97	-4.00	0.027
OTU 1336	Firmicutes	Lactobacillus	1.52	-3.08	0.028
OTU 0392	Proteobacteria	Pseudomonas	20.29	-1.87	0.029
OTU 0395	Firmicutes	Clostridium sensu stricto 1	12.55	-1.42	0.030
OTU 0403	Firmicutes	Lactobacillus	12.01	-2.10	0.030
OTU 0674	Bacteroidetes	Alloprevotella	1.78	-5.04	0.030
OTU 1368	Firmicutes	Streptococcus	1.64	-3.55	0.030
OTU 1619	Firmicutes	Lactobacillus	1.38	-3.68	0.030
OTU 0264	Fusobacteria	Fusobacterium	7.89	-3.67	0.031
OTU 0604	Proteobacteria	Desulfovibrio	6.92	-6.11	0.031
OTU 0821	Bacteroidetes	Muribaculaceae ge	6.75	-6.07	0.031
OTU 1304	Firmicutes	Lactobacillus	1.44	-4.21	0.031
OTU 0901	Proteobacteria	Brevundimonas	2.76	-2.30	0.035
OTU 1407	Proteobacteria	Enterobacteriaceae unclassified	1.25	-2.63	0.036
OTU 0367	Proteobacteria	Bilophila	7.37	-3.38	0.037
OTU 0419	Firmicutes	Faecalitalea	8.06	-5.15	0.039
OTU 0955	Proteobacteria	Psychrobacter	2.90	-2.58	0.039
OTU 1270	Firmicutes	Lactobacillus	1.51	-2.63	0.039
OTU 0173	Firmicutes	Dubosiella	37.63	-8.47	0.041
OTU 0899	Firmicutes	Lactobacillus	4.77	-3.43	0.041
OTU 0381	Bacteroidetes	Bacteroides	6.04	-4.57	0.041
OTU 0793	Firmicutes	Sharpea	3.15	-2.04	0.041
OTU 1858	Firmicutes	Lactobacillus	0.93	-3.74	0.041
OTU 0598	Firmicutes	Lachnoclostridium	11.69	-5.05	0.042

OTU 1152	Firmicutes	Intestinimonas	3.93	-4.05	0.042
OTU 0880	Bacteroidetes	Muribaculaceae ge	1.42	-4.28	0.042
OTU 0064	Fusobacteria	Fusobacterium	63.88	-4.36	0.042
OTU 0577	Bacteroidetes	Bacteroides	7.84	-4.46	0.043
OTU 0710	Actinobacteria	Actinomyces	4.96	-1.68	0.043
OTU 0809	Bacteroidetes	Chryseobacterium	3.45	-2.22	0.044
OTU 0569	Firmicutes	Ruminococcaceae UCG-014	10.55	-2.37	0.045
OTU 0198	Kiritimatiellaeota	WCHB1-41 ge	34.63	-3.18	0.045
OTU 0851	Firmicutes	Lactobacillus	3.61	-2.55	0.046
OTU 0951	Firmicutes	Ruminococcaceae UCG-014	5.31	-5.40	0.046
OTU 1137	Firmicutes	Intestinimonas	2.17	-4.19	0.046
OTU 0865	Firmicutes	Lachnospiraceae NK4A136 group	3.14	-4.13	0.046
OTU 0252	Fusobacteria	Fusobacterium	3.50	-4.71	0.047
OTU 0159	Firmicutes	Lactobacillus	95.35	-2.11	0.047
OTU 0946	Proteobacteria	Burkholderiaceae unclassified	2.79	-2.12	0.048
OTU 1041	Firmicutes	Clostridium sensu stricto 1	2.13	-2.25	0.049

Table D. 4 DeSeq2 analysis results for OTUs classified to the genera level that significantly changed between the colon and faeces at day 14, irrespective of environment, diet or sample type. P values are adjusted for multiple testing using the Benjamin-Hochberg correction. Fold changes represent Log2 fold changes.

	Phylum	Genus	Base Mean	LogFold Change	P value
Increased from colon to faeces					
OTU 0020	Bacteroidetes	Muribaculaceae ge	695.06	1.87	<0.001
OTU 0118	Actinobacteria	Eggerthellaceae unclassified	83.60	1.09	0.003
Decreased from colon to faeces					
OTU 0022	Proteobacteria	Gammaproteobacteria unclassified	736.96	-2.82	0.001
OTU 0028	Epsilonbacteraeota	Helicobacter	653.26	-2.49	0.001
OTU 0123	Proteobacteria	Sutterella	75.69	-1.54	0.001
OTU 0269	Proteobacteria	uncultured	4.18	-3.99	0.004
OTU 0169	Bacteroidetes	Alistipes	12.10	-5.23	0.004
OTU 0006	Proteobacteria	Escherichia-Shigella	6264.20	-2.71	0.009
OTU 0011	Epsilonbacteraeota	Campylobacter	1543.90	-2.03	0.049

Table D. 5 DeSeq2 analysis results for OTUs classified to the genera level that significantly changed between the mucosa and lumen of the jejunum, ileum and colon at day 14, irrespective of environment or diet. P values are adjusted for multiple testing using the Benjamin-Hochberg correction. Fold changes represent Log2 fold changes.

	Phylum	Genus	Base Mean	logFold Change	P value
JEJUNUM- Increased from lumen to mucosa					
OTU 0010	Epsilonbacteraeota	Helicobacter	6200.93	4.68	0.003
OTU 0009	Epsilonbacteraeota	Helicobacter	9371.52	3.77	0.007
OTU 0129	Proteobacteria	Enterobacteriaceae, unclassified	70.66	2.96	0.036
Decreased from lumen to mucosa					
OTU 0781	Actinobacteria	Actinomyces	10.65	-6.73	0.001
OTU 0248	Firmicutes	Lactobacillus	33.06	-2.63	0.012
OTU 0930	Firmicutes	Streptococcus	5.29	-3.81	0.032
ILEUM- Increased from lumen to mucosa					
OTU 0213	Proteobacteria	Stenotrophomonas	56.49	4.4	<0.001
OTU 4618	Bacteria, unclassified	Bacteria unclassified	1.04	20.58	<0.001
OTU 0153	Proteobacteria	Pseudomonas	94.48	2.54	<0.001
OTU 0104	Proteobacteria	Acinetobacter	175.78	2.65	<0.001
OTU 0177	Cyanobacteria	Cyanobium PCC-6307	12.26	4.95	<0.001
OTU 0344	Proteobacteria	Alphaproteobacteria unclassified	24.34	4.5	0.001
OTU 0170	Cyanobacteria	Cyanobium PCC-6307	17.4	4.16	0.003
OTU 0071	Firmicutes	Enterococcus	47.51	2.2	0.003
OTU 0098	Proteobacteria	Burkholderiaceae unclassified	37.7	3.93	0.007
OTU 0494	Firmicutes	Streptococcus	13.13	3.43	0.008
OTU 0825	Epsilonbacteraeota	Helicobacter	4.24	3.26	0.011
OTU 0187	Proteobacteria	Acinetobacter	139.91	2.57	0.015
OTU 0200	Bacteroidetes	Alistipes	5.24	5.09	0.016
OTU 0389	Actinobacteria	Flaviflexus	7.22	4.88	0.025
OTU 0124	Proteobacteria	Burkholderia-Caballeronia-Paraburkholderia	121.82	1.78	0.031
OTU 0221	Proteobacteria	Hydrogenophilus	22.3	3.17	0.031
OTU 0009	Epsilonbacteraeota	Helicobacter	3097.05	2.29	0.032
OTU 0145	Firmicutes	Ruminococcaceae unclassified	28.01	1.85	0.032
OTU 0294	Proteobacteria	Sphingobium	8.87	6.04	0.032
OTU 0306	Proteobacteria	Sphingobium	7.75	6.55	0.032
OTU 0383	Cyanobacteria	Synechococcus CC9902	3.81	4.17	0.032
OTU 0946	Proteobacteria	Burkholderiaceae unclassified	4.28	2.74	0.032
OTU 0842	Firmicutes	Clostridiales vadinBB60 group ge	4.35	3.86	0.033
OTU 0619	Proteobacteria	Delftia	11.82	2.48	0.046
OTU 0020	Bacteroidetes	Muribaculaceae ge	384.23	1.39	0.046
OTU 0702	Proteobacteria	Bosea	5.25	3.57	0.046

OTU 0887	Proteobacteria	Enhydrobacter	3.67	2.21	0.046
OTU 0333	Firmicutes	Staphylococcus	9.13	1.66	0.047
OTU 0291	Actinobacteria	Bifidobacterium	15.47	3.21	0.048
OTU 0544	Proteobacteria	Halomonas	11.29	2.44	0.048
Decreased from lumen to mucosa					
OTU 0854	Firmicutes	Lactobacillus	3.81	-3.00	0.031
OTU 0378	Firmicutes	Family XIII ge	12.53	-2.90	0.046
OTU 0512	Firmicutes	Lactobacillus	9.08	-2.71	0.046
OTU 1041	Firmicutes	Clostridium sensu stricto 1	2.41	-2.79	0.046
COLON – Increased from the lumen to the mucosa					
OTU 0028	Epsilonbacteraeota	Helicobacter	1380.51	3.05	0.002
OTU 0011	Epsilonbacteraeota	Campylobacter	3646.88	2.94	0.014
OTU 0166	Proteobacteria	Anaerobiospirillum	38.66	4.71	0.022

Appendix E

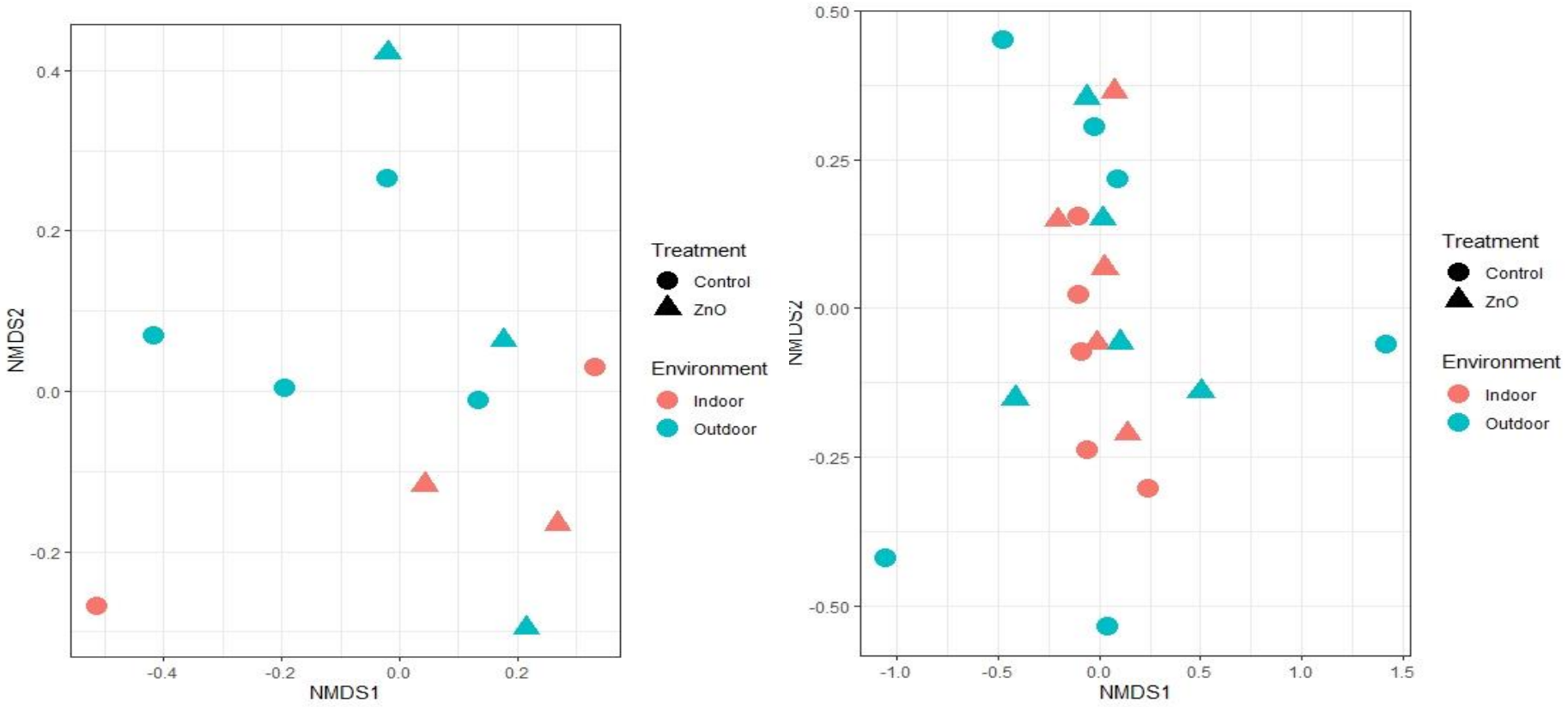

Figure E. 1 Non-metric multidimensional scaling (NMDS) plot of the bacterial community in the lumen (left) and mucosa (right) of the jejunum, obtained from indoor(red) or outdoor (blue) reared pigs that were provided control (~200 ppm; circle) or pharmacological (~2500 ppm; triangle) levels of ZnO for 14 days after weaning.

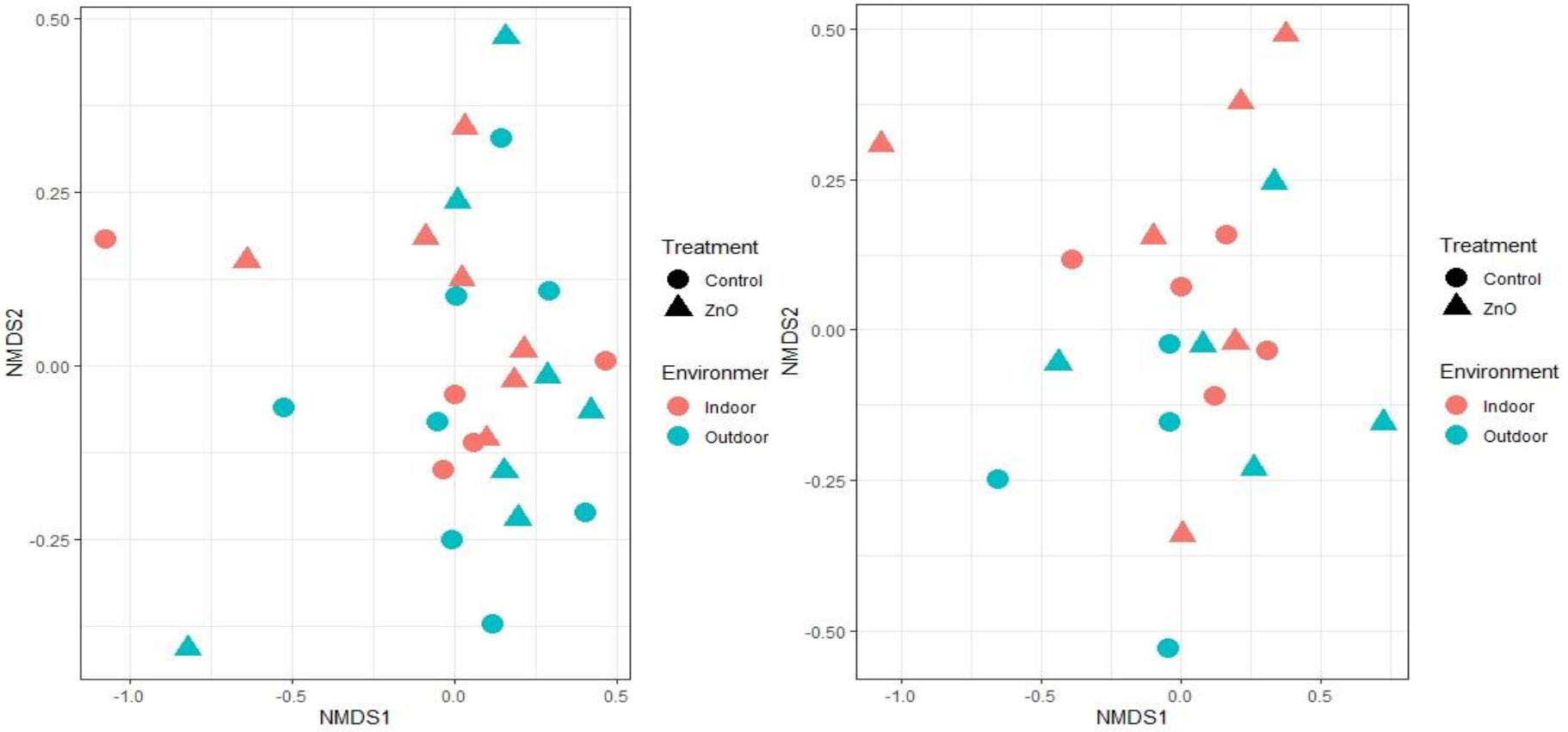

Figure E. 2 Non-metric multidimensional scaling (NMDS) plot of the bacterial community in the lumen (left) and mucosa (right) of the ileum, obtained from indoor(red) or outdoor (blue) reared pigs that were provided control (~200 ppm; circle) or pharmacological (~2500 ppm; triangle) levels of ZnO for 14 days after weaning.

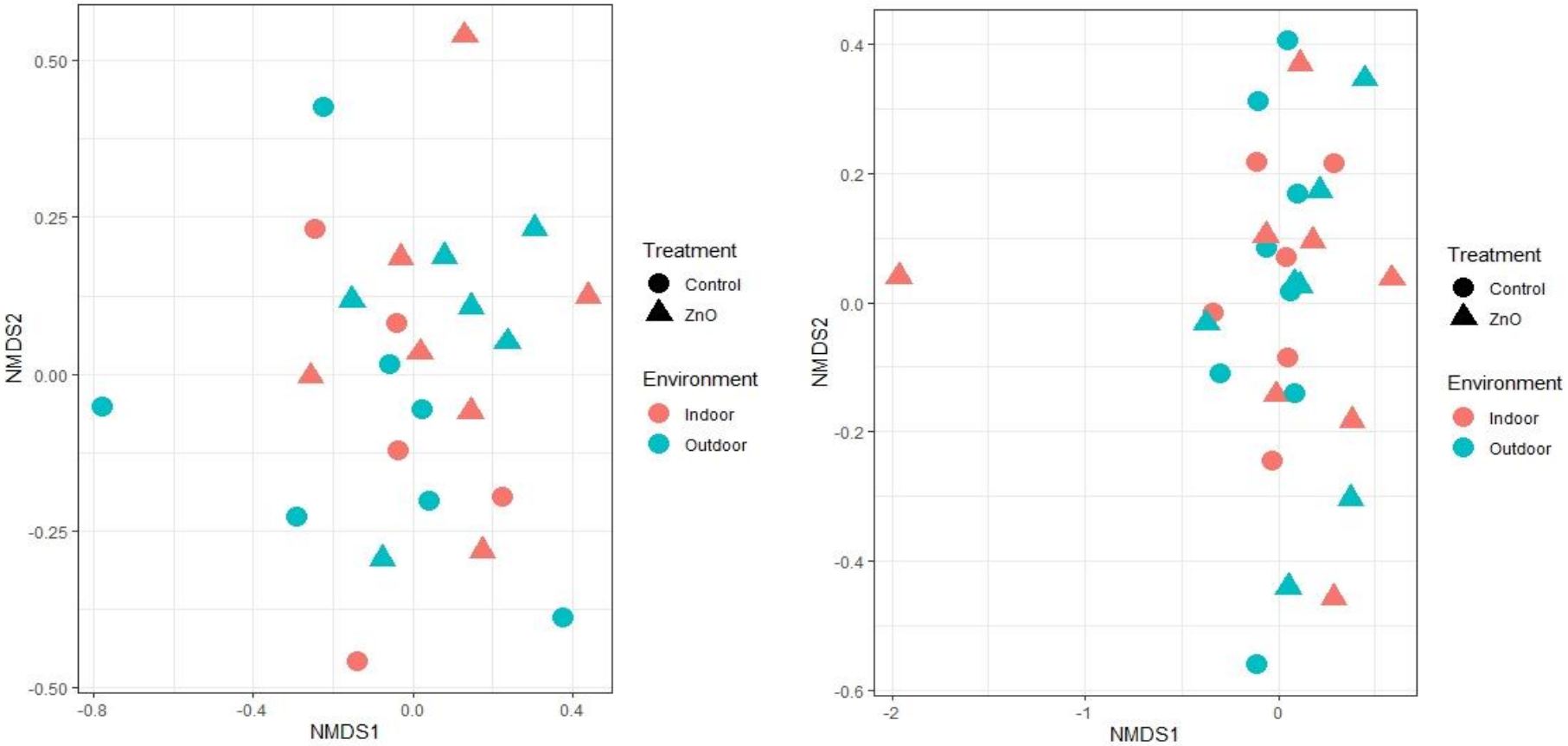

Figure E. 3 Non-metric multidimensional scaling (NMDS) plot of the bacterial community in the lumen (left) and mucosa (right) of the colon, obtained from indoor(red) or outdoor (blue) reared pigs that were provided control (~200 ppm; circle) or pharmacological (~2500 ppm; triangle) levels of ZnO for 14 days after weaning.

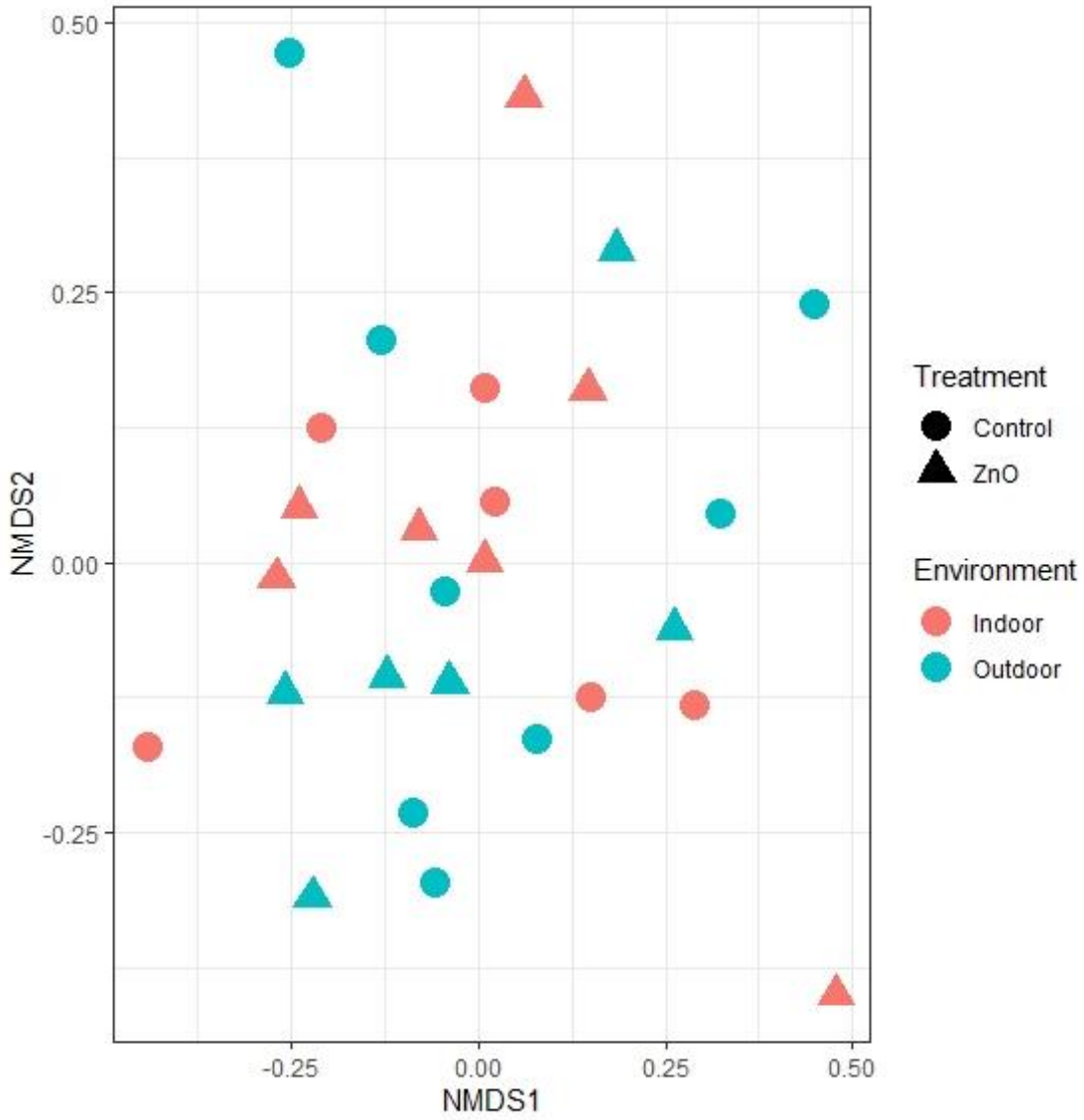

Figure E. 4 Non-metric multidimensional scaling (NMDS) plot of the bacterial community of the faeces, obtained from indoor(red) or outdoor (blue) reared pigs that were provided control (~200 ppm; circle) or pharmacological (~2500 ppm; triangle) levels of ZnO for 14 days after weaning.