Effects of therapeutic ZnO and Antibiotics and its withdrawal on the microbiome of weaned pigs

Efectos de los Antibióticos y Óxido de Zinc y su retirada en el microbioma de los cerdos al destete

Tesis doctoral presentada por: Juan Manuel Ortiz Sanjuán.

Dirigida por:

Juan José Garrido Pavón

Edgar García Manzanilla

Héctor Argüello Rodríguez

Tutor: Juan José Garrido Pavón

Programa de Doctorado: Biociencias y Ciencias Agroalimentarias.

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AUTOR: *Juan Manuel Ortiz Sanjuán*

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Ctra. Nacional IV, Km. 396 A
14071 Córdoba

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TÍTULO DE LA TESIS:
EFFECTS OF THERAPEUTIC ZnO AND ANTIBIOTICS AND ITS WITHDRAWAL ON THE MICROBIOME OF WEANED PIGS

DOCTORANDO/A:
JUAN MANUEL ORTIZ SANJUÁN

INFORME RAZONADO DEL/DE LOS DIRECTOR/ES DE LA TESIS

La tesis doctoral de D. Juan Manuel Ortiz Sanjuán es una tesis doctoral co-dirigida por investigadores de tres instituciones (UCO, Teagasc, Universidad de León), financiada por un proyecto de colaboración internacional (0376) en la cual el estudiante ha realizado tres estudios sobre el impacto de la retirada del óxido de zinc y de antibióticos en el posdeste de producción porcina.

Las tareas de la tesis doctoral se han realizado en parte en el departamento de Genética de la Universidad de Córdoba y en el Pig Development Department del Teagasc (Moorepark, Irlanda), con una estancia de larga duración comprendida entre el 01/05/2019 y el 12/11/2021. El estudiante se ha formado en técnicas de biología molecular, extracción y purificación de ácidos nucleicos, secuenciación masiva de ADN y análisis bioinformático de metagenomas.

La tesis doctoral está dividida en siete capítulos, de los cuales, los trabajos científicos realizados en la tesis se incluyen en los capítulos 2, 3 y 4. El capítulo 2, correspondiente al primer estudio, está publicado en la revista Microbiology spectrum (10.1128/spectrum.01597-22; IF: 9.043, posición 20/136 en el área de Microbiology JCR). El trabajo derivado del capítulo 3 está en revisión en la misma revista, mientras que el capítulo 4 está pendiente de envío, previsiblemente a la misma revista o a Animal Microbiome. La calidad de los trabajos presentados, queda reflejada por el impacto de la publicación o potencial impacto de aquellos que aun no han sido publicados.

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EFFECTS OF THERAPEUTIC ZnO AND ANTIBIOTICS AND ITS WITHDRAWAL ON THE MICROBIOME OF WEANED PIGS

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Summary

Post-weaning diarrhoea (PWD) is an infectious disease that causes significant productive and economic losses in pig production and often requires antimicrobial use. Antibiotic prophylaxis and metaphylaxis in animals are subject to more and more restrictions, especially in the EU, due to the risk of antimicrobial resistance. Zinc Oxide (ZnO) used in a range of 1500-3000ppm (dose referred as therapeutic or pharmacological) is also an effective treatment to prevent PWD. However, its therapeutic use was banned in the EU on the 28th of July 2022 due to environmental risk of soil pollution associated to its use. Finding alternative strategies to the use of antibiotics and ZnO to control PWD is crucial to ensure optimum levels of animal health and welfare and the economic viability of pig farms, ultimately resulting in high quality food production. A key step to find alternative strategies to the use of antibiotics and ZnO is to understand in detail their effects in the microbiome and the animal. This thesis focuses on the effects on the microbiome.

The main causative agent of PWD is enterotoxigenic *Escherichia coli*. Antibiotics and ZnO are effective controlling *E. coli* overgrowth during the post-weaning period, although the exact mechanism of action of ZnO is not completely understood. On the other hand, microbiome dysbiosis occurring immediately post-weaning is described as both a possible risk factor and consequence of PWD. ZnO stabilizes the gut microbiome avoiding this dysbiosis, yet the exact taxonomic and functional changes triggered by ZnO in the microbiome are not completely characterized. In this thesis, we used shotgun whole metagenome sequencing to explore the effects of ZnO and antibiotics both, at species and functional level, in pigs gut microbiome in the first weeks post-weaning.

In chapter 2, we studied the effects of ZnO and apramycin on the gut microbiome of the piglet a week post-weaning. Both, ZnO and apramycin had marked effects in gut microbiome taxonomy and functionality. Pigs fed the control diet with no ZnO or apramycin (Ct) exhibited high abundance of *E. coli* harbouring several virulence factors in animals not showing clinical

signs of diarrhoea. This study was carried out in a low disease, high hygiene and biosecurity experimental farm where effects of different cleaning procedures were assessed as well. Treatment was the factor with strongest effect on the microbiome, whereas cleaning procedures had no remarkable effects.

Given the results observed in the first study, the following studies described in chapters 3 and 4 were conducted in commercial farms to explore the effects of ZnO and antibiotics in commercial environments and between-farms variability in gut microbiome composition. Chapter 3 studied the impact of antibiotics and ZnO in pig microbiome comparing farms that had successfully removed ZnO and antibiotics to farms frequently using ZnO and antibiotic prophylaxis and metaphylaxis at weaning. Pig microbiome of farms using ZnO and antibiotics exhibited changes at days 7 and 14 post-weaning both at taxonomic and functional levels; these changes being more apparent in diarrhoea samples of 7 days post-weaning. Analysis of the environmental microbiome revealed a weak contribution of the environment to the gut microbiome of piglets, which shared few species early after weaning and within the 2 weeks post-weaning period studied.

Chapter 4 studied the effects of removing antibiotics and ZnO on the microbiome of farms regularly using ZnO and antibiotics as prophylaxis and metaphylaxis. Results showed that antibiotics, and especially ZnO, maintain a stable microbiome composition (taxonomical and functional), inhibiting *E. coli* overgrowth both in normal and diarrhoeic conditions. Removal of ZnO and antibiotics generated an increase of *E. coli* abundance, as well as virulence related genes associated to the higher abundance of *E. coli*.

Lastly, in chapter 5, we discussed the utility of shotgun sequencing in the study of microbiome changes caused by ZnO and diarrhoea, that could be triggered by antimicrobial and not-antimicrobial ZnO-associated effects (both taxonomical and functional), and the effects of ZnO maintaining gut microbiome stability during the most critical period of post-weaning stage.

From the results obtained in this thesis, the author concludes that weaning induces a sudden transition from a suckling pig microbiome to an adult like microbiome. The use of antibiotics and ZnO had a strong influence in this microbiome transition after weaning preventing piglet's gut microbiome dysbiosis by inhibiting *E. coli* overgrowth and hence the presence of its associated virulence factors related genes as well as promoting a stable transition to an adult-like microbiome. Finally, environmental microbiota (i.e., weaning room environment) exerts minor effects on the composition of the microbiome of the piglet.

Resumen

La diarrea pos-destete (PWD, del inglés post-weaning diarrhoea) es una enfermedad infecciosa que causa pérdidas productivas y económicas en producción porcina y que a menudo requiere el uso de antimicrobianos. El uso profiláctico y metafiláctico de estos antimicrobianos para el tratamiento de la PWD está sujeto a cada vez más restricciones, especialmente en la UE, debido al riesgo de resistencias antimicrobianas. El óxido de zinc (ZnO) usado en concentraciones de 1500 a 3000ppm (referidas como concentraciones terapéuticas o farmacológicas) también se usa como un tratamiento eficaz para prevenir la PWD. Su uso se prohibió el 28 de julio de 2022 en la UE debido al riesgo ambiental de contaminación del suelo asociado a su uso. Encontrar estrategias alternativas al uso de los antibióticos y del ZnO es crucial para mantener los niveles óptimos de salud y bienestar animal, así como la rentabilidad de las granjas, asegurando la producción de alimentos de alta calidad. Un primer paso clave para encontrar estas estrategias alternativas a los antibióticos y el ZnO es entender en detalle sus efectos de en el microbioma y en el animal. Esta tesis se centrar en los efectos en el microbioma.

El principal agente causal de la PWD es *Escherichia coli* enterotoxigénica. El ZnO y los antibióticos son efectivos para controlar el crecimiento excesivo de *E. coli* durante este período, aunque el mecanismo de acción exacto del ZnO no está totalmente claro. Por otro lado, la disbiosis del microbioma que ocurre en los días posteriores al destete uno de los nuevos posibles factores de riesgo y a su vez consecuencias descritas de la PWD. Se cree que el ZnO estabiliza el microbioma intestinal, pero hasta el momento, los cambios taxonómicos y funcionales exactos que provoca no están bien caracterizados. En esta tesis, utilizamos la secuenciación del metagenoma completo para caracterizar el efecto que tanto el ZnO como los antibióticos tienen en el microbioma intestinal del cerdo tanto a nivel taxonómico como funcional, en las primeras semanas posteriores al destete.

En el capítulo 2, estudiamos el efecto del ZnO y de la apramicina en la respuesta del microbioma intestinal del cerdo al destete una semana pos-destete. Ambos tuvieron efectos marcados en la

taxonomía y funcionalidad del microbioma intestinal. Los cerdos alimentados con dieta control sin ZnO ni antibióticos (Ct) exhibieron una gran abundancia de *E. coli*, que portaba varios factores de virulencia en animales que no mostraban signos clínicos de diarrea. Este estudio se realizó en una granja experimental de baja patología con altos niveles de higiene y bioseguridad en la que también se evaluaron los efectos de diferentes procedimientos de limpieza. El tratamiento fue el factor con mayor efecto en el microbioma, mientras que los procedimientos de limpieza no tuvieron efectos notables.

Dados los resultados observados en el primer estudio, los siguientes estudios descritos en los capítulos 3 y capítulo 4 se realizaron en granjas comerciales para explorar los efectos de los antibióticos y el ZnO en entornos comerciales y la variabilidad entre granjas en la composición del microbioma intestinal. En el capítulo 3, se compararon granjas que usaban antibióticos y ZnO con granjas que los habían retirado. El microbioma de las granjas que utilizaban ZnO y antibióticos exhibió diferencias en los días 7 y 14 posteriores al destete, tanto a nivel de taxonómico como funcional; diferencias más evidentes en muestras de diarrea de 7 días post destete. El análisis del microbioma ambiental reveló una contribución débil al microbioma de los lechones, que compartían algunas especies consideradas como “core” que permanecían en el ambiente limpio de la sala de destete y en muestras iniciales y recogidas a las 2 semanas pos-destete.

En el capitulo 4 se estudió el impacto de la retirada de los antibióticos y ZnO en el microbioma porcino en granjas que utilizaban habitualmente antimicrobianos de forma profiláctica y metafiláctica al destete. Los resultados mostraron que los antibióticos, y sobretodo el ZnO, mantienen la composición del microbioma estable (taxonómica y funcionalmente), inhibiendo el crecimiento excesivo de *E. coli* tanto en condiciones normales como en diarrea. La retirada de ZnO y antibióticos en estas granjas generó un aumento en la abundancia de *E. coli*, así como genes relacionados con la virulencia asociados a la mayor abundancia de *E. coli*.

Por último, en el capítulo 5, discutimos la utilidad de la secuenciación por medio de “Shotgun” en el estudio de los cambios en el microbioma causados por el ZnO y la diarrea, que podrían desencadenarse por efectos antimicrobianos y no antimicrobianos asociados al ZnO (tanto taxonómica como funcionalmente), y los efectos de ZnO manteniendo la estabilidad del microbioma intestinal durante el período más crítico de la etapa posterior al destete.

De los resultados obtenidos en esta tesis, el autor concluye que el destete induce una transición brusca de un microbioma de lechón lactante a un microbioma de cerdo adulto. El uso de antibióticos y ZnO tiene una fuerte influencia en la transición del microbioma después del destete, previniendo la disbiosis intestinal en el microbioma del lechón al inhibir el crecimiento excesivo de *E. coli* y, por lo tanto, la presencia de sus genes relacionados con factores de virulencia, así como promover una transición estable hacia un microbioma similar al de un animal adulto. Finalmente, la microbiota ambiental (la presente en la sala de destete) ejerció efectos menores en la composición del microbioma de los lechones.

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List of Abbreviations

€	Euro currency
16S rRNA	16S ribosomal RNA of the 30S prokaryotic ribosomal unit
Ab	Antibiotics
ADFI	Average daily feed intake
ADG	Average daily gain
AIDA	Adhesin involved in diffuse adherence
AMR	Antimicrobial resistance
AST	Arcsine square-root transformation
ATP	Adenosine Triphosphate
Caco2	Adenocarcinoma colon human cells (immortalized cell line)
CD	Crypt Depth
CIA	Critically Important Antimicrobials
CRF	Corticotropin Releasing Factor
Ct	Control (basal diet or treatment)
DGGE	Denaturing Gradient Gel Electrophoresis
DNA	Deoxyribonucleic acid
dpw	day post-weaning
EAST-1	Enteroaggregative E. coli heat stable enterotoxin 1
ELISA	Enzyme-Linked ImmunoSorbent Assay
ETEC	Enterotoxigenic Escherichia coli
EU	European Union
F18	Fimbriae
F4	Fimbriae
FD	Feeder-Drinker
G:F	Growth:Feed intake ratio
GPX1	Glutathione peroxidase-1
IFN-γ	Interferon gamma
Ig	Immunoglobulin
IgA	Immunoglobulin A
IGF-1	Insulin like Growth Factor 1
IGF-1R	Insulin like growth factor 1 receptor
IL	Interleukin
IL-10	Interleukin-10
IL-17	Interleukine-17
IL-1β	Interleukin-1β
Il-6	Interleukin-6
IL-8	Interleukin-8
ITS region	nuclear ribosomal internal transcribed spacer
KW	Kruskal-Wallis
LPS	lipopolysaccharide
mRNA	messenger RNA
N.S	Non-Significant
NF-κB	Nuclear factor-κB
NGS	Next Generation Sequencing
OECD-FAO	Economic Co-operation
OTU	Operational taxonomic unit
PC	Positive Control
PCR	Polymerase chain reaction
Ppm	Parts per million
PWD	Post-weaning Diarrhoea

qPCR	Quantitative PCR
RNA	Ribonucleic Acid
SCVMP	Standing Committee on Veterinary Medicinal Products
SD	Standard deviation
SDF	Stem Cell factor
SOD	Superoxide dismutase
SZ	Shield Zinc
TGFβ1	Transforming growth factor beta 1
TJ	Tight Junction
TNF-α	Tumour necrosis factor alpha
VH	Villus height
VH:CD	Villus height:Crypt depth ratio
WF	Wall-Floor
WHO	World Health Organization
wks	Weeks
Zn	Zinc (ZnO treated)
ZnO	Zinc Oxide
ZO-1	Zonula Occludens 1

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Chapter 1. Literature Review

1.1 Pig production

Pig meat accounts for the 33% of the global meat consumption (Lebret and Čandek-Potokar, 2022) and it is the most consumed meat in Asia and Europe, where is settled approximately 56% and 21% of the world pork production, respectively (Lebret and Čandek-Potokar, 2022). Global population is predicted to increase 11% and this population growth will be sustained mainly by animal protein with an expected increase of 15% in meat protein production by 2031. Within this meat protein demand, pork production is expected to grow by 17% in the same time period (OECD-FAO Agricultural Outlook 2022-2031, 2022), although factors such as current outbreaks of African Swine Fever (ASF) in China, the Philippines and Vietnam may limit pig livestock inventory growth to 1 billion heads (OECD-FAO Outlook 2021-2031).

As mentioned above, Europe is a major pig producer worldwide. Within the European Union (EU) figures in 2021, Spain was the leading producer of pork meat, with a pork population accounting for a 24.3% of the porcine census of UE-27 and producing 5,180,060 tons of meat, with 58,370,490 slaughtered pigs, ahead of Germany, which production was impaired due to the ASF outbreaks of 2020 (Eurostat, annual data., 2021) (Figure 1).

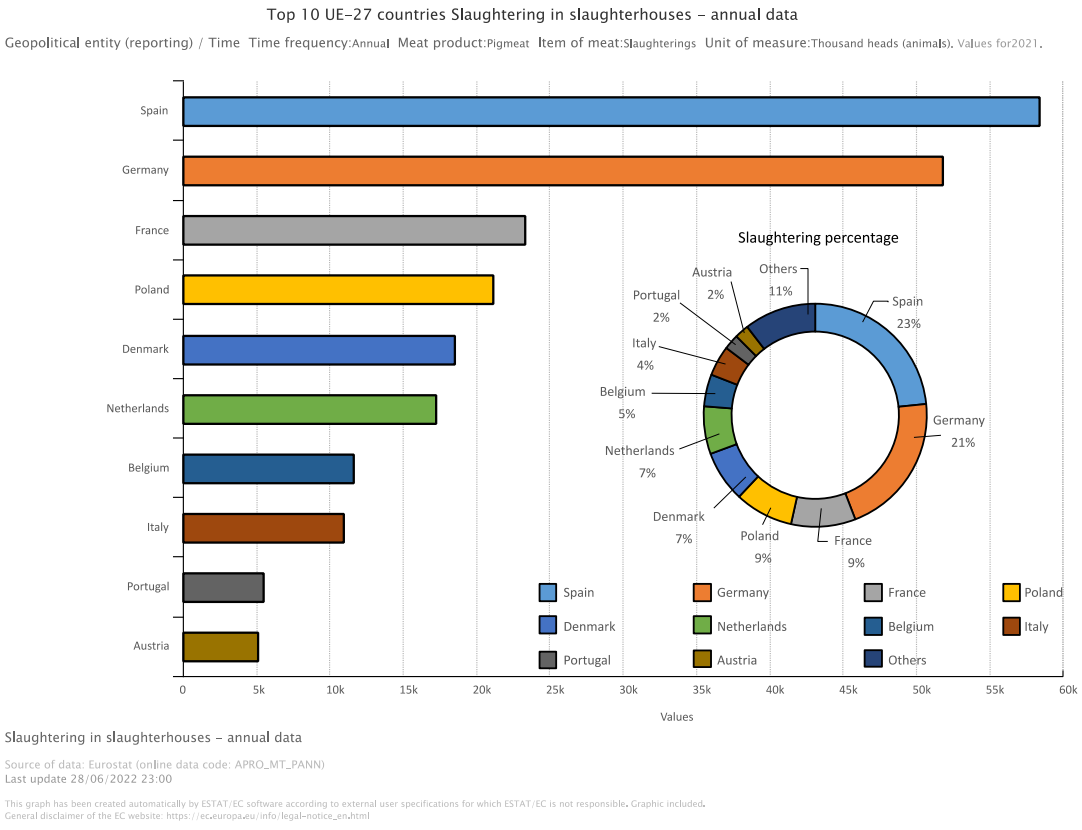


Figure 1. Top 10 countries slaughtering pigs (Thousand heads) and percentage of slaughtered pigs in EU in 2021. Source: Eurostat.

Pig husbandry is based on a production cycle with four main stages: gestation, lactation, nursery and growing/finishing rearing these animals until they reach the optimum weight for slaughter (Augère-Granier, 2020). Based on the stages just mentioned, predicted consumption levels of animal protein, animal husbandry needs to meet a trade-off among productivity, animal health and welfare and economic viability aspects are organized.

1.2 Pig weaning

Amongst the production stages, weaning is the most critical period. At weaning piglets are separated from the sow and abruptly forced to a dietary change and several environmental and social stressors (Zheng et al., 2021). In the wild, pigs are weaned at 3-4 months of age (Jensen, 1986; Kim, 2013). In intensive commercial farming, optimization of profitability has reduced the lactation period to 3 to 5 weeks in order to maintain production to satisfice demand at

reasonable prices (Revilla et al., 2019; Zheng et al., 2021). The stress that piglets have to face generates a challenging period in the production cycle, both for the animals and the farmer. The problems derived from the stressing factors result in an important cost, such as antibiotic therapy for infectious diseases and the economic loss caused by growth retardation in the weaned animal (Le Dividich and Sève, 2000). For instance, Sjölund et al., (2014) estimated a cost from €40 to €314 per sow for PWD outbreaks. Besides economic issues, weaning may also comprise public health due to the antimicrobial treatments needed to tackle the infections occurring at this point, post-weaning diarrhoea (PWD) (Revilla et al., 2019). These antimicrobial treatments contribute to the ongoing development and spread of antimicrobial resistant bacteria and PWD is highlighted as a hotspot in antimicrobial use in animal production (Gresse et al., 2017a; Gao et al., 2019; Revilla et al., 2019).

1.2.1 Stressors identified in pig weaning

Weaning is described as one of the most critical periods in pigs' productive cycle, in which they are abruptly exposed to several social, psychological, and environmental challenges that may generate acute or long-lasting health and productive problems. Among the main biological stressors that piglets have to face are the separation from the sow, the movement into a new place, a dietary change from liquid to solid feed, and the mixture with other piglets (Campbell et al., 2013). In nature, weaning is a process that occurs gradually, lasting approximately three months, during which the intestine and microbiota of the piglet adapts to several nutritional and environmental changes (Moeser et al., 2007). As aforementioned, weaned pigs are placed in a new environment, and usually mixed with other litters. At this point, piglets will explore the pen and start fighting with their pen-mates to establish a new social hierarchy. Mixed piglets can maintain this fight over 48 – 72 hours (Tong et al., 2020), or until the dominance is re-stablished (Bolhuis et al., 2005). In intensive farms, dietary changes occur suddenly and pig that are adapted to a highly digestible liquid milk diet, are subjected to an important dietary change, where they need to adapt to a more complex solid feed diet. Besides this dietary composition

change, sow's milk is rich in immunoglobulins A (IgA) that protects the intestinal mucosa of the piglet from potential pathogenic threats. Early after weaning, the content of IgA in the gut drops to non-protective levels and the piglet protection relies on generating its own defenses (Lallès et al., 2007; Rhouma et al., 2017). Unfortunately, the local immune response of the piglet in the intestine at three to five weeks of age is inefficient or at least not as efficient as the response in a mature intestine, thus, this moment creates a window of opportunity for pathogens to infect the animal.

1.2.2 Structural and functional intestinal alterations as a consequence of weaning

It is well reported that there is a drop in piglets feed intake in the first 24 to 48 hours, leading to an initial anorexia (McCracken et al., 1999; Campbell et al., 2013). Postweaning anorexia is considered a major factor triggering digestive, physiological and performance problems around weaning (Lallès et al., 2004; Wijtten et al., 2011). Two main inter-related alterations arise in piglet's intestine as a consequence of weaning anorexia: structural and functional alterations and inflammation in the small intestine (McCracken et al., 1999; Campbell et al., 2013).

The structural and functional changes are due to the fact that the absence of nutrients for enterocytes in the intestinal lumen first causes villus atrophy and crypt elongation. In line with this event, there is a depletion in brush border enzyme activity, alteration in amino acids metabolism as an intestinal adaptation mechanism to repair and protect the intestinal tissue. Several signs of tissue degradation are also reported, with the activation of metalloproteases as stromelysin (Lallès et al., 2004, Campbell et al 2013, Moeser et al 2007). Increases in the protein synthesis rate in the intestine and a drop in the muscle protein rate deposition have also been described (Pluske et al., 1997; Lallès et al., 2004; Campbell et al., 2013). All these factors mentioned lead to an intestinal barrier impairment and performance loses clearly evidenced by weight loss (Lallès et al., 2004; Wijten et al., 2011; Campbell., et al 2013).

The onset of an inflammatory status in the intestinal lumen has also been described as another response to weaning anorexia (McCracken et al., 1999; Campbell et al., 2013). The proposed mechanism so far to explain this phenomenon is associated to the compromised mucosal barrier alterations caused by anorexia, which allow food-antigens, bacteria and toxins to cross through the intestinal barrier into the lamina propria, where an initial inflammation is triggered (McCracken et al., 1999; Campbell., 2013). This event elicits the upregulation of pro-inflammatory cytokines such as IL-1β, IL-6, and TNFα (Pié et al., 2004), immune cell infiltration in lamina propria and further increase in intestinal permeability. On the other hand, other studies have addressed the importance of stress responses mediated by corticotrophin releasing factor (CRF) at weaning, which enhances the activity of mucosal mast cells, impairing intestinal impermeability. Pre-treatment of weaned pigs with mast cell stabilizer inhibited weaning intestinal barrier impairment (Moeser et al., 2007). In any case, the role of the immune system aggravating the already settled intestinal barrier structure and function disturbance seems to be a critical aspect of weaning-associated alterations.

1.2.3 Stages of weaning based on anatomical and functional alterations

Alterations occurring at weaning can be divided chronologically into two stages (Lallès et al., 2004). The early acute phase includes the already mentioned tissue atrophy and degeneration, inflammation (immune cells infiltration and cytokine upregulation), upregulation of cyto-protection system (mainly via heat shock proteins), which ends with a loss of intestinal barrier structure and function. The second phase involve an adaptative-regenerative phase, in which alterations cease upon normal feed intake resumption. Similarly, Pié et al (2003) also reported two phases of inflammatory response caused by weaning. The first occurring within the first two days post-weaning, which involves upregulation of proinflammatory cytokines IL-1, IL-6 and TNFα, and the second lasting to eight dpw, in which cytokines decrease to normal values, except for some intestinal locations such as TNFα at the distal small intestine and IL-8 in the proximal colon, and feed intake is restored.

1.3 Pig microbiota

Another factor to be addressed at weaning is the intestinal microbiota of piglets. Recent studies are pointing to this factor as one of the main critical factors impacting in a good lactation to weaning transition. Therefore, the study of the gut microbiota, its role in different diseases and processes in the organism has been gaining attention.

The gut microbiota is a complex community of microorganisms that inhabits the intestine of vertebrates, and which includes bacteria, archaea, fungi and viruses. The interaction between gut microbiota and the host, and its role in different processes in the organism has already been widely reported (Tremaroli and Bäckhed, 2012; Brestoff and Artis, 2013). However, microbiomes are really complex ecosystems which composition, functional traits, contribution to host health and interactions host-microbiota, are just at the dawn of knowledge. It is important to understand how the microbiota changes between these two phases of the productive cycle of pigs, in order to better comprehend which alterations are being conducted at weaning and, if possible, whether these changes can be redirected towards a specific state that allows piglets to cope with weaning and minimizing the alterations described before.

1.3.1 Piglets' microbial community development: Colonization and succession until weaning

The initial colonization of the gut of the pig by microbiota is thought to occur via maternal imprinting by transfer of bacteria through contact with birth canal (Patil et al., 2020). Sources as sows milk, faecal, vaginal, nipple microbiota have been described as the most important sources of colonizing microbiota in the suckling period (Chen et al., 2018; Liu et al., 2019). The first bacterial group described to colonize the gut within the first 2 days of age are facultative anaerobes within families Enterobacteriaceae, Enterococcaceae and Staphylococcaceae, followed by strict anaerobes such as *Bifidobacteria*, *Bacteroides* and Clostridiales (Patil et al., 2020). Other authors have reported the dominance of Streptococcaceae in all locations of piglet's gastrointestinal tract, between the first 3 days of age, that thereafter were displaced by Lactobacillaceae and Clostridiaceae (Inoue et al., 2005). Several strict anaerobic bacteria have

been described as shared among sow faeces, milk and faeces of neonatal piglets (Chen et al., 2018). Other authors also hypothesized that a whole microbial population is transferred to piglets since the first day after birth, and this community remains stable until exposure to other conditions, such as dietary changes, more favourable for the already present low abundant bacteria (Frese et al., 2015). However, a recent study (Liu et al., 2019) demonstrates that this statement is only valid for the small intestine, where the consortium of microorganisms present is highly shaped by milk consumption and remains relatively stable throughout lactation. In the large intestine, the microbial population suffers an evolution from this milk-oriented microbiota to a microbiota that, according to the ordination analyses performed by Liu and colleagues, resembles the faecal microbiota of their mothers.

At weaning, piglets gut microbiota is considered as a milk-oriented microbiome (Gresse et al., 2017), not well established yet to the next stages of pig's life. It is well known that weaning generates a sudden microbial shift in piglets' intestinal microbiota, not only because of the dietary replacement of milk by solid feed, but also due to the transient anorexia pointed before. Yet critical, the specific alterations in microbial population in this short window of first 24-48 hours after weaning are still not well established.

Post-weaning changes in microbial population and functions repertory after feed consumption have been characterized recently. In this regard, these studies report quick microbial and functional changes from an adapted milk-oriented microbiome, towards a highly rich and diverse community of bacteria capable of utilize complex plan-derived carbohydrates (polysaccharides) present in the feed (Frese et al., 2015; Wang et al., 2019b). Indeed, in weaned pigs, microbiota diversity and richness have been described to increase across time, along with a functional adaptation of the community in order to be able to degrade plant-derived carbohydrates (Frese et al., 2015; Guevarra et al., 2019). Among the main bacteria affected by the dietary feed inclusion, it has been reported a decrease in the abundance of Bacteroidaceae, Enterobacteriaceae, and Fusobacteriaceae; and an enrichment in Prevotellaceae,

Veillonellaceae and Lachnospiraceae families (Guevarra et al., 2019; Yang et al., 2019; Saladrigas-García et al., 2021). In addition to these bacterial groups, Lactobacillaceae family is a variable group of bacteria described to decrease or increase in several studies. *Lactobacillus* spp., are well known by their ability to utilize lactose. However, several bacteria within this group are not capable of metabolizing other complex olygossacharides present in milk, and so, it has been described to increase after weaning as a consequence of feed consumption and its ability to metabolize plant-derived carbohydrates (Frese et al., 2015; Wang et al., 2019b). These shifts in bacterial composition were accompanied by the already described functional changes, in which most of them coincide those increased bacteria with the ability to use plant-derived complex carbohydrates. On the other hand, in another study, suckling pigs' microbiota has been described to be dominated by *Bacteroides*, *Escherichia/Shigella*, *Fusobacterium*, *Lactobacillus*, and *Megasphaera*, whereas *Clostridium sensu stricto*, *Roseburia*, *Paraprevotella*, *Clostridium XIVa*, and *Blautia* were the predominant genera after weaning (Chen et al., 2017).

Less knowledge about the microbial composition state in the first 24-48 hours of weaning is available. Although microbiota diversity at weaning is supposed to decrease in the first days, increasing gradually as the animals start eating feed, little is known about the exact microbial composition and functions shifts occurring in this moment, when the typical weaning-associated alterations are taking place in the intestine, and pathogenic organism may infect the piglets. What is clear from the information gained so far is that weaning favours a microbial dysbiosis in the intestine.

1.3.2 The gut microbiota dysbiosis at weaning

As previously stated, the alterations occurring at weaning trigger an inflammatory state within the gut. This inflammation exacerbates the existing problem with intestinal permeability and, along with the loss of diversity caused by the absence of nutrients in the lumen, confers an evolutionary advantage to mucin-degrading bacteria, which leave accessible niches for pathogenic microorganism to colonize the gut (Gresse et al., 2017; Baümler and Sperandio,

2016). Weaning anorexia also has an important effect on piglet's microbiota, generating a disbalance/dysbiosis state that can favour the invasion and or proliferation of pathogens either primary such as enterotoxigenic *Escherichia coli* (ETEC) or secondary pathobionts such as *Citrobacter,* which take advantage of the disbalance created.

In any case, all these factors seem to have a feed-back-like pattern where an alteration stimulates another and *vice versa*. The pro-inflammatory environment elicited by weaning is boosted by enteric pathogens that elicit and exacerbate an inflammatory response that, in turn, benefits their growth (Baümler and Sperandio, 2016). In fact, this inflammation releases reactive oxygen species (Baümler and Sperandio, 2016; Gresse et al., 2017b) which alter the gut environment, boosting the growth of certain facultative anaerobes like the Enterobacteriaceae family (Zeng, Inohara and Nuñez, 2017) for instance, *E. coli* or *Salmonella* enterica serovar Tphymurium (Spees et al., 2013). The inflammation also impairs the growth of certain strict anaerobes within the Clostridia and Bacteroidaceae class (Winter et al., 2013).

Gut community perturbations suffered during the first 24-48h at weaning can cause a longer term disbalance in the gut microbial equilibrium if the mechanisms of resilience and resistance of the microbial community fail (Fassarella et al., 2021). In healthy animals, these alterations subside upon normal resumption/restoration of feed intake, following thereafter the described pattern of succession in gut microbiota. On the other hand, with all the risk factors mentioned before, weaning can result in the well-known post-weaning diarrhoea (PWD).

1.4 Post-weaning diarrhoea (PWD)

Post-weaning diarrhoea is an important multifactorial disease, affecting piglets by causing growth retardation, mortality and economic losses (Luppi, 2017; Rhouma et al., 2017). Although PWD may be caused by several pathogens (Katsuda et al., 2006; Eriksen et al., 2021), ETEC is the main etiological agent associated with it. ETEC has been extensively characterized in the context of PWD. This bacterium is generally ubiquitous on the farm environment and piglet's intestine.

However, an outbreak does not occur unless the pre-disposing factors are present, the pathogen reaches a high load within the intestine, and is able to adhere to the intestinal mucosa, where the expression of virulence factors finally end in the clinical disease (Luppi et al., 2017; Rhouma et al., 2017).

Different pathotypes (different ETEC strains harboring a combination of fimbriae and toxin genes) of ETEC have been described so far. For *E. coli* LPS antigen, several serotype antigens are described to cause PWD, but the predominant serogroup is O149, and overall, the most implicated pathotype in PWD is ETEC:LT:STb:F4. In this regard, the main virulence factors involved in the disease that define the bacterium ability to cause PWD are its adhesins and toxins (Luppi, 2017). Adhesins are membrane glycoproteins that bacteria use to adhere to surfaces, where it may develop several activities such as biofilm formation, release of toxins and virulence factors expression; and in some cases, it is a crucial factor for pathogenic bacteria to cause disease (Fairbrother et al., 2005; Klemm et al., 2010; Wu et al., 2013). These proteins are classified as fimbrial and non-fimbrial. Fimbriae are surface pili-like glycoproteins attached to the membrane of bacteria involved in bacterial adherence to specific surfaces such as certain tissues or cells (Jonson et al., 2005; Klemm et al., 2010). A thorough characterization of ETEC and its implications has been reviewed elsewhere (Luppi, 2017; Rhouma et al., 2017; García et al., 2020).

The most common fimbria involved in PWD are the F4 (former k88) and F18 (Fairbrother and Nadeau, 2019). Whereas F4 fimbriae is also involved in neonatal diarrhoea cases, F18 has been described only in cases of PWD. Both fimbriae have several antigenic variants. For F4, there are described the F4ab, F4ac and F4ad, being the F4ac the most frequent fimbriae involved in PWD; whereas for F18, there are 2 variants described: F18ac and F18ab, being associated the first with PWD and the second with edema disease. ETEC strains harboring non-fimbrial adhesin AIDA (adhesin involved in diffuse adherence) have also been reported in PWD cases (Fairbrother and Nadeau, 2019). The main enterotoxins associated to PWD are the heat stable (ST; STa and STb),

the heat labile (LT), and the enteroaggregative *E. coli* heat stable enterotoxin 1: EAST1. Although heat stable and labile toxins bind different receptors in the epithelium and activate different cell signaling cascades, the general mechanism of action for both of them is similar, causing a duodenal and jejunal secretion of electrolytes, increasing the active secretion of water to the lumen and causing a secretory diarrhoea and metabolic acidosis.

1.5 The AMR threat

The generalized missuse of antibiotics has contributed to the development and spread of antimicrobial resistance (AMR). These AMR are present, to a larger or lower extent, in almost all taxa, zoonotic, human/animal pathogens, or gut commensals, present in human, animals and environments (Woolhouse et al., 2015). In addition, many of these AMR determinants are located in mobile elements, such as plasmids, and can be transferred among bacteria, subsequently threating human health via food chain or animal contact, especially in farms workers (Djordjevic et al., 2013; Holmes et al., 2016). The raise and spread of AMR derminants together with the lack of new antimicrobial drugs is a great matter of concern (Plackett, 2020) and a study modelling the main attributable causes of deaths in the next future (O'Neill, 2014), estimated that the number of deaths caused by AMR could raise up to 10 million per year by 2050.

In animal production, the use of antimicrobials as prophylactic, metaphylactic and growth promoters to treat, prevent or yield better productive performance, especially at critical stages of lifecycle, have promoted the quick spreading of AMR (Rosengren et al., 2007). In 2018, more than half of all antimicrobials sold in the EU were used in livestock (ECDC, EFSA and EMA, 2021). Pig production is one of the main agriculture sectors using antimicrobials within the EU (Sarrazin et al., 2019). The main reasons forAMU overlap with pig's critical lifecycle stages such as birth, weaning and transfer to finisher stages. Antimicrobial treatments in these stages are as prophylaxis and metaphylaxis (Sarrazin et al., 2019). Undoubtedly weaning ranks as the main

stage in antimicrobial use (Lekagul et al., 2019; Sarrazin et al., 2019; Raasch et al., 2020), using the oral route (Sjölund et al., 2016; Sarrazin et al., 2019). Antimicrobial treatments usually are addressed to treat and control gastrointestinal and respiratory infectious diseases (De Briyne et al., 2014), with tetracyclines, polymyxins (colistin) and penicillins as the most frequent antimicrobials used in early production stages, where gastrointestinal problems are more common (Sjölund et al., 2016; Sarrazin et al., 2019).

1.5.1 Antimicrobial treatments used to control and treat PWD

Focusing on AMU in PWD, different antibiotics have been used as prophylactics and metaphylactics to prevent and treat PWD, respectively. Traditionally, ETEC has been susceptible to β-lactams, cephalosporines, aminoglycosides, aminocyclitols, sulphonamides combined with trimethoprim, quinolones, and polymyxins (Luppi, 2017). However, the development of AMR strains makes difficult to ensure the efficacy of the treatment (Fairbrother and Nadeau, 2019). If the animals are sick, antibiotics such as amoxicillin, trimethoprim/sulphametoxazole, ceftiofur, cefquinome or enrofloxacin must be administered parenterally (Luppi, 2017; Fairbrother and Nadeau, 2019). However, antibiotics such as ceftiofur (cephalosporin of 3rd generation), fluoroquinolones, polymyxin B (colistin) and macrolides are considered as "Critically Important Antimicrobials in human medicine" (CIAs) and its use must be reserved for last resource (Luppi, 2017; Fairbrother and Nadeau, 2019; WHO, 2021). Of particular concern is the ban of colistin use in pig production, the most effective antibiotics to treat and prevent PWD during the last few years (Rhouma et al., 2017). Other antimicrobial compounds have been used traditionally either in water or in-feed, as prophylactic or metaphylactic method, such as neomycin, apramycin (aminoglycosides) or spectinomycin (aminocyclitols).

The current situation of AMR and the negative future perspectives represent a challenge for economically viable and sustainable livestock production systems and sufficient animal protein supply for human population. Pig husbandry must adapt to the new restrictions to mitigate AMR spreading. Thus, antibiotics cannot be used anymore as prophylactic measure whereas in-feed

metaphylactic use of antibiotic will be reserved for situations with a confirmed diagnostic in well-defined groups of animals with severe infection or high risk of spreading the infection (REG EU 2019/4; REG EU 2019/6).

Besides the spread of AMR, antimicrobials affect the whole microbial community within the intestine, targeting both pathogenic and beneficial bacteria. The next subsection briefly summarizes the knowledge aquired about different antibiotics in pig microbiome in the last few years.

1.5.2 Effects of antibiotics on the gut microbiome of the piglet

Antibiotics exert their bactericidal or bacteriostatic effects by means of specific mechanisms of action depending on the antimicrobial class (Giguère, 2013). However, the effect is not targeted towards a specific bacterium but large groups of bacteria within the intestine (Neuman et al., 2018; Zeineldin et al., 2019a). Susceptibility or resistance to antimicrobials depends on factors such as the bacterial cell wall structure, the enzyme pool in bacterium genome, and the presence of specific ARGs or genome mutations that confer AMR. Characterizing the species composition and changes in the gut microbiome of the pig in response to each type of antibiotic is a challenging task. (Table 1). The variability among antibiotics used, routes of administration, antimicrobial concentration, age or productive stage studied, and bacterial groups found in each study makes really challenging to establish consistent links between the use of an antibiotic and a group of measurable changes, for instance in the intestine (Table 1). This task becomes even more complicated to track if we add the fact that there are billions of bacteria with the chance of exchange ARGs in each intestine (Sengupta et al., 2013; Zeineldin et al., 2019a). Beside these factors, the variability in the methods of sequencing should be considered. Most of these studies have been conducted using 16S rRNA sequencing, yet varying in each hypervariable region of the gene, which is sequenced, along with each sequence platform yielding different sequencing performance.

Overall, from the studies assessing the impact of antibiotics in pigs gut microbiome, it has been reported a decrease in microbial richness and diversity (Yu et al., 2018; Yang et al., 2022). But again, the specific changes in microbial population are inconsistent with some studies reporting a decrease in *Lactobacillus* spp populations (Mu et al., 2017; Gao et al., 2018; Yu et al., 2018; Yang et al., 2022) while others observe the contrary effect (Soler et al., 2018; López-Colom et al., 2020; Parois et al., 2020) and an increase in species within the phylum Bacteroidetes, particularly the family Bacteroidaceae and Prevotellaceae (Zhang et al., 2016). Other studies found increased levels of *E. coli* after antibiotic administration (Allen et al., 2011; Looft et al., 2012; Looft et al., 2014a; Connelly et al., 2018; Gao et al., 2018; Ghanbari et al., 2019) or the contrary (Looft, Heather K. Allen, et al., 2014; Sun et al., 2014; Zhang et al., 2016; Li et al., 2020; Rhouma et al., 2021). For instance, several studies using a mix of tetracyclines with other antibiotics reported increased levels of *Ruminococcus* spp., (Looft et al., 2012; Yu et al., 2018; Parois et al., 2020; Sun et al., 2022a) as well as in other studies using a different antibiotic (Zeineldin et al., 2019b). Other studies using chlortetracycline alone or in combination reported a decreased level of *E. coli* (Looft et al., 2014; Zhang et al., 2016) and decreased levels of *Sarcina* (Looft et al., 2012; Che et al., 2019). Further studies using colistin sulphate alone or in combination found lower levels of *E. coli* (Li et al., 2017c; Rhouma et al., 2021), or Proteobacteria (Soler et al., 2018). Other studies reported low strength or influence of gentamycin in gut microbiome (Poulsen et al., 2018) (Table 1).

In addition of tracking the specific taxonomic changes occurring in the intestine, there is a discussion about whether the alterations taking place within the intestine are reversible (Lourenco et al., 2021) or on the other hand have long-lasting effects (Schokker et al., 2014; Zeineldin et al., 2019a).

Chapter 1. Table 1. Summary of scientific studies that have analysed the impact of antimicrobial use in microbiota composition in pigs.

Ref	Weaning age/ sampling[1]	Sample type	Treatment/duration[2]	Sequencing Technique	Observed effects in the microbiota
Allen et al., 2021	14d[4]/3-7wks[7]	Faeces	In-feed Carbadox (subtherapeutic=10ppm[3]; therapeutic=50ppm) or ASP250®(chlortetracycline, 100 ppm; sulfamethazine, 100 ppm; penicillin, 50ppm)./ 1wk adaptation + 1wk Ab[14]	16S rRNA[5]	Shifts in the phage community of ASP250 fed pigs. Lower levels of *Coprococcus*, *Succinivibrio*, *Streptococcus*, *Treponema*, and *Turicibacter* spp and increased abundance of *Escherichia* in ASP250 treated animals. In-feed antibiotics induced prophages from gut bacteria
Looft et al., 2012	21d/18wks age. 0, 3, 14, 21 d post treatment	Faeces	In-feed AP250® (chlortetracycline 100ppm, sulfamethazine 100ppm, penicillin 50 ppm)/ 21 d	16S rRNA and Shotgun[6]	Greater abundance of *E. coli* and genes related to antimicrobial resistance and energy production. Decrease in the abundance of Bacteroidetes, *Anaerobacter*, *Barnesiella*, *Papillibacter*, *Sporacetigenium*, and *Sarcina* genera, and increased abundance of members of the Deinococcus-Thermus and Proteobacteria phyla as well as *Succinivibrio* and *Ruminococcus* genera. Increase in abundance of functional genes linked to energy production and conversion, as well as antibiotic resistance genes to antibiotics not administered and diversity Ab treated pigs.
Poole et al., 2013	28d/0, 14, 23, 28, 35, 42 and 49 dpw[16]	Faeces	In-feed chlortetracycline 50ppm/ 21 – 49dpw (4 wks)	16S rRNA	No differences in diversity or taxa composition.
Looft et al., 2014a	14d/3months age. (6 sampling days)	Ileum, cecum, mid-colon mucosa and content	In-feed chlortetracycline 100 ppm, sulfamethazine 100 ppm, penicillin 50 ppm (ASP250®)/ 2 weeks	16S rRNA and Shotgun	Enrichment of Bacteroidetes and phage-encoding genes in the ileum. Higher abundance of *E. coli* in ileum, *Lachnobacterium* in all gut locations, and resistance genes to antibiotics not administered in ASP250 treated animals.

Ref	Weaning age/ sampling[1]	Sample type	Treatment/duration[2]	Sequencing Technique	Observed effects in the microbiota
Looft et al., 2014b	21d/-5 (before), days 1-4 ("early carb"); 1, 2, 3, 4, 7, 14, 21d post treatment ("late carb"); 2,4, ("early withdrawal: WD); 21, 42 ("late withdrawal")	Faeces	Standard diet: 3 wks, then In-feed carbadox 50 ppm: methyl 3-(2-quinoxalinylmethylene) carbazate N1,N4 dioxide / 3 wks	16S rRNA	Increased relative abundance of *Prevotella* genus (which was unchanged measured by digital PCR) on Ab treated group. Increase of *E. coli* in non-medicated pigs. *Prevotella*, *Roseburia*, *Faecalibacterium*, and *Asteroleplasma* enriched in early carbadox-fed pigs. *Lactobacillus* enriched in early non-medicated pigs. Increased *Succinivibrio*, *Hallella*, and *Treponema* abundances in the late-withdrawal treated pigs, and *Spirochaetaceae* in late-withdrawal not medicated pigs.
Unno et al., 2015	21d. / weekly	Faeces	In-feed chlortetracycline, sulfathiazole, penicillin(2:2:1) at 0.2%/ 9 wks	16S rRNA	Inhibition of potential pathogens growth. No growth promoting effect in Ab treated pigs.
Zhang et al., 2016	30d/10d.	Jejunum, colon, and cecum contents	5mL Oral chlortetracycline (100 ppm)/ 10 days	16S rRNA	Increased abundance of Firmicutes and genus *Prevotella* in Ab treated animals. Proteobacteria and Firmicutes dominating jejunum and caecum, respectively, in Ab treated group. Lowest relative abundance of *Verrucomicrobia* and highest of *Actinobacillus* and decreased abundance of *Escherichia-Shigella*, *Lactobacillus* and *Streptococcus* in Ab pigs. *Akkermansia* not detected in Ab pigs (while so in Ct[15] animals).
Yu et al., 2017b	21d old/28dpw	Ileum and colon contents	In-feed chlortetracycline 300 ppm and colistin sulphate 60ppm/ 28days	16S rRNA	Increased abundance of *Spirochaetes*, *Tenericutes*, *Euryarchaeota*, *Verrucomicrobia*, *TM7* and decreased abundance of *Chlamydiae* in Ileal digesta.
Li et al., 2017a	35d/28dpw	Faeces	several in-feed treatments. CT: basal diet. CTC : 75 ppm chlortetracycline (CTC); BC: 40 ppm zinc bacitracin (ZB) and 20 ppm colistin sulphate (COL);CBC: 75 ppm CTC, 40 ppm	16S rRNA	Decreased α-diversity[20] in pigs fed decreased in the pigs fed CTC, OLA and ER/ VIR, and increased abundance of Bacteroidetes and *Prevotella* in these groups. Decreased relative abundance of *Succinivibrio* in pigs fed CTC, zinc bacitracin (ZB) and colistin sulphate (CT), a mixture of CTC, ZB and CT, CTC and OLA, or a mixture of CTC, OLA and ER.

Ref	Weaning age/ sampling[1]	Sample type	Treatment/duration[2]	Sequencing Technique	Observed effects in the microbiota
			ZB and 20ppm CT; CS: 75ppm CTC and 50 ppm Macleaya cordata extracts; CO: 75 ppm CTC and 100ppm olaquindox (OLA);COE: 75 ppm CTC, 100 ppm OLA and 20ppm enramycin (ER);COV: 75 ppm CTC, 100 ppm OLA and 15ppm virginiamycin (VIR)/ 28d		Decreased α-diversity in pigs fed ZB and CT, or a mixture of CTC, OLA and ER/VIR. Decreased relative abundance of *Faecalibacterium* in the group fed with a mixture of CTC, OLA and ER.
Yu et al., 2017a	25d/ 14dpw, 28dpw	Faeces	in-feed colistin sulfate (20 ppm)/ 28d	qPCR[18]	Higher abundances of *Lactobacillus* and decreased of *E. coli* in Ab treated pigs at day 14. Lower *E. coli* abudance and increased *Lactobacillus* in Ab treated animals compared to control at day 28.
Li et al., 2017b	0d (treatment after birth)/ 21, 49d post natal.	Intestine	Oral feeding of Amoxicilin 30ppm / twice a day from birth to 14day	16S rRNA	No effects in alpha diversity. Increased abundance of family Enterobacteriaceae and *Escherichia*, as well as *Erysipelotrichaceae*, and *Mitsuokella jalaudinii* at post natal day 7 (PND7) in Ab treated pigs.
Li et al., 2017c	28d/28dpw	Jejunum, ileum, caceum, and colon content	in-feed colistin sulfate (20 ppm) and bacitracin zinc (40 ppm)/ 28d	16S rRNA	No statistically significant differences. Decreased relative abundance of *Clostridium* in all locations except for colon, where it was increased. Decreased relative abundance of *Escherichia*, *Cyanobacteria_norank*, and *Streptococcus* in jejunum and ileum. Decreased relative abundance of *Lactobacillus* in jejunum and colon and increased at ileum and caecum. Increased relative abundance of *Ruminococcus* and *Lachnospiraceae unclassified* in jejunum and caecum.
Mu et al., 2017	23d/19dpw(42d old).	Duodenum, jejunum, ileum	In feed (50 ppm olaquindox, 50 ppm oxytetracycline calcium,	16S rRNA	Decrease of abundance of *Lactobacillus* in stomach and increase of abundance of *Streptococcus suis* in small intestine, *Treponema* in colonic lumen, and

Ref	Weaning age/ sampling[1]	Sample type	Treatment/duration[2]	Sequencing Technique	Observed effects in the microbiota
		contents and mucosa. Stomach, caecum and colon content.	50 ppm kitasamycin from day 7 to 42 of life.		*Faecalibacterium* in ileal mucosa of Ab treated animals. 10 (*Acetobacter*, *Aequorivita*, *Anaerococcus*, *B-42*, *Holdemania*, *HTCC*, *Mycobacterium*, *Sporanaerobacter*, *Tsukamurella* and *Veillonella*) and 1 (*Ramlibacter*) genera uniquely identified Ab treated pigs on d 14 and 28.
Li et al., 2017d	25d/0, 14, 18dpw	Faeces	In-feed Bacitracin Zn 40 ppm, aureomycin 75 ppm, and colistin 20 ppm/ 28 days	16S rRNA	No difference in richness and diversity in days 14 or 28 between Ab and Ct group. Tendency of lower abundance of Fibrobacteres in Ab treated grop at d28. Increased abundance of *Vibrio* and *Zhouia* and decreased levels of *Bacillus* and *Sphaerochaeta* in Ab treated pigs at d28.
Yu et al., 2017c	21d/28dpw	Caecum content	aureomycin (30 ppm), polymyxin E (12 ppm) and ZnO (3,000 ppm) (AZ group)/ 28d	16S rRNA	Differences in β-diversity[21] among groups. Increased abundance of Bacteroidetes and decreased abundance of Firmicutes and Proteobacteria in AZ group. The top four abundant genera in control group were *Prevotella*, *Succinivibrio*, *Lactobacillus*, and *Anaerovibrio*. Increased and decreased relative abundance of *Prevotella* and *Lactobacillus* in AZ group, respectively. Decreased relative abundance of *Succinivibrio*, *Anaerovibrio*, and *Desulfovibrio* in AZ group. Increased pathways related to energy metabolism, metabolism of terpenoids and polyketides, digestive systems and cell growth and death and decreased membrane transport. Most of the enriched pathways were linked to *Prevotella*.
Conelly et al., 2018	NA[17]/2 months old.-7, -4, 4 and 9 post treatment	Faeces	5mL PO[12] of 20ppm amoxicilin twice a day, 10mL IV[11] etarpenem 30ppm / 7 d	Shotgun	Differences in beta diversity. Decreased abundance of *Lactobacillus*, *Faecalibacterium*, *Megasphaera* and *Oxalobacter* genera (*O. formicigenes*)(*L. acidophilus, L. johnsonii*)(*F. prausnitzii*)(*M. elsdenii*), and increased abundance of *Escherichia*, *Bacteroides*, *Fusobacterium*,

Ref	Weaning age/ sampling[1]	Sample type	Treatment/duration[2]	Sequencing Technique	Observed effects in the microbiota
					Shigella and *Klebsiella* in Amoxicilin treated animals. Decreased abundance of *Faecalibacterium*, *Megasphaera* and *Oxalobacter*, and loss of *Desulfovibrio piger* and *E. coli* by day 9 of treatment with etarpenem. Increased abundance of *Pseudomonas*, *Bacteroides*, *Enterococcus* and *Acinetobacter* in etarpenem treated animals.
Gao et al., 2018b	42 d / -4, 2, 7, 13d of treatment.	Faeces and Ileal content.	Ileal cannulated: 200ppm Ampicillin, 5ppm gentamycin and 40 ppm metronidazole / 13 days.	16S rRNA	Increased abundance of *Escherichia/Shigella* and decreased abundance of *Lactobacillus* and *Bifidobacterium* in Ab treated animals.
Poulsen et al., 2018	28d/faeces: weekly. Rest of samples: 3, 28, 42 d of age.	Stomach, ileum, cecum, caecum and colon content, faeces	5mg gentamycin PO / 4, 5, 6d of age 3d	16S rRNA	No statistically significant differences between Ab group and Ctrl pigs. Higher diversity in AB treated pigs. Low effects of gentamycin in microbial population.
Soler et al., 2018	NA/ 0, 15 and 30dpw	Faeces	In-feed 3000ppm ZnO 1 wk after weaning + amoxicillin 15ppm + colistin sulphate 5ppm/ 30 days	16S rRNA	Increased abundance of *Bacillus* and *Lactobacillus* spp in additive. Increased abundance of Prevotellaceae and decrease of Bacteroidaceae in Ct animals from 0 to 15 days. Decrease of abundance of Proteobacteria and *Lactobacillus*, and increase of *Prevotella* abundance in Ab treated animals.
Yu et al., 2018	NA/23d old	Ileum and caceum content	In-feed 50 ppm olaquindox, 50 ppm oxytetracycline calcium, and 50 ppm kitasamycin / from 7d old to 22d 15d	16S rRNA	Decreased diversity and richness at the ileum, decreased abundance of *Lactobacillus* in ileum and cecum. Increased the abundance of *Streptococcus*, unclassified Enterococcaceae, unclassified Fusobacteriales, and Corynebacterium in the ileum, and the abundance of

Ref	Weaning age/ sampling[1]	Sample type	Treatment/duration[2]	Sequencing Technique	Observed effects in the microbiota
					unclassified Ruminococcaceae and unclassified Erysipelotrichaceae in the caecum.
Gao et al., 2018a	45d/ day 26 experiment	proximal ileum content and faeces	T-cannula Administration 150 ppm Ampicillin, 4 ppm gentamicin and 30 ppm metronidazole/ 25days	16S rRNA	No significant differences in ileal digesta. Markedly altered faecal microbial composition. Increased abundance of aromatic amino-acids metabolism
Che et al., 2019	21/28dpw	Ileum and colon content	chlortetracycline at 750 ppm (10% purity) and virginiamycin at 50 ppm diet (50% purity) 28	qPCR, 16S rRNA	Decreased bacterial load in ileum but not in colon. Higher F/B index, *Methanosphaera* species, and the pathway of "carbohydrate metabolism" in antibiotic group, indicating the better carbohydrate degradation and energy utilization. Ileum content: decreased abundance of *Sarcina*, increased abundance of *Anaerovibrio*. Colon content: Increased abundance of Firmicutes and Actinobacteria, decreased abundance of Bacteroidetes and several OTUs[19] classified as *Prevotella* in colon content of Ab treated groups. Increased abundance of "carbohydrate metabolism" and "membrane transporters" KEGG level 2.
Ghanbari et al., 2019.	28d/ 0, 8, 21 experiment.	Faeces	In feed 40 ppm oxytetracycline / 4d adaptation + 21d Ab	Shotgun	Overall reduction of Firmicutes and increase of Bacteroides and Proteobacteria in Ab treated pigs. Increased abundance of *Escherichia/Sighella*, Acidaminococcaceae, *Marvinbryantia*, *Prevotella*, *Blautia*, *Parabacteroides*, *Paludibacter, Megasphaera, Clostridium, Sporobacterium, Achromobacter, unclassified Lachnospiraceae* in Ab treated animals.
Li et al., 2019	21±1d/4wks	Stomach, duodenum, ileum, cecum, colon, and	in-feed 1:10,000 amoxicillin powder/ 4wks	16S rRNA	Increased abundance of Bacteroidetes in Ab treated group. Higher relative abundance of *Lactobacillus* and *Phaseoulus* in Ab treated pigs compared to Ct at day 14. Lower relative abundance of *Clostridium* and *Actinobacillus* in Ab treated pigs at day 28.

Ref	Weaning age/ sampling[1]	Sample type	Treatment/duration[2]	Sequencing Technique	Observed effects in the microbiota
		rectum content			
Zeineldin et al., 2019b	NA/Before weaning 0,5 and 20 days of age	Faecal	2.5ppm of IM tulathromycin (TUL)/ one shot directly after birth	Shotgun	Non-significant changes between control and TUL in α and β diversity. At day 5: Increased abundance of Erysipelotrichaceae, Bacteroidetes and *Mucilaginobacter*. At day 20, increased abundance of *Ruminococcus, Ethanoligenenes, Butyrivibrio*, Lachnospiraceae, *Dehaloccocoides, Thermoanaerobacterium, Abiotrophia, Cellulosilyticum*.
Zwirzitz et al., 2019	NA/0, 22, 25 day	Faeces, ileum mucosa, ileocecal lymph nodes(ICLNs)	Colistin sulfate 5ppm, lincomycin-spectinomycin 125 ppm premix / 25d.	16S rRNA	No effects on α-diversity. Enriched Methanobacteriaceae and decreased Veillonellaceae abundance in Ab treated group in faeces at the end of the study (faeces-end). In ileal mucosa reduced abundance of Clostridiaceae, Chlamydiaceae, and Halomonadaceae Ab treated group. Increased abundance of *Methanobrevibacter* and *Ruminococcus*-affiliated OTUs and reduced Clostridiaceae-affiliated OTU in faeces of Ab treated group. Lower abundance of Candidatus *Athromitus* and *Sharpea* in ileum mucosa of Ab pigs. Decreased abundance of family Helicobacteriaceae in ICLNs of Ab group.
Li et al., 2020	21d/5 (P1), 6 (P2) months.	Faeces	TG: No Ab. CG: 5ppm flavomycin, 15ppm enramycin. N1 and N2: No Ab 5 and 6 months, respectively. /A1 and A2: Ab 5 and 6 months, respectively.	16S rRNA	Overall higher abundance of *Prevotella, Streptococcus, Diallister, Coprococcus, Acidaminococcus, Olsenella, Megasphaera* in antibiotic treated animals. Greater abundance *of Phascolarctobacterium, Treponema, Clostridium, Escherichia/Shigella, Ruminobacter, Desulfovibrio, Enterococcus, Corynebacterium, Fibrobacter* in Antibiotic-free animals.

Ref	Weaning age/ sampling[1]	Sample type	Treatment/duration[2]	Sequencing Technique	Observed effects in the microbiota
López-Colom et al., 2020	21d/ 9 (PI),40(S),12dpw(F)	Faeces	in-feed Amoxicillin(300ppm), Oxytetracycline(1000ppm) and Lyncomicin(1100ppm) ZnO(2400ppm): 0-19dpw. /Ab: 19-42dpw	16S rRNA	Untreated pigs tended to have diarrhoea less time than AB. No significant differences were observed in richness, but followed a different evolution in AB treated animals (lower richness in PI and S periods). PI: Higher abundance of Bacteroidetes, Fibrobacteres, Streptococcaceae and *Streptococcus*, Succinivibrionaceae and *Succinivibrio, Lactobacillus*, and decreased abundance of Christensenellaceae in AB group. PI and S: Higher abundance of Porphyromonadaceae in AB groups (S); Increased abundance of *Catenibacterium* (PI and S), *Butyrivibrio*, and Enterobacteriaceae (S phase).
Massacci et al., 2020	31-38d/end of Ab administration (T1) 7 d after (withdrawal period) of Ab (T2)	Faeces	Amoxicillin: P (IM 15 ppm bodyweight, two administrations at 48-hr interval. O (oral, 12–20 ppm of suspension twice a day, for 5 days)	16S rRNA	Ordination of the microbiota at T0 mainly driven by MUC4 gene, by Ab treatment at T1, and Ab and faecal score at T2. Higher *Oscillospira* and *Actinobacillus porcinus* in *MUC4* resistant pigs. Decreased abundance of *Lactobacillus* spp in Ab treated pigs orally (O). Higher abundance of *Lactobacillus* spp. in Ct animals compared to the two Ab treatments. Lower abundance of *Prevotella copri, Ruminococcus* and *Lactobacillus* in orally Ab treated pigs.
Parois et al., 2020	18±4.2d/14 d	Ileum, caecum, colon mucosa and contents	in-feed 441 ppm of chlortetracycline + 38.6 ppm tiamulin/ 14d	16S rRNA	Higher abundance of *Ruminococcus2* in Ileum, caecum and colon, *Lactobacillus* in caecum and colon, *Blautia, Mitsuokella, Coprococcus*, in caecum; *Coprococcus, Dorea* and Veillonellaceae unclassified in colon at day 14.
Xu et al., 2020	28/d31 and d38	Faeces	Control group: oxytetracycline (20 ppm BW^{8}/day) IM trial 1/ 3d	16S rRNA	No comparison of Ab group to control. Lower diarrhoea index in oxytetracycline treated pigs in the second and third day after treated.

Ref	Weaning age/ sampling[1]	Sample type	Treatment/duration[2]	Sequencing Technique	Observed effects in the microbiota
Gaio et al., 2021.1	21d/weekly	Faeces	IM 0.1 mL per pig daily from a 200 mg/mL neomycin, starting on the 4th week of age/ 5 days	Shotgun	Higher representation of *Mollicutes* in Ab treated pigs. No significant differences in α diversity comparing Ab group with control. Lower α diversity in Ab treated pigs at 2 weeks after treatment measured by balance weighted phylogenetic diversity (PWPD).
Liang et al., 2021	28±2d/ 21dpw	Faeces	in-feed colistin sulphate 100ppm/ 21d	16S rRNA	Higher abundance of *Ruminococcaceae UCG 014* in Ab treated pigs compared to Control pigs.
Lourenco et al., 2021	21d/35, 49, and 63d	Faeces	in-feed carbadox at 55 ppm first days 21 to 35 of diet; followed by 27.5 ppm from days 36 to 49/ 4 wks	16S rRNA	Decreased Faith's phylogenetic diversity and abundances of abundance of *Slackia, Peptococcus, Catenibacterium, Coprococcus*, and *Blautia* in ab treated pigs on day 49d of treatment. Effects disappeared 2 weeks after treatment.
Rhouma et al., 2021	28d/ 1d before (D0), 1(D2), 3(D4), 6(D7), and 35(D36) after ETEC[9] challenge	Faecal swabs	colistin sulphate, 5mL, 50,000 IU[13]/kg twice a day / 5d	16S rRNA	No differences in α diversity between CS treated and non-treated of unchallenged pigs. Significantly higher abundance of *Escherichia/Shigella* in challenged non treated group compared to challenged treated. Lachnospiraceae was associated to unchallenged untreated pigs at D36.
Du et al., 2022	28d/7dpw	Caecum content	In-feed 300ppm oxytetracycline calcium/ 14d	16S rRNA	No significant effects in α diversity. Evolutionary diversity slightly higher than control group. Lowest abundance of top20 genera in Ab treated group. Higher abundance of Peptostreptococcaceae rc9 group, *Mollicutes RF39* and *Sphaerochaeta* in Ab treated group.
Gaio et al., 2022	21d/weekly	Faeces	IM[10] 0.1 mL per pig daily from a 200 ppm neomycin, starting on the 4th week of age/ 5 days	Shotgun	Differences in α diversity among Ab and probiotic groups during the first week of the study. Higher representation of *Mollicutes* in Ab treated group.
Han et al., 2022	NA/35dpw	Colon content	75 ppm Chlortetracycline/ 28d.	16S rRNA	No effects in colonic microbiota.

Ref	Weaning age/ sampling[1]	Sample type	Treatment/duration[2]	Sequencing Technique	Observed effects in the microbiota
Sun et al., 2022a	26d/21 and 28dpw	Faeces	in-feed oxytetracycline calcium 300 ppm + quinolone 50 ppm 28d	16S rRNA	The diarrhoea rate and score of piglets were the lowest in the PC. Higher abundance of *Faecalibacterium* and *Ruminococcaceae UCG 014* in Ab treated pigs at 21dpw. Lower abundances of *Prevotella_9, unclassified Lachnospiraceae*, and *Anaerovibrio* lower in Ab treated group at 28dpw.
Yang et al., 2022	21d/ 14, 42, 164dpw	Caecum content	In-feed 100 ppm olaquindox and 75 ppm aureomycin/ 164d	16S rRNA	ACE and Chao1[22] were highest in control group, but no significant differences were found among groups, no differences in β-diversity either. *Lactobacillus* was the predominant genus at 14dpw. Decreased abundance of *Lactobacillus* at 14dpw and increased at 164dpw in Ab treated group. *Subdoligranulum* and *Ruminococcaceae UCG 005* were the most dominant bacteria at 42dpw and 164dpw, respectively, in Ab treated animals. Increased abundance of *Lachnospiraceae XPB1014 group* in Ab treated group at 164dpw.
Zhu et al., 2022	28d/65, 95, 125 d	Colon content	50 ppm virginiamycin/ (sows fed during pregnancy and lactation) 134d	16S rRNA	No effects of treatments on α-diversity. Microbial communities clearly separated at day 65 of age. *Lactobacillus* as the main genus dominating colonic microbiomes in different treatments and stages. Increased relative abundance of Spirochaetes and Proteobacteria in Ab treated sows offspring at day 95. Increasing trend of relative abundance of *Blautia* and *Dorea* and decreased unclassified Clostridiales in Ab treated sows offspring at day 65. Ab group enriched in Actinobacteria, Gemmatimonadetes, Chloroflexi at d95; *Streptomyces* and *Dorea* at d65; and *Pediococcus, Campylobacter, Staphylococcus* at d95. Increased relative abundance of pathways related to immune disease, cardiovascular

Ref	Weaning age/ sampling[1]	Sample type	Treatment/duration[2]	Sequencing Technique	Observed effects in the microbiota
					disease, energy metabolism, and xenobiotics biodegradation and metabolism, and reduced abundance of cellular community-prokaryotes at d65 in Ab group. Toluene degradation enriched in Ab group at day 95.

[1]Weaning age (days of age)/Sampling period (day of experiment); [2]Treatment: antibiotic, dosage and route used in the study/duration of the treatment; [3]ppm: parts per million; 1ppm = 1mg/Kg = 1 g/t; [4]d: days of age/day of experiment; [5]16rRNA: 16S ribosomal RNA (ribonucleic acid) of the bacterial 30S ribosomal subunit; [6]Shotgun: Shotgun metagenomic sequencing. (Whole metagenome sequencing); [7]wk/wks: week(s); [8]BW: Body Weight; [9]ETEC: Enterotoxigenic *E. coli*; [10]IM: Intramuscular; [11]IV: Intravenous; [12]PO: *Per os*, oral route; [13]IU: International Units; [14]Ab: Antibiotic used in that study; [15]Ct: Non-treated pigs; [16]dpw: day post-weaning; [17]NA: not applicable; [18]qPCR: quantitative Polimerase Chain Reaction; [19]OTU: Operational taxonomic unit. Amplicon sequences of 16S rRNA clustered by a 97% (or 99%, depending of the bioinformatic pipeline followed in the study) of similarity, defining a taxonomic group (i.e., genus); [20]α-diversity: alpha diversity; [21]β-diversity: beta diversity; [22]ACE, Chao1: Indexes of alpha diversity.

1.6 Zinc Oxide

1.6.1 Zinc: an essential oligoelement

Zinc is the 27th most abundant metal in the earth. This element is considered as a transition metal and it is also described as a heavy metal. Due to its chemical and physical characteristics, it is used in an infinity of industrial, medical and food hygiene procedures and applications. In eukaryotes organisms, Zn is present and needed in more than 300 enzymes as structural stabilizers, cofactors and regulatory DNA-binding sites (Andreini et al., 2006; Wątły et al., 2016). The first report of zinc deficiency in human in 1963 opened an extensive research field to comprehend the role of this element in the organism (Prasad et al., 1963).

Although zinc has been largely used to treat and prevent diarrhoea in both human and pigs, the exact mechanisms of action of Zn in preventing PWD remain to be elucidated. Zinc is associated to multi-site effects in the host including its role as essential oligoelement (Suttle, 2010; Andreini et al., 2011; Wątły et al., 2016), its effects protecting gut morphological structure and function (Liu et al., 2014; Ping Liu et al., 2014; Shen et al., 2014b; Li et al., 2018), its local and systemic anti-inflammatory effect (Sargeant et al., 2010; Sargeant et al., 2011), its antimicrobial effect (Wang et al., 2004; Zhang and Guo, 2009; Pasquet et al., 2014) and its role in the specific immune response (Ma et al., 2016; Wątły et al., 2016; Sheldon and Skaar, 2020). The next subsections review the main reported mechanisms of action described for Zn, focusing particularly in ZnO use in pig weaning context and particularly in PWD prevention.

1.6.2 Therapeutic use of Zn as formulated as ZnO

In-feed therapeutic doses of ZnO have been used for decades to control or prevent PWD outbreaks (Hahn and Baker, 1993; Hill et al., 2001; Sales, 2013; Fairbrother and Nadeau, 2019; Bonetti et al., 2021). Added in the feed in a range of concentrations from 1500 to 3000 mg/Kg (Sales, 2013), ZnO is effective to control diarrhoea (Fairbrother 2019; Luppi et al., 2017). This range of concentration is referred to as “therapeutic” or “pharmacological” concentrations and

the beneficial effects on controlling PWD as well as improving growth performance have been demonstrated (Sales, 2013).

A large part of ZnO is dissociated in the acid pH of the stomach, and the resultant Zn^{2+} ions as well as the remaining not dissociated molecule are not well absorbed within the gut (Shen et al., 2014a; Bonetti et al., 2021). This low absorption may be an advantage for the animal avoiding possible toxic effects of overdose, however, it is an environmental problem because the non-absorbed zinc is excreted in faeces, generating manure loaded with high concentrations of zinc that will be accumulated in soil (Poulsen and Larsen, 1995; Meyer et al., 2002; Berenguer et al., 2008).

This environmental concern has led to its ban as therapeutic agent in the EU (SCVMP Commission Implementing Decision., 2017). This decision was redacted in line with previous reports of the EU stating that the benefits of using ZnO do not outweigh the hazards of environmental pollution and AMR spread associated with its usage (SCVMP., 2017). In addition to the environmental risks, several studies reported an increase in AMR genes due to ZnO use in absence of any other antimicrobial treatment (Bednorz et al., 2013; Yazdankhah et al., 2014; Vahjen et al., 2015). These AMR promoting effect is thought to occur via co-selection of ARGs which are very close to heavy metal resistance genes in the bacterial chromosome (Baker-Austin et al., 2006; Rossolini et al., 2017), as well as via utilization of protein to export zinc from the bacteria, with the ability to export several other molecules such as antibiotic (cross-resistance) (Chapman, 2003). The ban only affects therapeutic use of ZnO. Zinc is an essential element required at multiple organic sites and reactions of an organisms (Suttle, 2010; Wątły et al., 2016), and therefore, its use is allowed as feed additive up to a concentration of 150 mg/Kg (REG EU, 2016/1095) to ensure the inclusion of appropriate levels that enables the correct homeostasis maintenance in the animal.

1.6.3 Effects of ZnO in intestinal structure and function

Beyond controlling diarrhoea, a myriad of studies has reported several beneficial effects of in-feed therapeutic ZnO supplementation. Indeed, ZnO seems to act in a way that fixes each alteration occurring at weaning in the gut of the piglet. It increases feed intake and average daily gain (Sales, 2013), increases villus height and decreases crypt depth (Shen et al., 2014; Li et al., 2018). Similarly, it acts on the intestinal barrier structure and function through a decrease in intestinal permeability by means of an increased expression of tight-junction, zona-occludens 1 and occludin associated proteins (Hu et al., 2013; Shen et al., 2014b; Han et al., 2018). Moreover, it has also been reported to increase the activity of digestive enzymes in pancreatic tissue (Hedemann et al., 2006), to stimulate the secretion of ghrelin and cholecystokinin (Yin et al., 2009; Bonetti et al., 2021) and to upregulate insulin-growth factor 1 (IGF-1) and its receptor (IGF-1R) in intestinal mucosa (Li et al., 2006). Other studies have reported increased levels of several mucins in jejunal, ileal, and colonic mucosa (Liu et al., 2014a; Liu et al., 2014b).

1.6.4 Effects in the local immune response in the intestine

The effects of ZnO in the immune system at the intestine in the context of PWD prevention has been studied and reported widely. These effects of ZnO in host immunity have been reported elsewhere (for an overview of ZnO effects in piglets intestine and immune response see Table 2). The majority of these studies point towards a local effect of ZnO within the intestinal lumen and mucosa, having a great influence on the inflammatory response attenuating the inflammatory response elicited in the acute phase of weaning or during the ETEC infection (Sargeant et al., 2010, Sargeant et al., 2011; Bonetti et al., 2021). Studies conducted including levels up to 3000ppm of ZnO reported decreased levels of pro-inflammatory cytokines TNFα, IL-1β, IL-6, IL-8, IFNγ, and NF-κB (Sargeant et al., 2010; C. H. Hu et al., 2013a; C. Hu et al., 2013b; Ping Liu et al., 2014; Grilli et al., 2015; Xia et al., 2017; Zhu et al., 2017; Mukhopadhya et al., 2019). All these cytokines were associated in these studies with the inflammation and alterations observed at weaning. In fact, TNFα and IFNγ have been highlighted as the cytokines

responsible of an increase in intestinal permeability through an alteration in tight-junction proteins (Walsh, Hopkins and Nusrat, 2000; Roselli et al., 2003; Pié et al., 2004; Karakuła-Juchnowicz et al., 2017). It has been reported that ZnO counteracts TNFα effects and signalling by inhibiting the molecules implicated in the signalling pathway LPS-induced NF-κB cascade (von Bülow et al., 2007). On the contrary, in ZnO treated piglets, increased levels of anti-inflammatory cytokines have been reported as well, such as IL-10, and TGF-β (Hu et al., 2013b; Shen et al., 2014; Zhu et al., 2017).

Studies performed *in vitro* point to an attenuation of immune response and prevention of barrier alteration as a mechanism of ZnO against ETEC infection. Certainly, Sargeant et al., (2011) described an array of innate immune response genes upregulated in ETEC infection, all attenuated by ZnO treatment, perhaps via stress response mechanisms such as heat shock proteins. A study performed in Caco2 cell line by Roselli et al. (2003) reported decreased level of cytokine upregulation, ETEC adherence, and internalization in ETEC infected Caco2 cells. Interestingly, no differences were found in the number of viable counts of ETEC, and therefore, they suggested that the mechanism of action of ZnO might not be only or mainly based on antimicrobial effects. Further effects of ZnO related to immune effects on the intestinal lumen are related to antioxidant induction capability through an upregulation of metallothioneins, super-oxide dismutase (SOD) and glutathione peroxidase 1(GPX1) (Sargeant et al., 2010; Zhu et al., 2017; Xia et al., 2017).

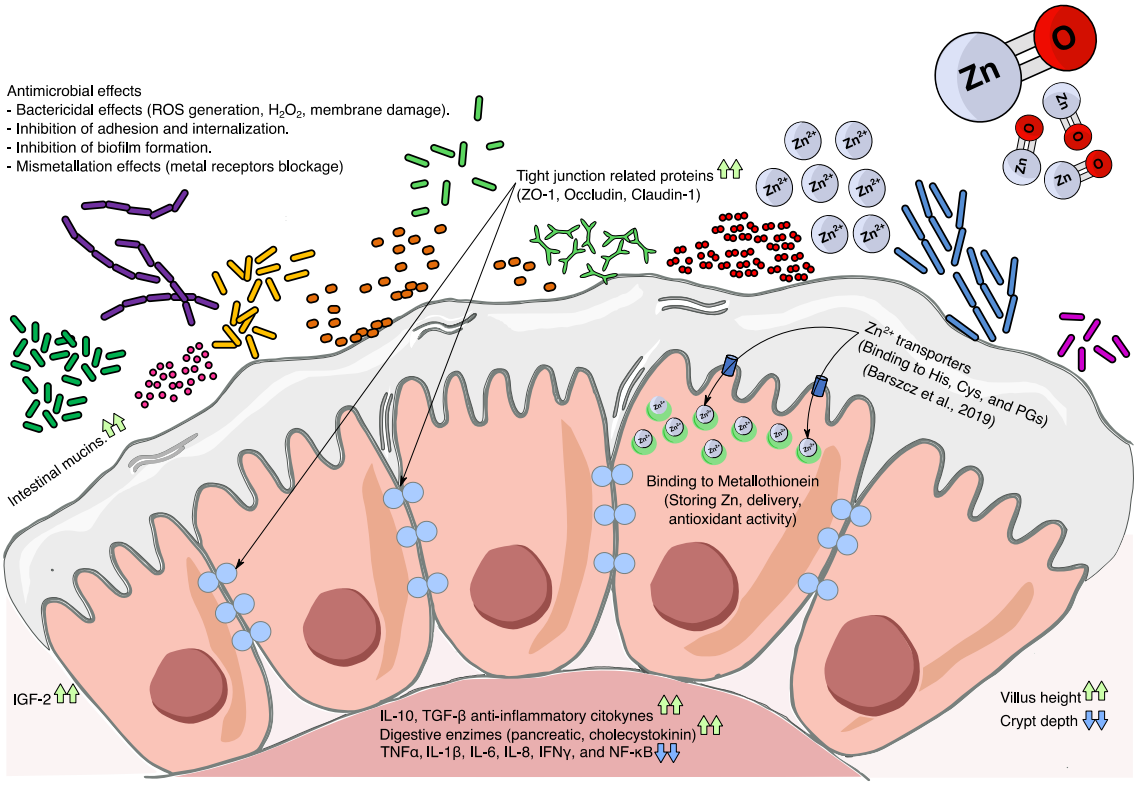


Figure 2. Local effects of Zinc Oxide within the intestine. Antimicrobial effects exerted through Reactive Oxygen Species and H_2O_2 generation from ZnO surface, and interaction of Zn^{2+} ions with bacterial surfaces. Inhibition of adhesion to the enterocyte and bacterial internalization, inhibition of biofilm formation, and mismetallation effects via metal receptors blockage by Zn. Increased expression of proteins Insulin-like Growth Factor-2 and tight junctions related proteins (Zonula occludens-1, Occludin and Claudin-1). Increased intestinal villus height and decreased crypt depth. Increased mRNA expression of anti-inflammatory cytokines such as *IL-10*, *TGF-β* and decreased expression of pro-inflammatory cytokines as *TNF-α*, *IL1-β*, *IL-6*, *IL-8*, *INF-γ* and *NF-κB.* Higher levels of pancreatic digestive enzymes and cholecystokinin. Increased levels of mucins in jejunum and colon. Increased levels of metallothionein.

Chapter 1. Table 2. Studies assessing effects of different sources of ZnO in immune responses at weaning

Ref	Weaning age	ZnO dosage/ duration[1]	Effects on ZnO treated animals
Sargeant et al., 2010	28 d[2]	3100ppm[3] + ETEC K88[5]/ 7days	Decrease expression of immune response genes: chemokines, antimicrobial peptides, NF-κB[6], Zn transporter (SLC39A4). Downregulation of *MUC4* gene expression (possible ETEC-binding receptor). Upregulation of metallothioneins (MTs).
Sargeant et al., 2011	*In vitro*		Upregulation of NF-κB targets post ETEC infection: chemokines, cytokines (*IL-1A, IL-6, IL-8*) and alveolar macrophage-derived chemotactic factor-II (*AMCF-II*). ETEC+ZnO: attenuation of inflammatory response by NF-κB inhibition through heat shock proteins (HSP), Heme-oxygenase, *AP1*-transcription factors, activating transcription factor 3 (*ATF3*) and early growth response genes (stress response genes).
Hu et al., 2013a	21d	Diosmectite-ZnO (DS-ZnO). ZnO 2250 ppm/ 7-14dpw[4]	Increased AGD[7] and average daily feed intake (ADFI), jejunal VH[8], VH:CD[10] ratio, Tight junction(TJ) protein expression of occludin, claudin-1 and Zonula-Occludens-1 (ZO-1) in jejunal mucosa. Decrease of Faecal Score (FSC), paracellular permeability, and mRNA expression of *TNFα*, *IL-6*, and *INF-γ* at 7 dpw. No effect at 14dpw
Hu et al., 2013b	21d	Zeolite-ZnO (Z-ZnO). ZnO 2250 ppm	Increased ADG, Daily feed intake (FDI) jejunal transepithelial resistance and expression of *TGF-β1* and *IL-10* at 7dpw. Decrease of colony counts of *E.coli* and *Clostridium* in SI contents, and expression of *TNFα*, *INFγ* in jejunal mucosa (Z-ZnO). No effect at 14 dpw.
Liu et al., 2014a	26d	3 levels: 57ppm, 164ppm and 2425ppm/ 4 weeks	Increased levels of sialomucins in distal jejunum villus and crypts. Decreased levels of sulfomucins in distal jejunum villus and crypts, expression of *B-defensin3* and abundance of $CD8^{+}\gamma\delta$ T-cells.
Liu et al., 2014b	26d	3 levels: 57ppm, 164ppm and 2425ppm/ 4 weeks	Increased amount of mixed neutral-acidic mucins and total number of mucin-producing goblet cells in colon Downregulation of *TLR4*[13] and *IL-8* in colon
Long et al., 2017	35d	Porous ZnO (HiZox), NanoZnO and ZnO 3000 ppm(PC)	Increased glutathione peroxidase (GSH-Px) activity, duodenal VH and jejunal CD[9]. Overall higher CD, trefoil factor 3 (TFF3) and erythroid 2-related factor 2 (Nrf2) levels. Decrease in serum serum malondialdehyde (MDA) and diamine oxidase (DAO) enzymes.

Ref	Weaning age	ZnO dosage/ duration[1]	Effects on ZnO treated animals
Han et al., 2018	25d	Shield Zn (SZ). ZnO 2500ppm (ZnO-2500)/ 14 days	Increased goblet cell density in duodenal crypt and VH:CD ratio in both treatments. Increased goblet cell density in jejunal crypts as well as higher VH:CD ratio in both treatments. Higher density in jejunal villus goblet cells in SZ treated animals. Increased density of ileal crypts goblet cells as well as VH and VH:CD ratio. Higher goblet cell density in colon in SZ treated animals. Upregulation of *ZO-1*[12] in ZnO-2500
Mukhopadhya et al., 2019	21d	3200 ppm ZnO. Yeast b-glucan	Decrease of faecal scores and downregulation NF-κB targeted cytokines (IL-1α, IL-1β, IL-18, IL-17,IFN-γ) in duodenal and ileal tissues
Lei & Kim., 2018	28d	PC: 2500 ppm ZnO/ 0-21d. 21-42d	Higher duodenal villus height and VH:CD ratio in PC[16] animals. Decrease of faecal scores
Grilli et al., 2015	28d	microencapsulated ZnO (mZnO) (various levels). 3000 ppm/ 14 d	Higher AF, Feed to Gain ratio (F:G), and Body Weight(BW) at 14dpw. Increased VH and VH:CD in ileum at day 42dpw. Increased Occludin protein levels. Decreased TNFα protein level in both treatments. ZnO treated group with the highest value of zinc plasma levels.
Zhu et al., 2017	21d	3000ppm/ 28days	Decreased diarrhoea incidence in the whole study. Lower CD in duodenum. Decreased levels of MDA blood concentration. Downregulation of IL-1β and IFN-γ in jejunum mucosa. Higher levels of blood total superoxide dismutase (T-SOD) at 14dpw. Increased VH in duodenum and ileum, and upregulation of *ZO-1* and *occludin* and *TGF*-β mRNA in jejunum mucosa
Ou et al., 2007	28d	3000ppm/ 10 days	Lower expression of Stem Cell Factor (*SCF*) gene at mRNA and protein levels. Decrease of number of mast cells in the mucosa and submucosa of small intestine and histamine release from mucosal mast cells. Lower incidence of diarrhoea
Jang et al., 2014	21d	2500ppm/ 14days	Higher hepatic and serum zinc concentration. No differences in both intestinal architecture traits and digestive enzymes
Xia et al., 2017	27d	2000ppm/ 14d	No differences in ADG. Lower F:G ratio in ZnO treated group at 14dpw. Lower incidence of diarrhoea, and decreased levels of IFNγ, IL-1β, TNFα and NF-κB. Increased jejunal VH and VH:CD, mRNA expression of intestinal antioxidant enzymes and TJ proteins.
Bouwhuis et al., 2017	24d	3300ppm/ 31dpw	Higher ADG, ADFI. Higher G:F at week 1 of experiment. Decreased Faecal scores between days 0 and 31 of experiment

Ref	Weaning age	ZnO dosage/ duration[1]	Effects on ZnO treated animals
Broom et al., 2006		3000ppm/ 20dpw	Differences in performance. Statistical tendency($p<0.1$) of increased levels of intestinal IgA at 20dpw.
Shen et al., 2014	28d	2250ppm/ 14 days	Lower diarrhoea index. Increased duodenal VH and VH:CD, Insulin like Growth Factor 1 (*IGF-1*) and its receptor (*IGF-1R*), *ZO-1*, occludin, *IL-10* and *TGFβ1*. Increased concentration of secretory IgA in jejunal mucosa. Higher concentration of Zinc in liver, kidney, and faeces
Pei et al., 2019	28d	3000ppm/ 21 days	Higher ADG and ADFI. Increased VH:CD in duodenum and jejunum. Increased levels of serum IgA concentration, *IL-6*, *TNFα*. Higher levels of zinc in serum, liver, spleen, and kidney. Lower diarrhoea incidence. Decreased IgM concentration.

[1]ZnO dosage/duration: Zinc Oxide dosage /duration; [2]d: days of age; [3]ppm: parts per million; [4]dpw: days post-weaning; [5]ETEC K88: enterotoxigenic *E. coli* harbouring fimbriae F4 (former K88); [6]NF-κB:Nuclear factor kappa-B; [7]ADG: Average daily gain; [8]VH: Villus height; [9]CD: Crypt depth; [10]VH:CD ratio: Villus height:crypt depth ratio; [11]TJ: Tight junction; [12]ZO-1: Zonula Occludens-1; [13]TLR4: Toll-like receptor 4; [14]GSH-Px: Glutathione peroxidase; [15]MDA: Malondialdehyde; [16]PC: Positive Control.

1.6.5 Effect of ZnO in the intestinal microbiota

In the context of enteric diseases, commensal microbiota plays a pivotal role against colonization by enteric pathogens, acting as an additional protective layer, occupying and competing for niches in the intestine and avoiding pathogens to access to the intestinal barrier. This concept is known as colonization resistance (Buffie and Pamer, 2013). Besides exerting as a physical barrier, bacteria in the gut are able to fight pathogen colonization by producing bacteriocins, modulating the immune response of the host by microbiota-immune system crosstalk, limiting the access to nutrients and generating an adverse environment for pathogenic bacteria as a result of their own metabolite excretion (Brestoff and Artis, 2013; Pickard et al., 2017; Argüello et al., 2019; Bain and Cerovic, 2020). Thus, the composition of the gut microbiota at weaning is one of the factors involved in the onset of PWD. Some previous studies have hypothesized that ZnO may exert a local effect within the intestinal lumen and particularly in the micorbiota (Bonetti et al., 2021; Sales, 2013). The question is what specific effect has ZnO in the gut microbiome at weaning, and whether this effect can be emulated using other approaches. Overall, the effect of ZnO in intestinal microbiome has not been studied in detail enough, and there is still a lack of a common understanding of what ZnO does. In the following section, the reported effects of ZnO in gut microbiota will be briefly reviewed.

1.6.6 Effects of ZnO in pigs gut microbiota at weaning

A summary of effects of different doses of ZnO in pigs gut microbiota can be found in Table 3. In the same way as described previously for antibiotics, there is not a clear effect of ZnO in specific taxa within gut microbiota. The age of the animals used in these studies is limited to weaning period, with differences in study design, samples analysed and ZnO dosages. Most of these studies report that inclusion of pharmacological levels of ZnO in the diet are associated to a decrease in richness and diversity indexes (Shen et al., 2014b; Xia et al., 2017; Yu et al., 2017b; da Silva et al., 2021) but this seems to be dependent on the intestinal location studied. For

instance, Pieper et al., (2012) and Xia et al., (2017) found increased levels of richness and diversity in ileum content, while these were decreased in caecum and colon.

At taxonomic level, different effects on the abundance of microbial groups are reported. Among the groups showing increases are *Enterobacteria* and *Enterococci* (Højberg et al., 2005; Broom et al., 2006; Pieper et al., 2012; Yu et al., 2017b), *Weisella* spp, *Streptococcus* spp. and Clostridiales (Hojberg et al., 2005; Vahjen et al., 2010; Xia et al., 2017; Juhász et al., 2022; Xu et al., 2022). Further species reported to be favorably affected by ZnO are those within the familiae Prevotellaceae, Bacteroidaceae, Tannerellaceae or Lachnospiraceae (Xia et al., 2017; Pieper et al., 2020; Rattigan et al., 2020; Wei et al., 2020; Liu et al., 2021; Juhász et al., 2022; Tang et al., 2022; Xu et al., 2022; Sun et al., 2022b; Zhang et al., 2022). On the other hand, species negatively affected by ZnO are those within Veillonellaceae family (specially *Megasphaera* spp), *E. coli* and Enterobacteriaceae, such as *Desulfovibrio piger* (Starke et al., 2014; Kim et al., 2015; Pei et al., 2019; Wei et al., 2020; Pieper et al., 2020; Rattigan et al., 2020; da Silva et al., 2021; Hou et al., 2021; Li et al., 2021; Xu et al., 2022; Juhász et al., 2022; Venardou et al., 2022). These differences demonstrate inconsistent results in Enterobacteriaceae family. Similarly, a decrease in *Lactobacillus* spp. seems to be consistent among studies (Højberg., et al., 2005; Vahjen et al., 2011; Starke et al., 2014; Yu et al., 2017b; Peng et al., 2019; Wei et al., 2020; Li et al., 2021; Juhász et al., 2022) although there are discrepant results as well (Xia et al., 2017; Dowley et al., 2022; Sun et al., 2022b).

Chapter 1. Table 3. Effects of ZnO (used as study group, or as positive control) at different pigs gut microbiota locations.

Ref	Weaning / sampling[1]	Sample type	Treatment/ duration	Technique	Effects of ZnO on microbial population.
Højberg et al., 2005	28d[2]/ 14dpw[3]	Stomach, cecum, colon and rectum contents	2500 ppm[4] ZnO/14dpw	Culture, T-RFLP[5], ATP analysis	2500 ppm ZnO: reduced bacterial activity (ATP activity), lactic acid bacteria and Lactobacilli in digesta. Increased abundance of coliforms and Enterococci. *Enterococci* dominating high ZnO dose treated pigs.
Broom et al., 2006	22.9 ± 3.6d/ 6 and 20dpw	Ileal mesenteric Lymph nodes, distal ileal tissue and luminal contents	3100 ppm ZnO, *E. faecium* SF68/20dpw	Culture, ELISA[6].	Reduced anaerobic counts in ZnO treated animals. Tendency to decrease lactic acid bacterial translocation to mesenteric lymph nodes, and to increase intestinal IgA concentration on day 20.
Vahjen et al., 2010	26-28d/ 14dpw	Ileal content	200 ppm or 3000 ppm of ZnO/14dpw	16S rRNA[7] pyrosequencing	Increased relative abundance of *Weissella* spp., *Leuconostoc* spp., *Streptococcus* spp. Non-significant increase in Gram-negative facultative anaerobic genera (Proteobacteria, enterobacteria and relatives). Decreased relative abundance of *Sarcina* spp., and *Neisseria* spp.
Vahjen et al 2011	28d/ 12-14dpw	Ileum content	3042 ppm ZnO/12 to 14dpw.	16S rRNA pyrosequencing	Reduced relative abundance of *Lactobacillus reuteri*, and increased relative abundance of *Weissella cibaria*, *W. confusa*, *Leuconostoc citreum*, *Streptococcus equinus* and *S. lutetiensis* in ZnO medicated pigs.
Pieper et al., 2012	25±1d/ 21dpw	ileum content	(7d adaptation: basal), then ZnO, 50, 150, 200, 1000, and 2500 ppm, /	qPCR, PCR-DGGE[8]	Higher levels of species richness, Shannon and evenness[18] at high levels of ZnO. Higher levels of enterobacteria and lower levels of *Clostridial cluster XIVa* at high levels of ZnO (2500 ppm)

Ref	Weaning / sampling[1]	Sample type	Treatment/ duration	Technique	Effects of ZnO on microbial population.
			14d		
Starke et al. 2014	26±1d/ 32, 39, 46, 53 day old	stomach, mid-jejunum, terminal ileum and colon ascendens content.	2425 ppm/ 30d	qPCR[9]	Reduced abundance of Enterobacteriaceae group and *Escherichia*, and *Lactobacillus* spp. group. Impact of ZnO in Enterobacteria diminished with increasing age. Increased molar ratios of acetate/propionate in the proximal intestine. Reduced lactate concentrations in ZnO treated animals throughout the study.
Kwon et al., 2014	28d/ (+7d adaptation) 1,3,7 post-challenge	faecal, jejunum, ileum, colon, and rectum	ZnO+ETEC[10], 2000/ 7d	culture: relative occupancy of colonies	Reduced faecal consistency score (harder faeces) in ZnO treated pigs challenged with ETEC (K88[11]). Reduced shedding and presence of ETEC in the sampled tissues in animals treated with ZnO and challenged with ETEC.
Shen et al., 2014	27±1d/ 14dpw	Jejunal digesta, faeces	5d adaptation (basal), then 2250ppm ZnO/ 14d	PCR-DGGE of 16rRNA gene, and qPCR of *Lactobacillus* and *E. coli*.	High Zinc group: Decreased Shannon diversity in and Observed richness Jejunal digesta
Kim et al., 2015	25d/ 31, 34, 37 dpw	Rectal stool samples	Infection with ETEC K88 + 120ppm Apramycin or 2400ppm ZnO/ 7d	Culture	Decrease in faecal shedding of *E. coli* in AB and ZnO treatments at day 1, 4 and 7 post-infection.

Ref	Weaning / sampling[1]	Sample type	Treatment/ duration	Technique	Effects of ZnO on microbial population.
Xia et al., 2017	27±1d/ 14dpw	Digesta: Ileum, Cecum, Colon	2000 ppm/ 14d	16S rRNA sequencing	Increased Diversity and richness in Ileum and decreased in cecum and colon. Increased relative abundance of Lactobacillaceae, Streptococcaceae, Clostridiaceae, Lachnospiraceae, Veillonellaceae and Erysipelotrichiaceae families and higher abundance of *Lactobacillus*, *Prevoltella* and *Oscillospira* in ZnO and Nano-ZnO groups.
Yu et al., 2017b	21d/ 7dpw	Ileum and colon	3000ppm ZnO. 300ppm Chlortetracycline and 60ppm Colistin sulphate/ 28d	16S rRNA sequencing	Decreased microbial richness and diversity in colon contents of Ab[15] and ZnO[16] treated animals. Overall decrease of Clostridiales and *Lactobacillus* percentage in Ab and ZnO treated animals. Greater abundance of Enterobacteriales in ZnO vs Ab, and ZnO vs Ct[14]. Decreased Campylobacteriales and increased Enterobacteriales in Ileum content of ZnO and Antibiotic treated animals. Higher relative abundance of *Euryarchaeota*, and *Methanobrevibacter smithii* in colon of ZnO treated animals. Increased abundance of Enterobacteriales and decreased abundance of Campylobacteriales and Pseudomonadales in Ileum of ZnO treated animals.
Li S et al., 2018	21d/ 28dpw	ileum, colon, and caecal content	ZnO, 3000ppm/ 14d	qPCR(1), 16S rRNA pyrosequencing	No comparison of ZnO to a basal diet, ZnO diet used as Negative control.
Mukhopadhya et al., 2019	21d/ 10dpw	Caecum and colon digesta	3200 ppm ZnO/ 10dpw	16s rRNA gene qPCR	Increased abundance of Bacteroidetes in ZnO and 5kDaR diets. Decreased abundance of *Bifidobacterium* genus in ZnO treated pigs.
Pei et al., 2019	28d/ 21dpw	Caecal, colonic and rectal content	3000ppm ZnO/21d	Culture	Both 450 ppm nano-ZnO and 3000ppm ZnO decreased population of *E. coli* in the contents of cecum, rectum and colon. Abundance of *Salmonella*, *Lactobacillus*, *Bacillus bifidus*: N.S.

Ref	Weaning / sampling[1]	Sample type	Treatment/ duration	Technique	Effects of ZnO on microbial population.
Peng et al., 2019	21d/ 28dpw	caecal and ileum content	ZnO, 2400ppm/ 28d	qPCR	Reduced faecal score in ZnO treated animals. No comparison of ZnO (positive control) to Control animals. Lower levels of *Lactobacillus* spp. In ZnO treated levels (difference of 1 log10 unit of 16S rRNA gene copies per g wet weight).
Wang et al., 2019a	20d/ 15dpw	Small intestine (proximal and distal)	Basal + 110 ppm ZnO, 2400ppm, 2 alternatives/ 15dpw	Culture and 16s rRNA gene qPCR .	Distal small intestine: 2400ppm decreased counts of *E.coli* and coliforms (vs Control). Bacterial groups assessed by qPCR were not affected. Shannon diversity significantly higher in alternative treatment HiZox (110 ppm).
Rattigan et al., 2020	26d/ 9dpw	Cecum and colon contents.	SCP: Standard Crude Protein + 3100ppm ZnO; LCP: Low CP + 3100ppm ZnO; Supp.3100ppm Zn/ 9dpw	16S rRNA sequencing	Overall reduced indices of Alpha diversity, and increased abundance of Ruminococcaceae, *Frisingicoccus*, *Lachnoclostridium*, *Peptoclostridium*, and decreased abundance of *Murimonas*, *Psudobutyruvibrio* and *Desulfovibrio* in ZnO treated pigs. Increment of Firmicutes and reduction in Proteobacteria abundance of LCP ZnO treated pigs. Greater abundance of Tannerellaceae and Bacteroidaceae of SCP ZnO compared with SCP. Decreased abundance of *Roseburia* in SCP ZnO compared to SCP.
Kociova et al., 2020	28d/ 0, 10, 20dpw	Faecal samples	Nanoparticles (ZnA and ZnB) and ZnO (500-2000ppm)/ 10d	Culture, MALDI-TOF MS[12]	No basal diet (Ct). Decrease in coliform counts on day 5 vs day 0 and decrease on day 10 vs day 0.
Wang et al., 2020	28d/ 0-70d of age	Faecal Samples	Various groups. Basal diet + 3000ppm of ZnO (CON)/ 42d	Culture	No significant differences in *Lactobacillus* and *E. coli* counts between ZnO treatments.

Ref	Weaning / sampling[1]	Sample type	Treatment/ duration	Technique	Effects of ZnO on microbial population.
Pieper et al., 2020	25d/ 3wks pw		2500ppm of ZnO/ 3wks	Shotgun[13]	Significant decrease in Shannon diversity and Evenness in ZnO group. Increase in abundance of *Intestinimonas*, *Lachnoclostridium*, *Blautia*, *Subdilogranulum*, *Faecalibacterium*, *Coprococcus*, *Pseudoflavonifractor*, *Acetivibrio*, *Bacteroides*, *Holdemania*, *Collinsella*, *Parabacteroides*, *Holdemannella*. Decrease in abundance of dominating genera: *Megasphaera*, *Diallister*, *Acidaminococcus* and *Ruminococcus* in 2500ppm ZnO.
Wei et al., 2020	21d/ 30d,42d, 60d	faecal	ZnO, 2500 ppm (HZ), Standard zinc (SZ, 195 ppm Zn)/ 21d-60d	16S rRNA sequencing	Differences in beta-diversity between SZ (low zinc) and HZ(high ZnO) groups. Reduced relative abundance of *Lactobacillus* and *Megasphaera* in ZnO treated animals. Higher relative abundance of *Streptococcus* at day 42 in ZnO treated animals. Higher abundance of *Prevotella* at day 30 in ZnO treated animals. Day 42: Higher abundance of *Streptococcus*, *Prevotella*, and *Clostridium sensu stricto* in ZnO treated pigs and higher abundance of *Lactobacillus*, *Megasphaera*, *Campylobacter*, and *Holdemanella* in control animals. Day 60: Higher abundance of different OTUs corresponding to *Prevotella*, *Streptococcus*, *Clostridium sensu stricto*, *Phascolarctobacterium*, and *Terrisporobacter* compared to the SZ (low Zinc) group, with higher abundances of a different OTU corresponding to *Prevotella*, *Megasphaera*, *Lactobacillus*, and *Acidaminococcus*.
Yoon et al., 2020	28d/ 7 and 14dpw	ileum content	ZnO, 2500 ppm/ 14d	Culture	No difference in total anaerobic bacteria, *Bifidobacterium* spp., *Lactobacillus*, and coliforms among treatments. Decreased counts of *Clostridium* spp. in the ileum compared with pigs in control treatment at days 0 to 7 post-weaning.
Da Silva et al., 2021	21-23d/ 65days of age	caecal content	ZnO 2500 ppm/ 65d	16S rRNA sequencing	Lower incidence of diarrhoea in ZnO and/or benzoic acid + probiotic treated animals. Lower Chao1 and Observed richness[18] in ZnO treated pigs. Differences in beta-diversity. Numerical reduction of relative abundance of *Megasphaera elsdenii* in caecal content of ZnO treated animals.

Ref	Weaning / sampling[1]	Sample type	Treatment/ duration	Technique	Effects of ZnO on microbial population.
Dowley et al., 2021	28d/ 45dpw	colon a caecal content	ZnO, 2500ppm, 1550ppm/ 15dpw(2500ppm), 35dpw (1550ppm)	16S rRNA sequencing	Increased abundance of *Lactobacillus* in caecal contens of ZnO treated animals. Decreased abundance of *Prevotella* and Prevotelllaceae family in ZnO treated animals.
Li et al., 2021	23d/ 21, 35dpw	faecal	ZnO, 2000ppm/ 35d	16S rRNA sequencing	Tendency of decreased diarrhoea rate in ZnO treated animals. Day 21dpw: increased relative abundance of *Clostridium sensu stricto 1*, *Terrisporobacter*, *Succinivibrio*, *Olsenella*, and *Agathobacter* and decreased relative abundance of *Prevotella*, *Lactobacillus*, *Sarcina*, *Methanobrevibacter*, *Megasphaera*, and *Treponema* in ZnO treated group. Day 35dpw: Increased relative abundance of *Clostridium sensu stricto 1*, *Methanobrevibacter*, and *Succinivibrio* and decreased relative abundance of *Prevotella*, *Lactobacillus*, *Sarcina*, *Megasphaera*, *Treponema*, *Rikenellaceae RC9 obacter group*, and *Agathellaceae RC9 obacter*.
Liu et al., 2021	NA/ 28dpw	faecal	ZnO, 22500ppm/ 28d	16S rRNA sequencing	Increased abundance of *Lachnospiraceae UCG 004* and decreased abundance of *Ruminococcus flavefaciens* in ZnO treated pigs.
Hou et al., 2021	21d/ 17dpw	ileum content	ZnO 1600ppm (H-ZnO); Basal (CON)/ 17d	16S rRNA sequencing	No significant differences in alpha diversity between H-ZnO and control diet. Distinct clustering of groups in ordination (β-diversity). No significant differences in *Lactobacillus* spp abundance. Lower abundance of *Streptococcus*, *Escherichia-shigella*, *Actinobacillus*, and *Clostridium sensu stricto 6* in Chitosan-chelated Zn treated group compared to CON piglets. Decreased abundance of *Streptococcus* and *Actinobacillus* compared to CON pigs.
Conway et al., 2022	28d/ 1-21dpw (ZnO)		ZnO+Se, ZnO 2500 ppm/ 21d(ZnO)	16S rRNA sequencing	Overall, greater faecal scores and improved performance parameters in ZnO treated animals from day 1dpw to 21dpw, no study of microbiota on ZnO treated animals.

Ref	Weaning / sampling[1]	Sample type	Treatment/ duration	Technique	Effects of ZnO on microbial population.
Juhász et al., 2022	28d/ 3, 6, 12 wks age	faecal	ZnO 3100ppm (PC)/ 3wks age - 12wks age	Culture, 16S rRNA sequencing	Lower Lactic acid bacteria (LAB) and coliforms in 6W and 12W samples in PC. Lower Enterococcaceae and *E. coli* abundance in PC (and another treated group). Higher abundance of Clostridiales. Ruminococcaceae, Lachnospiraceae, Clostridiaceae 1 and Prevotellaceae in ZnO group. Decreased relative abundance of Veillonellaceae in PC.
Tang et al., 2022	28d/ 28dpw	environmental, colonic content	ZnO 1445ppm/ 28d	16S rRNA sequencing	Decrease in diarrhoea incidence throughout the study in PC and HI treatments, with PC[17] having the lowest incidence. Decrease in ACE, Chao1[18] and Shannon in colonic content of PC. Differences in β-diversity (PC). Increased abundance of Muribaculaceae, Bacteroidaceae, *Bacteroides*, and *Coprococcus*, and decreased abundance of *Agathobacter* in PC. Higher abundance of *Parabacteroides*, *Bacteroides*, *Coprococcus*, and *Prevotellaceae UCG 003*
Venardou et al., 2022	28d/ 25,27, 34dpw	faecal, colonic and caecal digesta	ZnO 2500ppm/ 21d	qPCR	Reduced faecal score on ZnO treated animals in 0 to 21dpw. 25-34dpw: reduced counts of Enterobacteriaceae in fucoidan-rich *Ascophyllum nodosum* extract (ANE) group, and ZnO-residual groups. Higher abundance of *Bifidibacterium* in ANE group compared to ZnO-residual.
Xu et al., 2022	28d/ 20dpw	colonic content	ZnO 1600ppm/ 20d	16S rRNA sequencing	Lower incidence of diarrhoea in ZnO treated pigs. Lower Shannon and Simpson index of α-diversity in ZnO treated pigs. No comparison of ZnO to Control group. Higher abundance of *Parabacteroides goldsteinii*, *Clostridium sp culture 27*, *Faecalicoccus pleomorphus*, *Clostridium sp culture 54*, and *Clostridium leptum* and lower abundance of *Desulfovibrio*, *Megasphaera elsdenii* and family Veillonellaceae in ZnO treated pigs compared to coated tannin treated pigs.
Zhang et al., 2022	NA/ 28dpw	faecal	ZnO 1600ppm/ 28d	16S rRNA sequencing	Lower incidence of diarrhoea in ZnO treated animals. greater ($P < 0.05$) acetate and total short-chain fatty acids concentrations. Increased abundance of *Prevotellaceae NK3B31 group*, *Parabacteroides*, *Succinivibrio*, and *Lachnoclostridium* in the ZnO group compared with the other groups. Statistical tendency of higher Prevotellaceae and lower Veillonellaceae in ZnO treated pigs.

Ref	Weaning / sampling[1]	Sample type	Treatment/ duration	Technique	Effects of ZnO on microbial population.
Sun et al., 2022b	21d/ 42dpw	ileum and colon content	ZnO 2000ppm/ 42d	16S rRNA sequencing	Reduced diarrhoea incidence in ZnO treated groups. Higher Chao1 index and increased *Lactobacillus* and *Blautia*, and Lactobacillaceae family abundance in in ileum of ZnO treated pigs. Colon: Higher abundance of Streptococcaceae in CON group. Increased abundance of Lachnospiraceae and Lactobacillaceae and decreased abundance of Clostridiaceae 1 in ZnO treated groups (ZnO and C-ZnO: Coated ZnO). Higher abundance of *Faecalibacterium*, *Prevotellaceae NK3B31 group*, *Intestinibacter*, and *Coprococcus 1* and Prevotellaceae in ZnO treated pigs.

[1]Weaning/Sampling: Weaning age (days of age)/Sampling period (days of age, days post-weaning, or day of treatment); [2]d: day of age/ day of experiment; [3]dpw: day post-weaning; 4ppm: parts per million; [5]T-RFLP: Terminal Restriction Fragment Length Polimorphism; [6]ELISA: Enzyme-Linked ImmunoSorbent Assay.; [7]16S rRNA: 16S ribosomal RNA (ribonucleic acid) of the bacterial 30S ribosomal subunit; [8]DGGE: Denaturing Gradient Gel Electrophoresis; [9]qPCR: quantitative Polimerase Chain Reaction; [10]ETEC: Enterotoxigenic *E. coli*; [11]K88: F4 fimbriae of *E. coli*; [12]MALDI-TOFF MS: matrix-assisted laser desorption/ionization time-of-flight mass spectrometry; [13]Shotgun: Shotgun metagenome sequencing, whole metagenome sequencing; [14]Ct: Control diet (basal, non-treated); [15]Ab: Antibiotic provided in the study (if any); [16]ZnO: group fed pharmacological doses of ZnO; [17]PC: Positive Control, group fed pharmacological doses of ZnO; [18]ACE, Chao1, Observed richness, Species richness, Shannon, Evenness: Indexes measuring alpha diversity.

1.7 Microbiome profiling through Next Generation Sequencing techniques

Next generation sequencing (NGS) techniques have permitted the characterization of microbiomes, allowing describing the complex composition and phylogenetic relationships among the microorganisms community, often non-culturable bacteria, that builds each microbiome.

Overall, the main approaches used in NGS are based on marker gene studies and whole metagenome sequencing (Boers et al., 2019; Knight et al., 2018). Approaches based on marker gene analysis rely on the sequencing and identification of specific regions of genes of interest with the sufficient variability between bacteria while being present in all of them. The sequencing of these regions is achieved by targeting flanking regions to the gene of interest that remain stable across bacteria, thus allowing to amplify the same genetic region of several groups of bacteria. Such is the case of the hypervariable regions of 16S rRNA in prokaryotes, as well as the 18S rRNA (the equivalent to 16S rRNA in eukaryotes) and ITS region (nuclear ribosomal internal transcribed spacer) in eukaryotes and fungi, respectively (Boers, 2019; Knight et al., 2018). This approach enables a cost-effective taxonomic profiling rendering acceptable resolution level up to genus level in low biomass samples, regardless of host DNA contamination (Boers, 2019; Knight et al 2018). Despite taxonomic identification is improving, 16S rRNA normally does not reach an accurate species level identification, due to the similarity of the hypervariable region between similar species (i.e., species of the same genus or families) (Jovel et al., 2019; Wensel et al., 2022). Recently, it has been demonstrated that species and strain level resolution can be reached through the sequencing of the full 16S rRNA gene (Johnson et al., 2019), but this is more expensive and time consuming than sequencing 16S rRNA hypervariable regions, which is one of the main advantages of 16S rRNA sequencing. The bias to which this method is subjected normally involves PCR linked artefacts such as choice of hypervariable regions to sequence (primers), overamplification of contaminant bacteria, affinity

of the primers towards the targeted regions in the different bacteria within the sample, amplicon size, and number of PCR cycles (Knight et al., 2018).

On the other hand, whole metagenome sequencing, or whole metagenome shotgun sequencing (Perez-Cobas et al., 2020) allows to sequence all the genomic DNA within a sample, enabling to identify the taxonomic community of the sample as well as its functional repertory, based on the functional annotation of the genes encoding proteins in the sample (Boers et al., 2019). This approach allows reaching species and straining level resolution (Quince et al., 2015). Regarding the preprocessing of the sequence reads and its identification, there are two main approaches with pros and cons: Short-reads based approach (or assembly-free), and assembly approach. The first one consists of identifying the taxa or functions of all reads within a sample using different algorithms to ensure identification of species or functions, whereas the second one consist of assembling or joining partially overlapping reads in longer fragments called “contigs” (contiguous reads). Likewise, these contigs, can be further assembled into longer sequences called scaffolds, and these scaffolds, if sufficient read coverage (ie., amount of DNA sequence), into genomes (Quince et al., 2017). This last approach enables an accurate identification of species and strains, as well as functions, virulence factors, antimicrobial resistance genes, allowing to screen larger fragments of sequences or genomes. However, it also requires complex algorithms and computational resources to assemble the correct reads in the correct order from the correct organism, and overcome certain complications such as inter-genomic repetitive elements among bacteria, discernible from sequencing errors (Boers et al., 2019), difference of coverage depending the microorganism present in the sample (depending on the genome size of each bacterium) and the presence of different strain within the same species (Boers et al., 2019; Quince et al., 2017). Although shotgun metagenomics offers such an outstanding set of advantages in resolution and description of microbial composition and genetic content within a sample, this technique is more expensive than marker analysis, due to the higher coverage needed (Perez-Cobas et al., 2020).

Objectives

This doctoral thesis is part of a project funded by Teagasc Walsh Scholarships Programme of Ireland, which aimed to determine how pig's faecal microbiome is impacted by ZnO over time in commercial conditions.

The objectives of this thesis were:

1) To describe the taxonomic and functional changes on the faecal microbiome of the piglet during the 2 weeks post-weaning using NGS.
2) To describe the effect of in-feed antibiotics and pharmacological doses of ZnO on the faecal microbiome of the piglet during the 2 weeks post-weaning.
3) To study the relative effect of weaning, ZnO and antibiotic treatment, environmental factors and farm of origin on the faecal microbiome of the piglet during the 2 weeks post-weaning.
4) To describe the differences on the faecal microbiome of the piglet during the 2 weeks post-weaning between farms that need to use in-feed antibiotics and ZnO on a regular bases and farms that do not.
5) To describe the differences on the microbiome of the piglet one week post-weaning in normal and diarrhoeic faeces.

Chapter 2. Study I: Using Shotgun Sequencing to Describe the Changes Induced by In-Feed Zinc Oxide and Apramycin in the Microbiomes of Pigs One Week Postweaning

Using shotgun sequencing to describe the changes induced by in-feed zinc oxide and apramycin in the microbiome of pigs one week post-weaning

Juan M. Ortiz Sanjuán[a,b], Edgar G. Manzanilla[a,c], Raúl Cabrera-Rubio[d,e], Fiona Crispie[d,e], Paul D. Cotter[d,e,f], Juan J. Garrido[b], Héctor Argüello[g].

INSTITUTIONS

[a]Pig Development Department, Teagasc Grassland Research and Innovation Centre, Moorepark, Fermoy, Co. Cork, Ireland.
[b]Grupo de Genómica y Mejora Animal, Departamento de Genética, Facultad de Veterinaria, Universidad de Córdoba, Córdoba, Spain.
[c]School of Veterinary Medicine, University College Dublin, Dublin, Ireland.
[d]Teagasc Food Research Centre, Moorepark, Fermoy, Co. Cork, Ireland.
[e]APC Microbiome Institute, University College Cork, Co. Cork, Ireland.
[f]VistaMilk SFI Research Centre, Fermoy, Co. Cork, Ireland.
[g]Animal Health Department, Veterinary Faculty, Universidad de León, León, Spain.

Running Head: ZnO and apramycin effects in weaned pig's microbiome.

#Address correspondence to Juan M. Ortiz Sanjuán, Juan.Ortiz@teagasc.ie.

Using shotgun sequencing to describe the changes induced by in-feed zinc oxide and apramycin in the microbiome of pigs one week post-weaning

ABSTRACT

Post-weaning diarrhoea (PWD) is a relevant problem associated with early weaning in pig farms. For decades, in-feed antibiotics and therapeutic zinc oxide (ZnO) have been widely used to prevent piglets PWD. The EU is banning both strategies in 2022 due to antimicrobial resistance and environmental contamination concerns respectively. Understanding the effects of these products on the pig's microbiome is crucial to correct potential microbial disbalances that would prompt PWD. Using shotgun sequencing, three trials were carried out to explore the impact of in-feed apramycin and ZnO, combined with different on farm hygiene protocols, on the faecal microbiome of piglets 7 days post-weaning. In trial 1, 28-day old piglets were allocated to one of three groups: Control diet (Ct), Ct + ZnO (Zn), and Ct + apramycin (Ab). In trial 2 and 3, piglets were allocated to the same treatments, but the trials also included different cleaning protocols achieving different hygiene levels. In-feed treatments impacted richness, diversity, and relative abundance more than hygiene. Pigs in the Ct group showed higher species richness than pigs in Ab and Zn groups. Clustering analysis evidenced the link between Enterobacteriaceae in Ct; Lactobacillaceae and Veillonellaceae mainly in Ct; and Bacteroidaceae, Ruminococcaceae, Oscillospiraceae, Acidaminococcaceae and Lactobacillaceae in Ab and Zn groups. Functional data analysis revealed higher abundance of virulence genes in Ct microbiomes, heavy metal and antimicrobial resistance-related functions in Zn treatment. Results demonstrate that alternatives to Ab and ZnO should balance microbial abundance and stimulate the growth of commensals to outcompete potential pathogens.

IMPORTANCE

Weaning is a critical moment for piglets, during which time potentially harmful bacteria can increase in abundance in the intestine, creating digestive problems and diarrhoea by pathogens such as *Escherichia coli*. In-feed antibiotics, the main antibiotic use in livestock, and therapeutic doses of zinc oxide (ZnO) help to control diarrhoea but prompt secondary problems such as

antimicrobial resistance and soil pollution heavy metals. Understanding how these strategies impact the gut microbiota is crucial to establish health biomarkers and design successful replacement strategies. Using shotgun sequencing, this study compares the microbiota of pigs after early-weaning when treated with in-feed antibiotics, ZnO or treatment-free diets to describe differences that could define the susceptibility to infections, providing the basis of future research on improving intestinal resilience through microbiota-based strategies.

KEYWORDS: antibiotics, diarrhoea, metagenomics, swine, weaning, zinc oxide

INTRODUCTION

Weaning is a critical period during which piglets are abruptly exposed to stressors such as an early separation from the mother, a sudden dietary change from milk to solid feed, and/or transport to a new environment where they are mixed with other litters (Campbell et al., 2013). Two major consequences of weaning are the piglets becoming immunocompromised (Lallès et al., 2007) and a transient gut dysbiosis promoted by the sudden nutrient changes (Gresse et al., 2017). This is an opportunity for pathogens to colonise the gut and establish an infection, leading frequently to post-weaning diarrhoea (PWD), a multifactorial disease causing economic and productive losses on pig farms. PWD usually involves the presence of enterotoxigenic *Escherichia coli* (ETEC) and requires antimicrobial therapies to control the problem (Luppi., 2017; Fairbrother and Nadeau., 2019).

Within the context of enteric diseases, the commensal gut microbiota can play a pivotal role, acting as an additional protective layer by competing with pathogens for niches in the intestine, a concept known as colonization resistance (Dou et al., 2017). Indeed, an early establishment of a desirable microbiota after weaning can promote intestinal health and prevent enteric diseases (Gresse et al., 2017, Argüello et al., 2019). This post-weaning microbiota is influenced by factors such as the mother's microbiota, the environment and cleaning protocols used (Law et al., 2021) and, in particular, diet (Wang et al., 2019b). Despite this generally accepted view, the importance of each individual factor is still not well understood.

Antibiotics and therapeutic Zinc Oxide (ZnO) have been widely used in feed for prophylaxis and metaphylaxis to prevent, and/or treat PWD. Although antibiotics directly inhibit the growth of pathogens such as ETEC, they also contribute to an increase on antimicrobial resistance (AMR), which is a major public health concern. In contrast, the mechanisms of action of ZnO are not well known. Zinc is an essential element required as a co-factor in multiple biological processes (Suttle., 2010) and piglets may undergo a zinc deficiency at weaning (Davin et al., 2013). The effects of ZnO also include modulation of gut morphological structure, mucus composition and intestinal permeability (Li et al., 2006, Liu et al., 2014b), as well as potential antimicrobial impacts (Fröhlich and Fröhlich., 2016; Sawai et al., 1996). Altogether, the use of ZnO results in reduced inflammation and diarrhoea (Shen et al., 2014; Xia et al., 2017) and improves growth performance (Sales., 2013). However, the high dosages of therapeutic ZnO (over 2000 ppm) required and its poor absorption in the gut leads in environmental pollution of soil when pig slurry is used as a fertilizer (SCVMP., 2017). Due to these concerns, ZnO and antimicrobial prophylactic medication will be banned in the EU from 2022 (EC 2019/4) and other countries/regions have or will soon introduce similar regulations.

By studying the modulation of the intestinal microbiota by ZnO and antibiotics, the potential exists to gain insights into the key changes that contribute to the control of PWD with a view to identify other microbiome modulators than could be employed in the forthcoming post-ZnO and AB-free era. Thus, the present study used shotgun sequencing to determine the impact of in-feed apramycin and therapeutic in-feed ZnO on the faecal microbiome of early-weaned pigs, while also assessing the relative importance of the background hygiene protocols in the facilities.

RESULTS

Three trials were carried out to assess the faecal microbiome of pigs supplemented with an in-feed antibiotic (apramycin) (Ab group) or in-feed ZnO (Zn group) relative to a control (Ct) group. The productive performance of the animals was not affected by the in-feed treatments or cleaning protocols in any of the three trials although pigs on treatment Ct showed softer faeces. The average daily gain was 193, 169 and 173 g/day, and the average daily intake was 240, 184 and 215 g/day for trials 1, 2 and 3 respectively resulting in feed conversion rates of 1.37, 1.31 and 1.33.

All data related to the microbiome was analysed from an overall global perspective as well as for each individual trial considering the cleaning protocols used in each trial. Trial 1 only had standard cleaning. Trial 2 included two cleaning protocols (Table 1), a substandard protocol to study the effects of a deficient hygiene and an optimum cleaning to study the effects of excellent hygiene. Dietary treatments had a much more marked effect on the microbiome than cleaning protocols. Trial 3 included a group of pigs with no cleaning and a substandard cleaning protocol to study the effects of deficient hygiene compared to a standard protocol (Table 1). Again, most effects observed were a consequence of dietary treatments and not of the cleaning protocols.

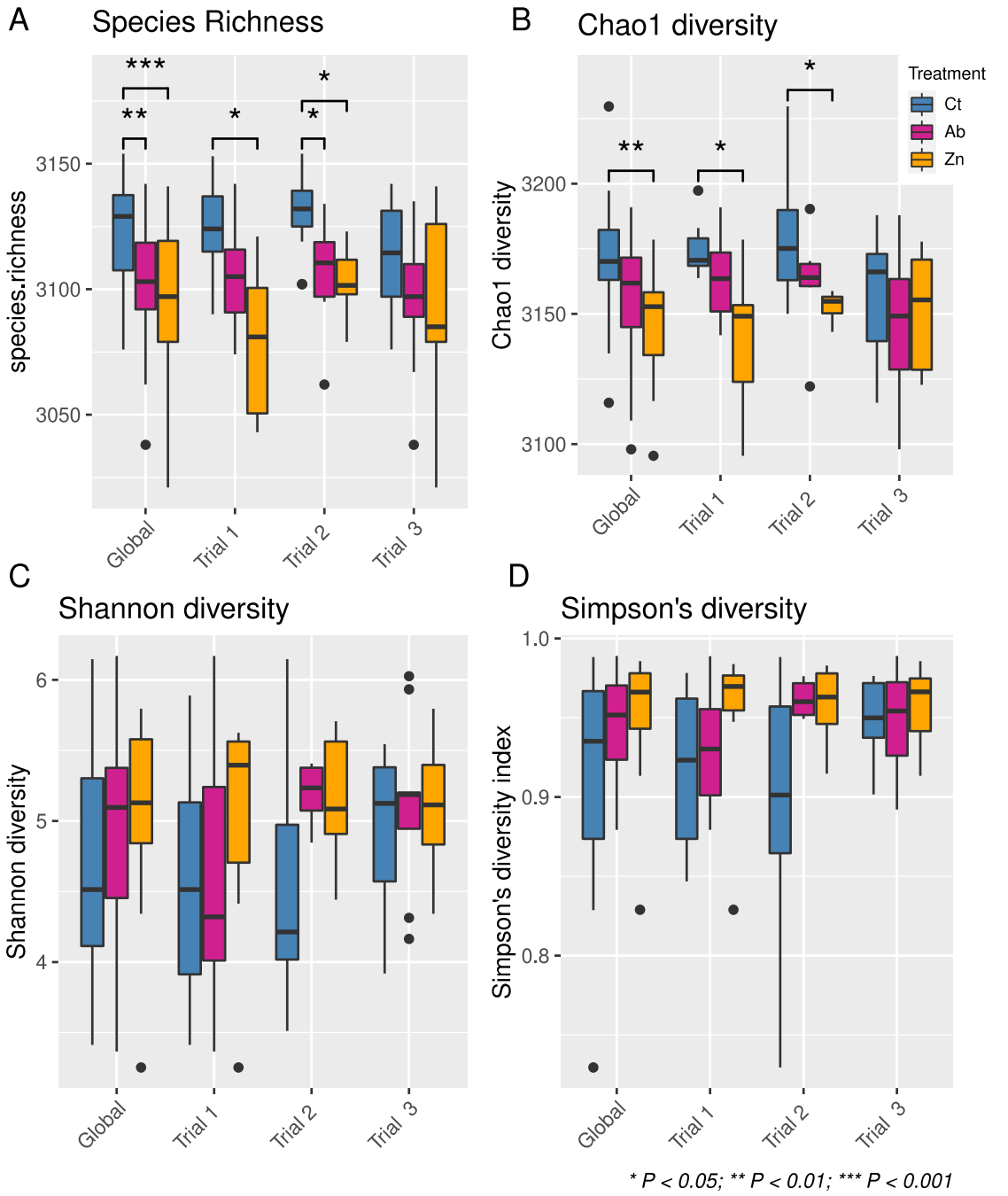
Chapter 2. Study I. Table 1. Cleaning protocols used in the 3 trials.

	Cleaning Protocol						
Trial	Code	Description	Pre-soak[a]	Detergent[b]	Power wash[c]	Disinfectant[d]	Dry
1	A	Standard	Yes		Yes	Yes	Yes
2	B	Substandard			Yes	Yes	
2	C	Optimal	Yes	Yes	Yes	Yes	Yes
3	D	No cleaning					
3	E	Substandard	Yes		Yes		Yes
3	F	Standard	Yes		Yes	Yes	Yes

[a]Pre-soak: Rooms are equipped with sprinklers in the ceiling. Sprinklers with cold water (10-15°C) are turned on for approximately 1h 40min. Then the room is powerwashed. [b]Detergent: Kenosan used at 0.5% (CID lines, Belgium) applied to all surfaces in the rooms for approximatelly 1h 30min after pre-soaking. Then the rooms are powerwashed. [c]Powerwash: Cold water (10-15°C) is applied using a power hose to all surfaces in the room. Rooms are then left to dry for at least 24h. [d]Disinfectant: After washing, Hyperox (Virkon used at 1%, LANXESS Deutschland GmbH, Germany). Rooms are then left to dry at least 48h.

In feed treatment with ZnO or apramycin limits species richness at weaning. The outputs from microbial richness (species richness, Chao1 indexes and Shannon) and evenness (Simpson index) analyses are summarised in Figure 1 (further details in supplementary file S1). The global analysis revealed a higher species richness in the faeces of the Ct group pigs compared to pigs in the antibiotic Ab treatment ($p = 0.003$) or Zn treatment groups ($p < 0.001$; Figure 1A), while Chao1 diversity values were also higher in control compared to Zn-treated pigs ($p = 0.002$; Figure 1B). No differences among groups were observed for Shannon or Simpson indexes (Figures 1C and 1D).

The differences in global analysis were consistent for trial 1 and trial 2, where samples from control pigs had greater species richness (trial 1, $p = 0.007$; and trial 2, $p = 0.019$) and Chao1 diversity (trial 1, $p = 0.007$; trial 2, $p = 0.043$) than Zn as well as higher richness than Ab group in samples from trial 2 ($p = 0.033$). No differences were observed among dietary treatments in trial 3 or among cleaning protocols in trials 2 or 3 (Supplementary Figure S1).

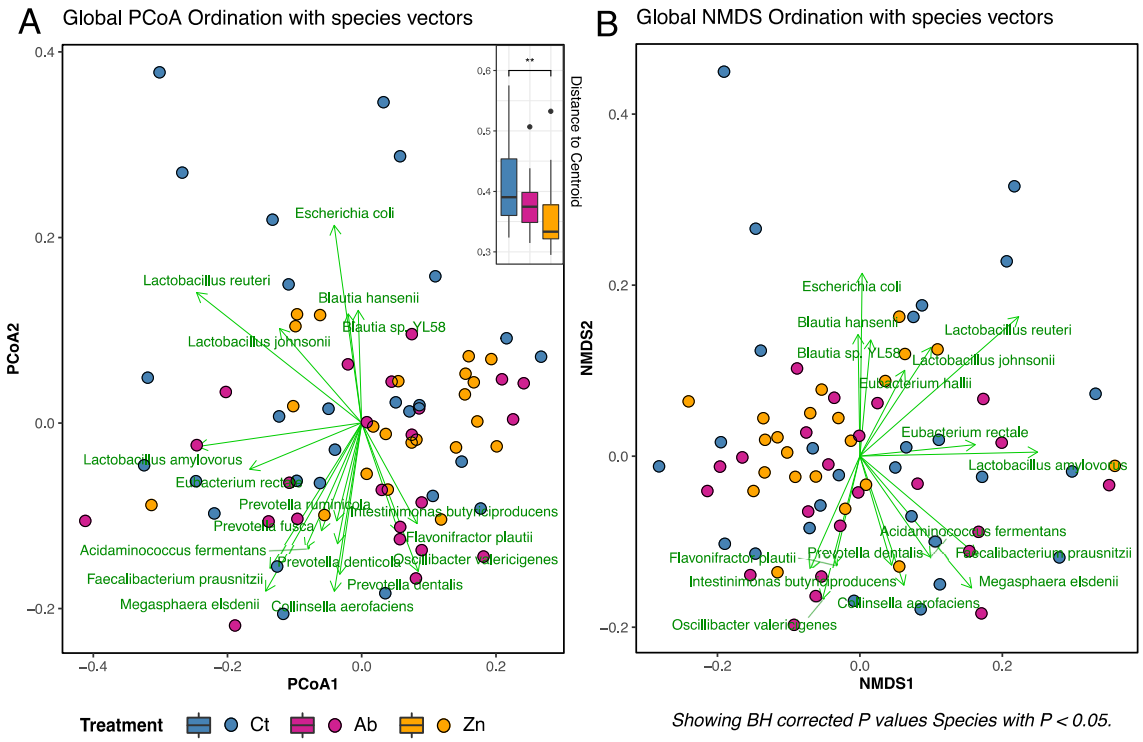


Chapter 2. Study I. Figure 1. Alpha diversity richness (Species richness, Chao1 indexes, and Shannon) and evenness (Simpson index) of samples from different dietary treatments. Information is split by global analysis, trials 1, 2 and 3. species. The lower, medium, and upper horizontal box lines correspond to the first, second and third quartiles (the 25th, 50th and 75th percentiles). Upper and lower whiskers include the range of the upper and lower points within the 1.5 interquartile range. A) Species richness; B) Chao1 diversity index; C) Shannon diversity index; D) Simpson's diversity index. *P < 0.05, **P < 0.01, and ***P < 0.001, respectively. Taxonomic identification of sequences was performed using Kraken2.

Microbiota ordination is impacted by in-feed treatments rather than environmental cleaning.

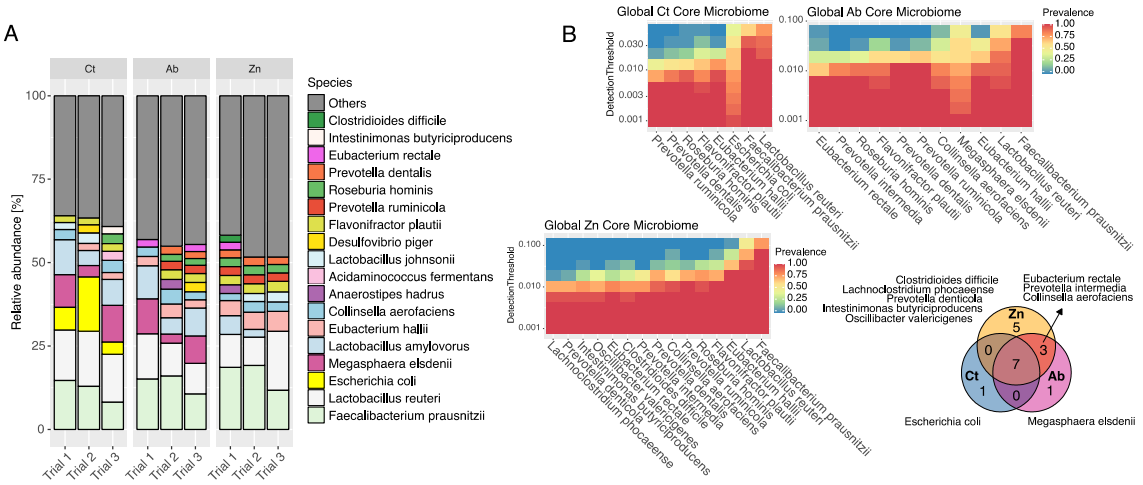
Analysis of within group dispersion revealed differences between control and ZnO treatments (Figure 2A). The influence of dietary treatments on the global analysis ordination was confirmed by envfit function when visualized by PCoA (Supplementary Table S1; $p = 0.041$). The sample ordination was clearly influenced by the abundance of species as shown in figures 2A and 2B.

PERMANOVA analyses revealed differences in dietary treatment variable (Supplementary Table S2; $p = 0.001$). Further analyses by pairwise PERMANOVA depicted the differences among all dietary treatment levels (Supplementary Table S2; $p < 0.01$). Ordination of samples and PERMANOVA analysis of the respective trials revealed no differences between trial 1 treatments (Supplementary Table S1 and S2, Fig S2A and S2D). In trial 2, microbiomes from Ct diet pigs showed higher dispersion than Ab and Zn dietary treatments pigs (Figure S2E); and ordination and PERMANOVA revealed differences among cleaning and dietary treatments (Supplementary Table S1 and S2, Fig S2B and S2E). In trial 3, ordination and PERMANOVA revealed differences between Zn and the other two dietary treatments (Supplementary Table S1 and S2, Fig S2C and S2F).



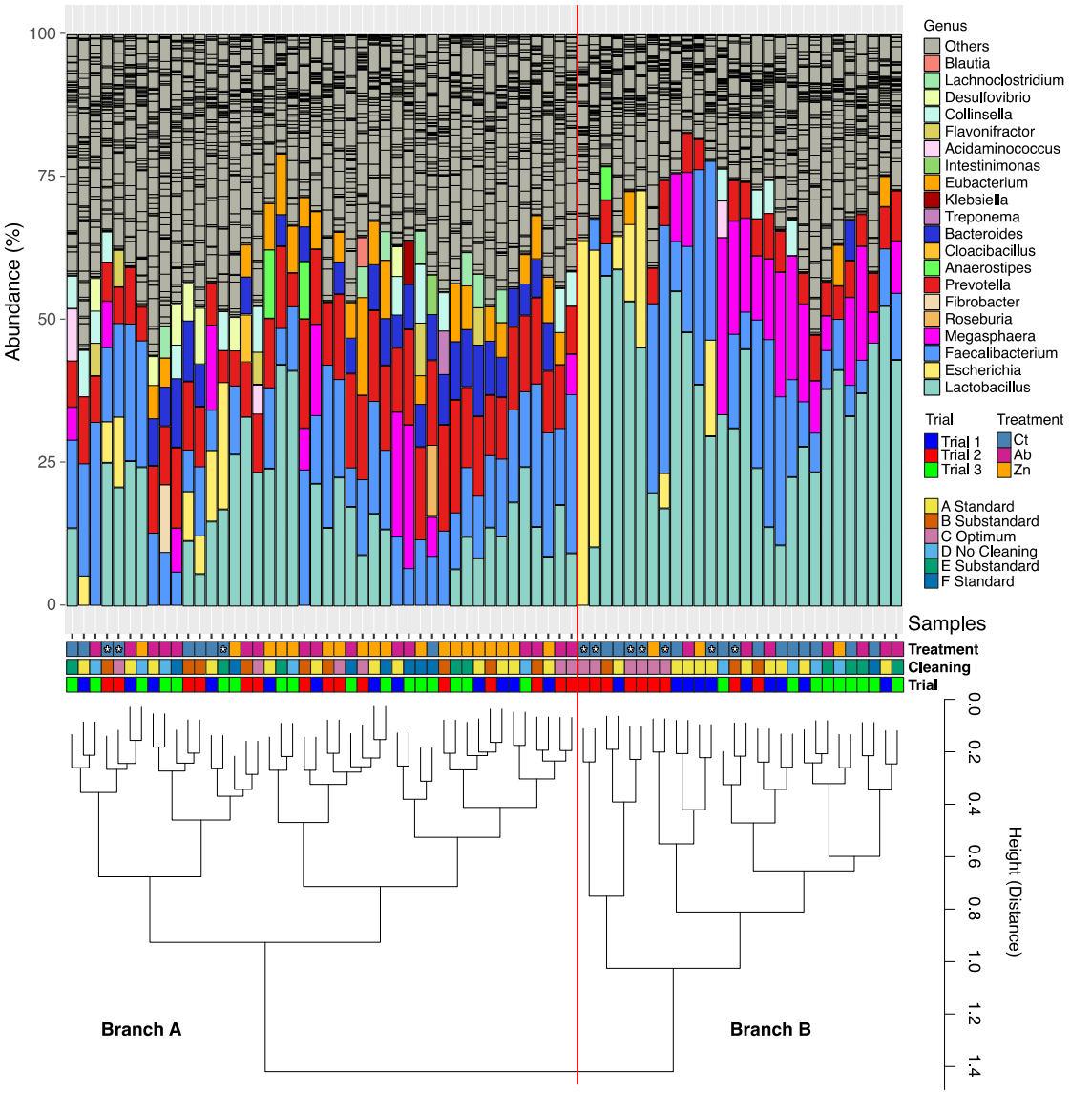
Chapter 2. Study I. Figure 2. Ordination analysis of faecal samples from weaned pigs with different dietary treatments (Control, apramycin and ZnO). (A) PCoA ordination of global analysis of faecal samples, along with a boxplot of within group dispersion analysis of each dietary treatment. **P < 0.01. (B) NMDS ordination of global analysis. Figures 2A and 2B show the significant species returned by "*envfit*" model (Arrows showing species with significant BH-adjusted p values; Arrows length represents the strength of each specie influencing the ordination of samples). Taxonomic identification of sequences was performed using Kraken2.

Dietary treatments shape microbiota composition and impact the dominant core species. Microbiota abundance was dominated by 18 species that accounted for approximately the 60% of the faecal relative abundance (Figure 3A). *Faecalibacterium prausnitzii* and *Lactobacillus reuteri* were consistently the dominant species regardless of the treatment group (Figure 3A; Supplementary file 2). Other dominant species were *Megasphaera elsdenii*, and *Lactobacillus amylovorus* among others (Supplementary file 2). Most of these species defined the core microbiota of each group (Figure 3B).



Chapter 2. Study I. Figure 3. Faecal microbiota composition in weaned pigs with different dietary treatments (control, apramycin and ZnO). (A). Mean relative abundance of the main species in each group and trial. "Others", refers to those species accounting for less than a 2 percent of abundance. (B) Core microbiome of pigs from each dietary treatment. Inclusion criteria for species were an abundance detection threshold of 1% in at least a 70% of samples of each group. Taxonomic identification of sequences was performed using Kraken2.

Abundance of different species clearly grouped samples by in-feed treatment using Ward clustering and Bray Curtis distances. Thus, 18 out of 27 control samples clearly grouped in one of the two branches composed by 28 pigs (Figure 4, Branch B), dominated by the high relative abundance of *Escherichia coli*, *Lactobacillus* spp, *Faecalibacterium* spp and *Megasphaera* spp. Indeed, core microbiome analyses, performed at an abundance threshold of 1% of the total abundance in the 70% of the samples of each group, identified *E. coli* as singular taxon in the core microbiota of control animals (Figure 3B). The other branch (named branch A) in Figure 4 included mainly pigs from the Ab and Zn treatment groups (a total 35 out of 44 pigs, Branch A), where the most representative species were *Lactobacillus* spp, *F. prausnitzii*, *Bacteroides* spp, or *Prevotella* spp., the abundance of which accounted, in most cases, for approximately 50% of the relative abundance.

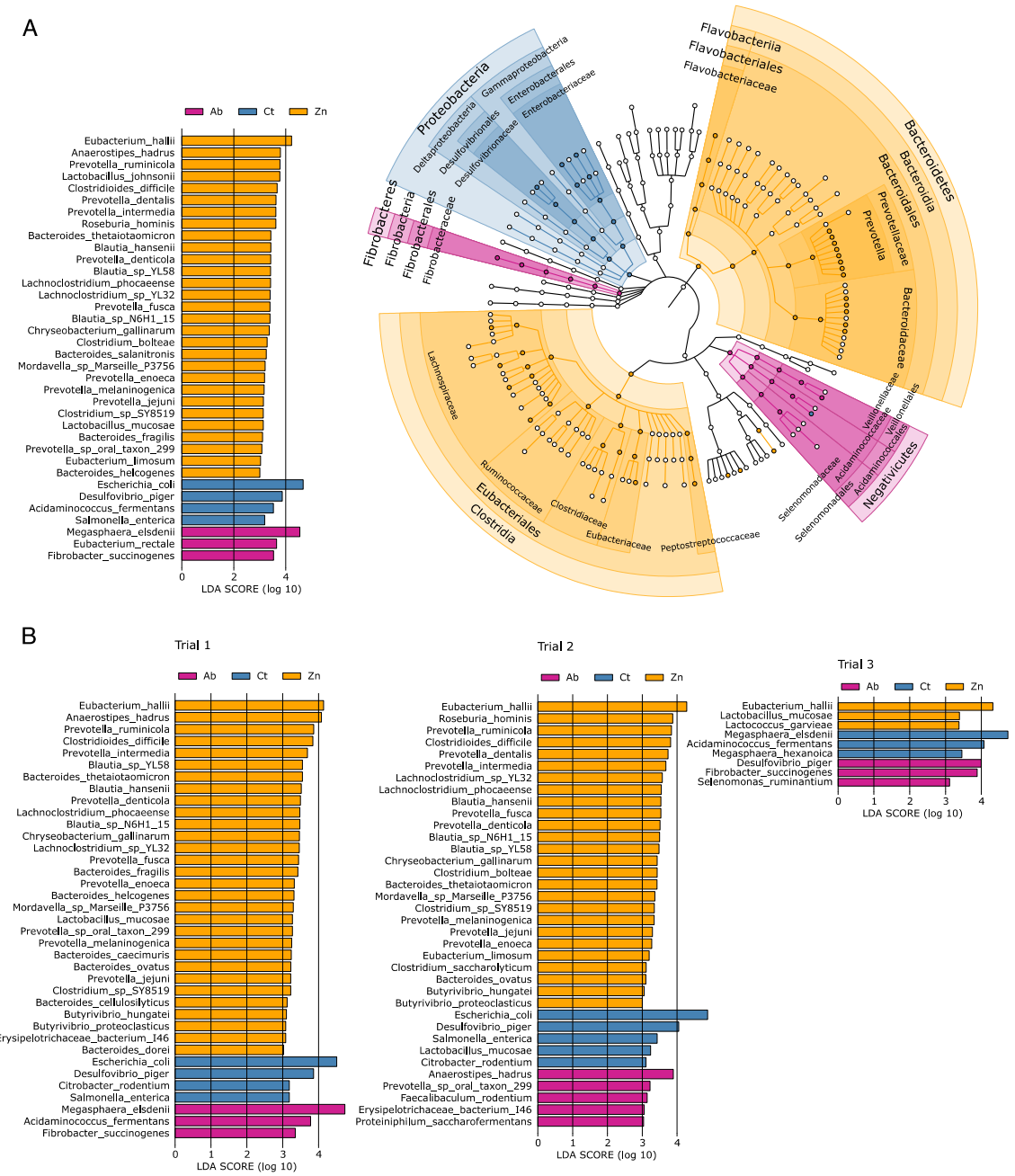


Chapter 2. Study I. Figure 4. Stacked bar plot of the relative abundance of the main genera in faecal samples from weaned pigs with different dietary treatments. Profiles of samples are ordered by Ward clustering of the squared root Bray Curtis distances between samples. Cluster dendrogram represents the similarity between samples regarding its microbial composition. Variables information in each sample (from lower to upper level: Trial, Cleaning, Treatment) are indicated in the coloured squares below the bars. For the purpose of enabling a better visualization, a line is dividing the relative abundance profiles in the two patterns stablished by the Ward Clustering. "Others", refers to those genera accounting for less than a 5 percent of abundance. Taxonomic identification of sequences was performed using Kraken2.

A cladogram of bacterial groups linked to any of the treatments by Linear discriminating score analysis (LDA) evidenced an association of Clostridia and *Bacteroides* to Zn group, Negativicutes and Fibrobacteres in Ab group and Proteobacteria to Ct group (Figure 5A). Differences in species abundance as determined by Linear discriminating score analysis again revealed a higher

abundance of *E. coli*, *Desulfovibrio piger*, *Acidaminococcus fermentans* and *Salmonella enterica* in Ct pigs, while *M. elsdenii*, *Eubacterium rectale* and *Fibrobacter succinogenes* were more abundant in Ab animals. Zn animals were enriched in species belonging to the following families: Bacteroidaceae, Prevotellaceae, Flavobacteriaceae, Clostridiaceae, Eubacteriaceae, Lachnospiraceae, Peptostreptococcaceae and Ruminococcaceae (Figure 5A). The singular species in Zn diet core microbiome were represented by *Clostridioides difficile*, *Lachnoclostridium phocaeense*, *Prevotella denticola*, *Intestinimonas butyriciproducens* and *Oscillibacter valericigenes*. Species found uniquely at the established levels of abundance and prevalence in Ct and Ab animals were *E. coli* and *M. elsdenii*, respectively. The species *E. rectale*, *Prevotella intermedia* and *Collinsella aerofaciens* were shared between Ab and Zn core microbiomes (Figure 3B).

Similarly, the analysis of abundance by trial (Figure 5B), revealed the systematic higher abundance of *E. coli* in Ct animals together with other Proteobacteria (*D. piger*). A higher abundance of *F. succinogenes* was linked to Ab in trials 1 and 3. We also observed a link between Zn treatment and a consistently higher abundance of *Eubacterium hallii* in the 3 trials, and different species belonging to Bacteroidaceae, Prevotellaceae, Lachnospiraceae family in trials 1 and 2 while Lactobacillus mucosae was higher in abundance in Zn animals in trial 1 and trial 3, and *Lactococcus garvie* in trial 3. *M. elsdenii* was higher in abundance in Ab animals in trial 1 and control pigs in trial 3, showing inconsistent results. As well as different species as *Anaerostipes hadrus* shifting from Zn to Ab in trial 1 and 2, or *A. fermentans* between Ab and Ct in trials 1 and 3.



Chapter 2. Study I. Figure 5. Differences in species abundance, returned by LEfSe analysis, most likely explaining the differences among dietary treatments. (A) Global analysis, Species (right) and taxa (left) associated to each dietary treatment (B) Differences in species abundance, returned by LEfSe analysis, most likely explaining the differences among dietary treatments in trials 1, 2 and 3. Horizontal bars are coloured according to each dietary treatment and represent the effect size of each species in each particular group. Background shades and clades (circles) of cladogram of figure 5A are coloured according to its associated dietary treatment found by LEfSe.

Finally, we obtained 138 Metagenome-Assembled genomes (MAGs) from 61 species (Supplementary Figure S3). Some species MAGs were ascribed to particular dietary treatments groups. Thus, the seven *E. coli* MAGs were present in control samples while 13 *Prevotella* spp. were linked to 6 samples of Ab animals, 6 samples of Zn animals, and 1 of Ct animals, this last genome clustering in a different group.

ZnO and apramycin increase gene functions associated to metabolism while treatment-free diets increase virulence factors. Supplementary Figure 4 summarises the microbial functions by the genes differentially abundant identified in each dietary medication. Global and by trial analyses highlighted a higher abundance of genes associated with carbohydrate metabolism in faeces from Zn supplemented pigs. These animals exhibited higher abundance pathways associated with the metabolism of mono-, di-, oligo- and polysaccharides. The Ab group had few differentially abundant functions related to protein metabolism, pyrimidines, or amino acid assimilation (Figure S5E), while animals in Ct group exhibited a higher abundance of functions associated with virulence such as siderophores, oxidative stress, type III, IV and VI secretion systems (Figure S4F) and other virulence factors (Figure S4J).

DISCUSSION

The use of in-feed medications such as antibiotics and ZnO have supported the control of PWD over the last few decades. Among potential antibiotics to treat PWD, apramycin is indicated to treat porcine colibacillosis and is widely used since the 1980s' (Luppi., 2017; Papich and Riviere., 2018). It is a bactericidal antibiotic within the group of aminoglycosides that inhibits the bacterial protein synthesis by irreversibly binding to the receptor proteins on the 30S subunit of the bacterial ribosome (Yang et al., 2020). Both treatments not only directly inhibit the growth of pathogens but also modulate the piglets gut microbiota composition (Pieper et al., 2020; Rattigan et al., 2020). However, more knowledge is needed about the exact impact of these medications in the microbiota, thus clarifying which bacteria and functional roles are misplaced

when these treatments are removed. This information is of paramount relevance to design strategies that successfully replace antibiotics and ZnO in PWD control. In this study, we used shotgun metagenomics to explore the changes in the faecal microbiota composition and functionality of weaned piglets arising from the use of these medications.

Alpha diversity measures the richness and diversity of microbiota composition. We observed lower richness values in groups with in-feed medication, particularly in the Zn group. Indeed, several studies that employed 16S rRNA sequencing have reported a loss of richness in faecal microbiota of therapeutic ZnO treated animals (Shen et al., 2014; Xia et al., 2017; Yu et al., 2017b). The other diversity measure, β- diversity, allows comparing the microbiota across samples. In this study, the microbiota of the Ab and Zn groups had a higher within group similarity than Ct animals, which showed higher intra-group dispersion. Indeed, the lower dispersion in microbiota samples from Zn pigs, regardless of whether the samples were collected from trials 1, 2 or 3, suggests that ZnO shapes and homogenizes the intestinal microbial composition even in different batches of pigs. Not only the treatments shaped a more homogeneous microbiota, but further abundance analyses also revealed a richer core microbiota in Ab and Zn groups compared to control pigs (see figures 3B and 4). The restrictive parameters used to calculate species core ensured the finding of consistently present species in each group, thereby removing rare species and keeping the dominant ones (which are usually the well-known microorganisms). Ward clustering allowed to clearly differentiate samples into two major branches (Figure 4), one formed mainly by control animals (Branch B), dominated by *E. coli*, *Megasphaera* spp, *F. prausnitzii*, and *Lactobacillus* spp., which contrasted with the Ab and Zn treated groups (Branch A) that were dominated by a more diverse pattern of abundance of the genera *Eubacterium*, *Bacteroides*, *Prevotella*, *F. prausnitzii* and *Lactobacillus* (altogether about a 60% of the faecal relative abundance) and where *E. coli* accounted for less than 5% of the relative abundance in the animals treated with ZnO or apramycin. In this study, animals did not show clinical signs of PWD and there were no significant differences in productive

performance among the treatments. However, pens on diet Ct were the only ones where softer faeces were observed at sampling. Samples showing a softer consistency are annotated with an asterisk in Figure 4. Not all soft faeces had a high abundance of *E. coli*, but most of them are associated to Branch B (Figure 4). E. coli is the principal pathogen involved in PWD (Gresse et al., 2017) and in-feed medication, both ZnO and antibiotics, aim to reduce the growth of this bacteria in the post-weaned piglet gut (Starke et al., 2014; Kim et al., 2015). Accordingly, removal of in-feed medications resulted in an increase of *E. coli* abundance and virulence functions related to secretion systems, siderophores, haemolysins and fimbriae in the microbiota of non-medicated pigs. These results on functional profiling reveal different mechanisms linked to colonization and pathogen survival and demonstrate that potential functional biomarkers of intestinal disbalance or gut dysbiosis can be obtained in functional analyses of the microbiome.

Similar to previous studies (Li et al., 2020), overgrowth of *E. coli* was linked to the rise of other Proteobacteria such as *D. piger* in control samples, a bacteria linked to unhealthy enteric status (Karasova et al., 2021) and which seems to be controlled by ZnO (Rattigan et al., 2020). The clear association of these and other Proteobacteria to Ct animals clearly evidences that either both ZnO and apramycin limited their growth or that the microbiota in these treated animals exerted a competitive exclusion against these bacterial groups.

A specific aim of this study was to shed light in which particular bacteria are influenced by these in-feed treatments, particularly the specific impact of ZnO on the gut microbiota still remains a topic of debate. For this reason, we designed a study with three different trials, and we used shotgun metagenomics to characterise the taxonomy up to species level. The results consistently evidenced the association of Zn treatment with species belonging to *Ruminococcus*, *Eubacterium*, *Clostridium*, *Blautia*, *Lachnoclostridium* (*Lachnoclostridium phocaeense* exclusively in the core microbiota of the Zn group) and *Roseburia*, all from Eubacteriales order (Phylum Firmicutes, Class Clostridia). Previous studies support the presence of members of Lachnospiraceae in ZnO based diets (Pieper et al., 2020; Rattigan et al., 2020). Clostridia class

members such as those enriched in ZnO treated animals are considered as part of the anaerobic desirable microbiota, renown fibre degrading bacteria and short chain fatty acids producers (Cao et al., 2016; Holman et al., 2017). Some of these functions were clearly highlighted in functional profiles of Zn animal metagenomes. From the taxa that were enriched in Zn supplemented animals, several species such as *Roseburia hominis* and *L. mucosae*, are already linked to improvement intestinal barrier and host immune response modulation (Patterson et al., 2017; Han and Kim., 2019).

The systematic use of antibiotics in livestock both for animal treatment and as growth promoters have favoured that some commensals are particularly adapted to this challenging environment, exemplified for instance by the genus *Prevotella* (Li et al., 2020; Looft et al., 2014b; Soler et al., 2018) or *M. elsdenii* (Stanton and Humphrey., 2011; Stanton et al., 2011; Wang et al., 2011). Both bacteria are important gut inhabitants after weaning (Wang et al., 2019b), responsible of complex saccharides degradation and SCFA production (Li et al., 2020; Soler et al., 2018). Interestingly, while *Prevotella* adapted to both Zn and Ab treatments, not only *M. elsdenii* but the growth of Negativitutes class in general (including Veillonellaceae and Acidaminococcaceae) was only favoured by the treatment with apramycin. A previous study already stressed the limitations of Veillonellaceae and some members of Acidaminococcaceae to colonise and growth in the gut with ZnO (Pieper et al., 2020) and it may explain the delay of these groups in intestinal colonisation after weaning. However, as stated above, these commensals seem to have adapted to different antimicrobials exposure (Li et al., 2020; Ghanbari et al., 2019).

Different cleaning protocols with variable degree of hygiene were applied in the trials performed. Livestock is usually managed in intensive farming conditions under the so-called all-in/all-out system, in which facilities are emptied and cleaned between batches. Considering the potential effect of the environment in the establishment of the microbiota after weaning (Law et al., 2021) and the lack of specific studies addressing it in piglets at weaning, we decided to evaluate to which extent the cleaning protocol influenced the microbiota of the pigs re-shaping

a week after weaning. Analyses of factors influencing microbiota ordination clearly reflected, regardless the cleaning applied or the trial, that the in-feed treatment (ZnO, apramycin or control diet) was the factor shaping the gut microbiota. Effects of cleaning and environment may be observed when other variables are under control. Our results clearly evidence that the potential effect is negligible compared to the influence of the in-feed medications.

The current study is one of the first addressing PWD and Zn use by shotgun metagenomics. Shotgun sequencing reaches species taxonomy and current analysis pipelines improve taxonomic identification. Additionally, it allows to explore functional profiling and microbial genomes by MAGs analyses. On the other hand, its limitations are the bias towards model organisms and cultivable bacteria (which is constantly improving by increased accuracy in databases), the inability to distinguish between live and dead microorganisms or active genes, and the compositionality problem linked to microbial datasets, which does not reflect the real absolute molecule concentration within the studied sample. Nevertheless, most of these caveats are being solved through a large number of published bacterial reference genomes, high-quality metagenomic assemblies, and newly computational and statistical approaches (Quince et al., 2017).

CONCLUSION

Here we used shotgun sequencing to offer a detailed characterisation of the microbiota composition early after weaning in piglets medicated with in-feed ZnO, antibiotics and medication-free. As it occurs in real conditions in commercial farms, variability between batches is reflected across the three trials performed in this study. ZnO and apramycin modulate the microbiota composition and functionality, irrespective of batch and cleaning protocols, enabling a better bacterial carbohydrate utilization and amino acids synthesis, generating a different relative abundance pattern that tends to resemble a more mature microbiota. Future preventive

measures to control PWD should involve strategies to enhance the establishment and growth of beneficial gut inhabitants that outcompete potential pathogens.

MATERIALS AND METHODS

Experimental design. Three trials were carried out to study the role of in-feed ZnO and antibiotics, in combination with different cleaning protocols, on the faecal microbiota of piglets one week after weaning. The three experiments conducted in this study were carried out in the Teagasc Pig Research Facility and were licensed by Teagasc Animal Ethics Committee. This research facility was established in 2016 and has since been free of the main infectious diseases affecting pig farms (i.e. PRRS, enzootic pneumonia, pleuropneumonia, swine flu, ileitis, dysentery, oedema disease and streptococcal meningitis). The animals are regularly vaccinated for those diseases present in the herd (*Erysipelas*, parvovirus, porcine circovirus and neonatal *E. coli* and *Clostridium* spp.) and therapeutic zinc oxide and in-feed antibiotics are not used regularly. Piglets were weaned at 4 weeks of age in all the trials and moved to the experimental rooms. The treatment groups were balance by weight and the pigs were weighted at pen level on day 0 and 7 post-weaning. The rooms were equipped with fully slatted plastic floors with automatic environmental control, each pen having a single space wet-dry feeder and a nipple drinker. Animals used in this study were Danish Duroc × (Large White × Landrace). Pigs were fed a pelleted starter diet in dry form that met commercial nutritional requirements (Table 2). Feed was provided in bags and intake was recorded at pen level by weighing the bags weekly. Trial 1 studied the influence of the dietary treatments (Control diet: Ct, Control diet + 3000ppm of ZnO: Zn; Control diet + 100ppm of Apramycin as Apralan G200: Ab). Trials 2 and 3 studied the same in-feed treatments in combination with different cleaning protocols (Table 1) for the rooms that were part of another study (Misra et al., 2020). A detailed description of each trial is provided further below.

In trial 1, 264 piglets (8.3 ± 1.22 kg) from 24 litters were weaned and allocated into 24 different pens in the same room. The room was previously pre-soaked, power-washed, disinfected and left to dry as per standard practice in the farm (Table 1). Pens were allocated to the three dietary

treatments (Ct, Zn or Ab; n = 8 pens per treatment) balanced by pen weight and pigs were fed the treatment for 1 week after weaning.

In trial 2, 180 piglets (8.5 ± 0.80 kg) from 18 litters were weaned and allocated into two rooms with nine pens each. Before introducing the pigs into the rooms, the rooms were cleaned with a substandard or optimum protocol. In the substandard protocol, pre-soaking and drying were not carried out and in the optimum protocol, an extra step using detergent was added (Table 1). Within each room, pens were randomly allocated to one of the three dietary treatments resulting in 3 pens per diet per room balanced by pen weight (Ct, Zn or Ab; n = 6 pens per treatment, 3 in each room).

In trial 3, 324 piglets (8.6 ± 1.26 kg) from 27 litters were weaned and allocated into three rooms with nine pens each. Before introducing the pigs into the rooms, the rooms were either not-cleaned or cleaned with a substandard or standard protocol. For this trial, the substandard did not include disinfection (Table 1). Within each room, pens were randomly allocated to one of the three dietary treatments, resulting in 3 pens per diet per room balanced by pen weight (Ct, Zn or Ab; n = 9 pens per treatment, 3 in each room).

Chapter 2. Study I. Table 2. Ingredients and nutrient composition of the diet used in the three trials in this study on an as-fed basis

Dietary constituent	Amount (%)
Maize	30.0
Soybean meal 48	18.7
Whey permeate	15.0
Wheat	10.0
Full fat soy	7.00
Barley	6.74
Skimmed milk	5.00
Vegetable Oil	3.85
Calcium carbonate	0.80
Dicalcium phosphate (anhydrous)	0.70
Sodium chloride	0.30
L-Lysine HCl	0.672
L-Threonine	0.342
DL-Methionine	0.318
L-Tryptophan	0.127
L-Valine	0.126
Phytase (5000 FTU/g)[a]	0.01
Vitamin and trace mineral mix[b]	0.30
Calculated composition (% as fed or as specified)	
Dry Matter	89.44
Net Energy (MJ/kg)	10.95
Crude Protein	19.00
Standardized Ileal Digestible Lysine	1.41
Fat	6.84
Neutral Detergent Fiber	8.06
Calcium	0.77
Digestible Phosphorus	0.42

[a]FTU, Phytase units.

[b]Provided per each kg of feed: 400 mg Copper sulphate, 450 mg Ferrous sulphate monohydrate, 60 mg Manganese oxide, 150 mg Zinc oxide, 1 mg Potassium iodate, 0.6mg Sodium selenite, 6 MIU Vitamin A, 1 MIU Vitamin D_3, 100 MIU Vitamin E, 4 mg Vitamin K, 15 mg Vitamin B_{12}, 2 mg Riboflavin, 12 mg Nicotinic acid, 10 mg Pantothenic acid, 2 mg Vitamin B_1, 3 mg Vitamin B_6.

Sample collection, DNA extraction and library preparation. At 7 days post-weaning, one random freshly voided faecal sample from one pig per pen replicate was collected, transferred to 1.5mL microcentrifuge tube and straight stored at -80° C until processed. The samples were collected using a 140x7mm conical steel spatula avoiding the part in direct contact with the floor and a new sterile spatula was used for each sample. The DNA from the faecal samples was extracted using the QIAamp PowerFecal Pro DNA Kit (Qiagen, Crawley, West Sussex, UK) following the manufacturer's instructions. A Qubit fluorimeter (Qubit 3, Invitrogen) was used to determine the total DNA concentration. Paired-end sequencing libraries were prepared from the extracted DNA using the Illumina Nextera XT Library Preparation Kit (Illumina Inc., San Diego, CA) followed by sequencing on the Illumina NextSeq 500 platform using high-output chemistry (2 × 150 bp) according to the manufacturer's instructions. Library size from each sample was assessed on an Agilent Technology 21000 Bioanalyzer using a High Sensitivity DNA chip.

Bioinformatics analysis. Pre-processing of raw reads by sequence quality and length was performed with PRINSEQ-Lite v0.20.4 (Schmieder and Edwards., 2011). A mean quality lower than Q25 in a 10 base pair sliding window was the criteria used for trimming low quality reads at the 3' -end. A minimum length of 150 base pairs was ensured for all reads. The Illumina sequences clean were screened against the Pig reference genome (Sus scrofa UCSC) downloaded from Illumina iGenomes (https://support.illumina.com/sequencing/sequencing_software/igenome.html, 2019) to remove host reads using Bowtie2 (version 2.2) (Langmead and Salzberg., 2012) using the default values, in order to identify and remove host DNA sequences and reads derived from human DNA contamination. The unmapped reads were then used for the downstream analysis.

Read duplicates were removed using the Picard MarkDuplicates tool (https://broadinstitute.github.io/picard/, 2016) to create fastq files with unique reads only. Afterwards, reads were subjected to a further quality-filtering step. In brief, sequences were

trimmed for low quality score using a modified version of the script trimBWAstyle.pl that works directly from BAM files (TrimBWAstyle.usingBam.pl, 2010; https://github.com/genome/genome/blob/master/lib/perl/Genome/Site/TGI/Hmp/HmpSraProcess/trimBWAstyle.usingBam.pl). The script was used to trim off bases with a quality value of three or lower. This threshold was chosen to delete all the bases with an uncertain quality, as defined by Illumina's EAMMS (End Anchored Max Scoring Segments) filter.

The analysis of the microbial composition was carried out using the Kraken2 species classifier (Wood et al., 2019). Functional profiles were assigned using SUPER-FOCUS (Silva et al., 2016). Metagenome assembly was performed using MEGAHIT (Li et al., 2015).

Statistical Analysis. Analyses were carried out in R v4.0.2 separately for each trial, studying differences between diets and cleaning protocols. Average daily gain, average daily feed intake and feed conversion rate were calculated and differences between treatments were analysed using general linear models. Alpha and beta diversities were both computed at the species level using the R package vegan v2.5-7 (Oksanen et al., 2020). For alpha diversity estimation, Species richness, Chao1, Simpson, and Shannon indices of diversity were calculated. Statistical differences in alpha diversity indexes were tested, after confirming their normal distribution, with ANOVA and pairwise compared with Tukey (car v3.0.10 (Fox and Weisberg., 2019), multcompView v0.1.8 (Graves et al., 2019) and lsmeans v2.30.0 (Length., 2016) R packages) or by Kruskal-Wallis and pairwise tested with Wilcoxon test (stats v4.0.2 (R Core Team., 2020) R package), when data did not follow a normal distribution. Beta diversity and ordination of samples were performed by PCoA and NMDS of previously calculated Bray-Curtis distances between samples. Within groups dispersion was calculated by 'betadisper' function and tested with ANOVA. The separation between groups was tested with PERMANOVA (*adonis2* and pairwise adonis (Martinez Arbizu., 2020)). Factors and species influencing the ordination were assessed by linear models fitting on the ordination results (*envfit* function in Vegan R package). All p-values were adjusted by Benjamini-Hochberj (BH) approach. For fitting species

in ordination space, taxa were filtered, removing those species not present in at least 30% of samples and with less than a minimum threshold of total relative abundance of 0.005%. To produce a neat representation of fitted species on the ordination graphic, species of trial 3 were subjected to a filter of 0.008% of total relative abundance.

Bacteria and function abundance analyses among treatments were performed using Linear Discriminant Analysis Effect Size (LDA LEfSE (Segata et al., 2011)), with an alpha cut-off of 0.05 and a LDA threshold of 3, selecting the strictest analysis (all against all), and treatments as classes. Species explaining differences between classes were determined by LEfSE using Kruskal-Wallis test ($P < 0.05$) followed by linear discriminant analysis. Cladogram of dietary treatment associated taxa was produced using Graphlan (Asnicar et al., 2015). Core microbiomes of each dietary treatment group were calculated for a minimum threshold of the abundance of 1% in at least 70% of samples of each group using the microbiome R package (Lahti and Shetty., 2019). Venn diagrams were built using “venn” and “get.venn.partitions” functions from gplots v3.1.1 and VennDiagram v1.6.20 packages, respectively (Warnes et al., 2020; Chen., 2018). Plots were built using ggplot2 v3.3.3 and pheatmap v1.0.12 (Wickham., 2016; Kolde., 2019). Figures were produced using inkscape software v1.0.2 (Inkscape Project., 2020).

Data availability. The full data sets have been submitted to NCBI Sequence Read Archive (SRA) and is available at https://www.ncbi.nlm.nih.gov/bioproject/PRJNA821641 under Bioproject accession PRJNA821641.

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Authors Contributions: JMOS participated in the study design, samples collection and processing, data analysis and manuscript writing. EGM participated in study design, samples collection and manuscript correction. RCR performed the bioinformatic analysis, FC participated in data sequencing and final version of manuscript, PDC participated in the data sequencing and manuscript revision. JJG participated in study design, data analysis and manuscript revision. HA participated in data analysis, manuscript writing and revision. All authors revised and approved the final version of the manuscript.

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Chapter 3. Study II: Comparing the microbiome of post-weaning pigs in farms using, or not using, in-feed zinc oxide and antibiotics

Comparing the microbiome of post-weaning pigs in farms using, or not using, in-feed zinc oxide and antibiotics

Juan M. Ortiz Sanjuán[a,b], Edgar G. Manzanilla[a,c], Raúl Cabrera-Rubio[d,e], Fiona Crispie[d,e], Paul D. Cotter[d,e,f], Juan J. Garrido[b], Daniel Ekhlas[a,c], Héctor Argüello[g].

INSTITUTIONS

[a]Pig Development Department, Teagasc Grassland Research and Innovation Centre, Moorepark, Fermoy, Co. Cork, Ireland.
[b]Grupo de Genómica y Mejora Animal, Departamento de Genética, Facultad de Veterinaria, Universidad de Córdoba, Córdoba, Spain.
[c]School of Veterinary Medicine, University College Dublin, Dublin, Ireland.
[d]Teagasc Food Research Centre, Moorepark, Fermoy, Co. Cork, Ireland.
[e]APC Microbiome Ireland, Cork, Ireland.
[f]VistaMilk SFI Research Centre, Fermoy, Co. Cork, Ireland.
[g]Animal Health Department, Veterinary Faculty, Universidad de León, León, Spain.

#Address correspondence to Juan M. Ortiz Sanjuán, jmos11.1993@gmail.com

Comparing the microbiome of post-weaning pigs in farms using, or not using, in-feed zinc oxide and antibiotics

Abstract

Post-weaning diarrhoea (PWD) is a multifactorial disease that affects piglets at weaning, contributing to productive and economic losses. Its control includes the use of in-feed prophylactic antibiotics and therapeutic zinc oxide (ZnO), treatments that, since 2022, are no longer available in the European Union. This study uses shotgun metagenomic sequencing to investigate the environmental and faecal microbiome on 10 farms using (Treated) or not using (ZnO-free) in-feed antibiotics and ZnO during the first 14 days post-weaning (dpw). Clean pen environmental samples were collected on the day of weaning (0 dpw), and faecal samples at 0, 7 and 14dpw. Microbiome structure and taxonomy was impacted by microbiome maturation and transition after weaning rather than the environment. Treatment with antibiotic and ZnO showed no effects in diversity indexes while analysis of microbiota taxonomy and functionality revealed increased abundance of taxa such as *Phascolarctobacterium succinatutens* or *Prevotella* spp.in Treated farms, and *Megasphaera elsdenii* and *Escherichia coli along with both bacteria-associated functions*, more abundant in ZnO-free farms. The analysis of diarrhoea samples revealed that the treatment favoured an easier microbiota transition from 0dpw to 14dpw in Treated farms, being more similar to healthy animals, compared to diarrhoea from ZnO-free farms, which were linked in composition to 0dpw. Altogether, the results highlight the beneficial role of ZnO and antibiotics in PWD from the perspective of the maturation of microbiota maturation and preventing the outgrowth of pathogens such as pathogenic *E. coli*.

Key words: piglets, post-weaning diarrhoea, environment, microbiome, zinc oxide.

Introduction

Post-weaning diarrhoea (PWD) is a multifactorial disease affecting piglets at weaning. It threatens piglet survival at the most critical stage of their life, and often requires antimicrobial treatment (Luppi, 2017; Fairbrother and Nadeau, 2019). Weaning in commercial farms is associated with numerous challenges such as early age mother separation, sudden transition from milk to solid feed or environmental stressing factors. This can result in immunological, physiological and microbial imbalances that create a window of opportunity for enteric pathogens, either primary or opportunistic, which invade and/or overgrow in the gut (Rhouma et al., 2017; Bonetti et al., 2021). Enterotoxigenic *Escherichia coli* (ETEC) is the main aetiological agent associated to PWD (Rhouma et al., 2017; Fairbrother and Nadeau, 2019). Prevention and control of PWD has been based on the use of prophylactic and metaphylactic in-feed antibiotics and/or therapeutic zinc oxide (ZnO) during the past decades (Poulsen, 1995; Sales, 2013). However, the rise of antimicrobial resistance and soil pollution with zinc (Zn) has forced a severe restriction in antibiotic use and a ban on ZnO for public health and environmental reasons, respectively, in the European Union, both implemented in 2022 (EC(2019/4); EC(2019/6); Standing Committee on Veterinary Medicinal Products, 2017). In contrast to the well-described mechanisms of action for antibiotics, the exact mechanism of action of ZnO in PWD control is not completely understood yet, despite its proven efficacy. Zinc participates as a co-factor for multiple enzymes and is required for multiple biochemical reactions, both for eukaryote and prokaryote organisms (Wątły et al., 2016; Sloup et al., 2017). Within the intestine, zinc is involved in different physiological processes such as digestion and immune response, which finally impact animal performance (Sales, 2013; Bonetti et al., 2021). In addition, zinc has antimicrobial activity against certain groups of bacteria (Söderberg et al., 1990; Pasquet et al., 2014). With respect to the broader importance of microorganisms, the role of the gut microbiome in contributing to and controlling enteric diseases is receiving ever more attention. Indeed, the gut microbiota may play a pivotal role in providing colonization resistance against

pathogens, occupying niches, competing for substrates or producing inhibitory molecules such as bacteriocins (Spees et al., 2013; Dou et al., 2017). Antibiotics and, to a lesser extent ZnO, have been reported to modify the microbiome composition (Li et al., 2020; Pieper et al., 2020). Despite the potential microbial shift observed in these studies, we are still far from developing a comprehensive understanding about the effects of the regular use of antibiotics and ZnO in the environment of commercial farms and in the pig gut microbiome from the perspective of PWD. Undoubtedly, further research in this area can provide relevant insights relating to the microbial changes that occur at weaning, how the antibiotic and ZnO treatments shape the microbiome and its potential impacts on PWD outcome, offering new research lines to explore alternatives to in-feed antibiotics and ZnO use by modulating microbial populations.

The present study provides an in-depth analysis of microbiome composition and functionality in the first 2 weeks post-weaning, both on farms using in-feed prophylactic antibiotic and ZnO (Treated) and farms free of these treatments (ZnO-free). Through the analysis of faecal composition, the on-farm environment and composition of microbiome in PWD diarrhoea samples, we extend the knowledge relating to this critical period of the pig's life.

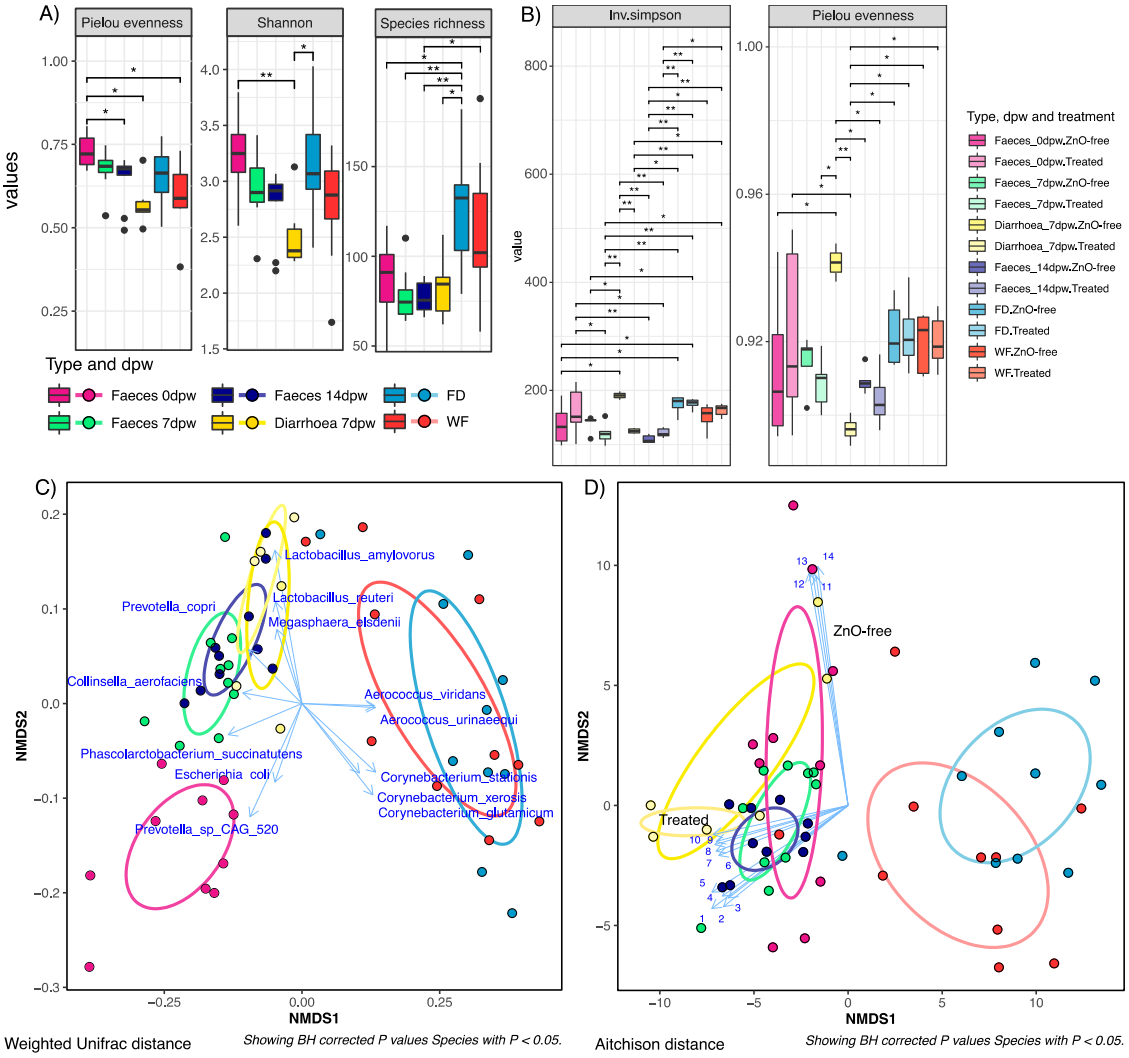
Results

The porcine gut microbiome shifts during weaning and, in cases of PWD, is impacted by antibiotic and ZnO treatment

Results obtained from computing richness (Species richness) and diversity (Shannon, Inverse Simpson, Pielou) did not reveal any difference by the treatment (ZnO-free vs Treated) or farm (farms 1 to 10) variables, while there were differences when data was analysed by sample-type and days-postweaning (dpw) factors. Figure 1A summarises the α-diversity taxonomic results while functional analyses are shown in Figure 1B. Faecal diversity evenness measured by Pielou index decreased from weaning (0dpw) to the last sampling performed at 14dpw ($P < 0.05$). Interestingly, the lowest diversity value across the three indexes analysed corresponded to the Diarrhoea 7dpw sample, which was lower than Faeces 0dpw in both Pielou evenness and

Shannon indexes (P = 0.022 and P = 0.009, respectively). Diversity values in environmental samples collected at weaning revealed a large species richness in FD (Feeder-Drinker) and to a lower extent in WF (Wall-Floor). While taxonomic α-diversity was not impacted by treatment, the analysis of diversity in metabolic pathways revealed differences between Treated vs ZnO-free samples that were more evident in Diarrhoea 7dpw samples. These functional differences were also obvious with respect to sample-type and days postweaning variables (Figure 1B).

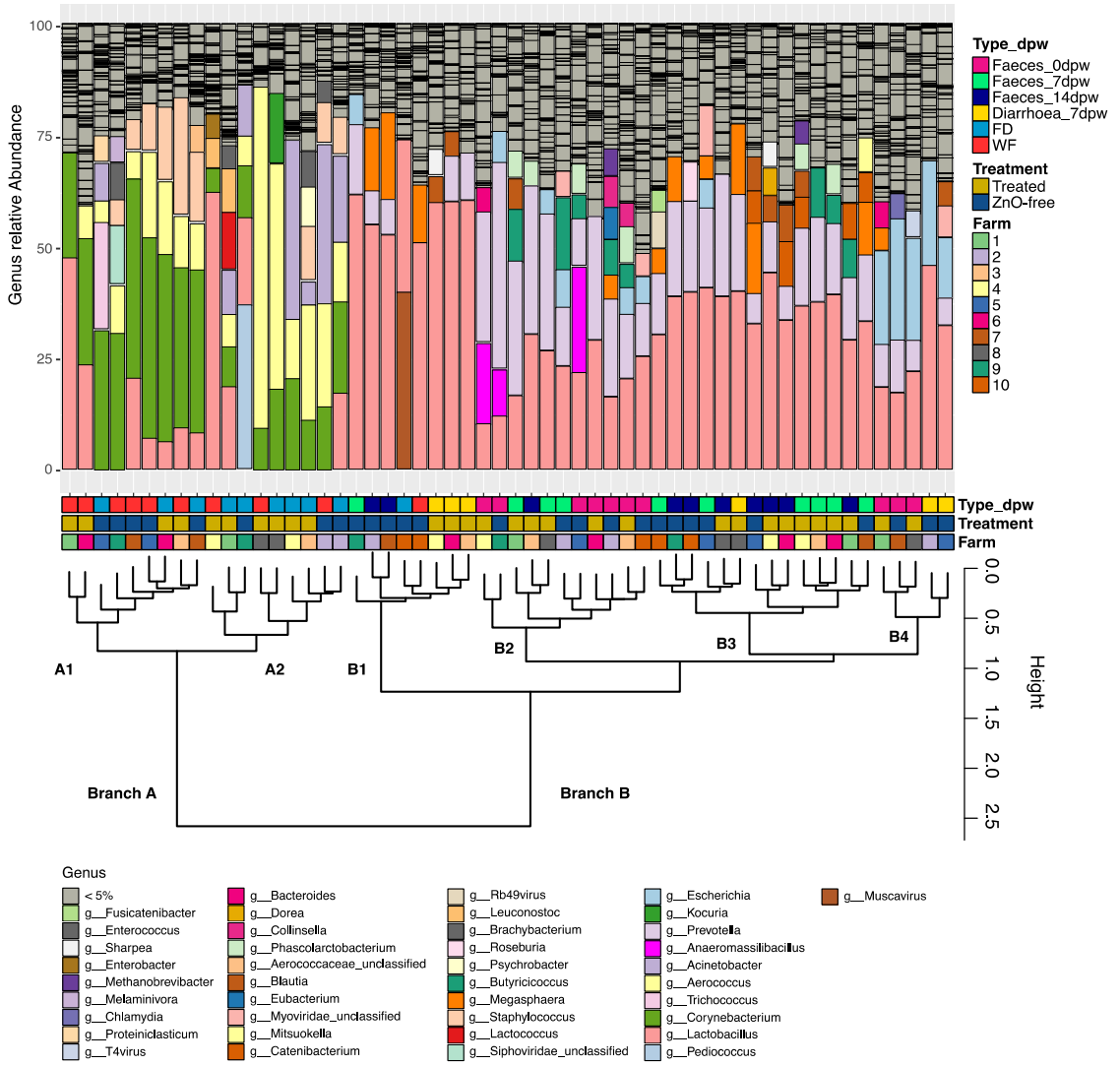
Similar to the results observed in α-diversity, treatment or farm did not impact the ordination of the microbiomes (Supplementary table 1). Both sample-type and days post-weaning variables contributed to microbiome structure (Supplementary table 2, $P < 0.05$), both at species composition and functional levels, showing marked diversity differences between faecal and environmental samples (Figure 1C and 1D). Ordination analyses revealed a clear separation on the basis of treatment group (Treated vs ZnO-free) in Diarrhoea 7dpw samples, which were more apparent in with respect to the functional than taxonomic data. Species and pathways that drive microbiome ordination are depicted in Figures 1C and 1D.



Chapter 3. Study II. Figure 1. Analysis of microbiome diversity by type of sample, day post-weaning and treatment. A) Results of alpha diversity: Pielou evenness, Shannon and Species richness diversity indices. (* P < 0.05; ** P < 0.01). B) Alpha diversity values by treatment sample type and day post-weaning variables. C) Samples ordination using Weighted Unifrac distances at species level and ploted in a NMDS. D) Samples ordination based on functional pathways using Aitchison distances. Blue arrows display the top 15 species and pathways with the highest mean abundance returned by "envfit" model, influencing the ordination of samples (Arrows showing BH p.adjusted significant species; Arrows length shows the strength of each specie influencing the ordination of samples). Pathways fitted onto ordination are indicated as numbers, ordered according to its NMDS coordinates. 1. Nucleoside and Nucleotide Biosynthesis: *Lactobacillus amylovorus*, 2. Cell Structure Biosynthesis: *L. amylovorus*, 3. Carbohydrate Biosynthesis: *L. amylovorus*, 4. Cofactor, Carrier, and Vitamin Biosynthesis: *L. amylovorus*, 5. Amino Acid Biosynthesis: *L. amylovorus*, 6. Fatty Acid and Lipid Biosynthesis: *Lactobacillus reuteri*, 7. Cell Structure Biosynthesis: *L. reuteri*, 8. Amino Acid Biosynthesis: *L. reuteri*, 9. Nucleoside and Nucleotide Biosynthesis: *L. reuteri*, 10. Cofactor, Carrier, and Vitamin Biosynthesis: *L. reuteri*, 11. Amino Acid Biosynthesis: *E. coli*, 12. Fatty Acid and Lipid Biosynthesis: *E. coli*, 13. Nucleoside and Nucleotide Biosynthesis: *E. coli*, 14. Cofactor, Carrier, and Vitamin Biosynthesis: *E. coli*. Ellipses drawn on Figures C and D represent each type-dpw level, along with the Diarrhoea 7dpw ZnO-free samples, with their shape being defined by the covariance within each group. Taxonomic and functional profiling of sequences was performed using Metaphlan3 and HUManN3 respectively.

Differences in microbial structure composition between environmental and faecal samples are associated with the most representative genera, while species compositon further differenciates between environmental categories and sampling time-points in faeces

We further explored the sample metagenome structure on the basis of their similarity in microbial abundance and the species weighted unifrac phylogenetic distance (Figure 2, Supplementary figure S1). The analysis clearly split samples in two branches, i.e., consisting of environmental (branch A) and faecal (branch B) samples, respectively. Branch A composition, similarly to the ordination results (Figure 1C), was impacted by the genera *Aerococcu*s and *Corynebacterium*. This branch was split into two sub-branches; i.e., sub-branch A1, dominated by species of the genera *Lactobacillus*, *Corynebacterium*, *Aerococcus* and *Staphylococcus* and where six of the nine samples were from WF and sub-branch A2, with *Aerococcus*, *Corynebacterium* and *Acinetobacter* as the main representatives, with lower *Lactobacillus* abundance and including mainly FD samples (six of nine). Most of samples in branch B were faecal samples and further subclustering in this branch was influenced by the species present rather than differences in genera composition and clearly impacted by the time point factor (Supplementary figure S1). Thus, Faeces 0dpw exhibited higher similarity with part of Faeces 7dpw (branch B2), and Diarrhoea 7dpw samples from ZnO-free group (branch B4). The rest Faeces 7dpw and Faeces 14dpw were allocated in the branch B3.

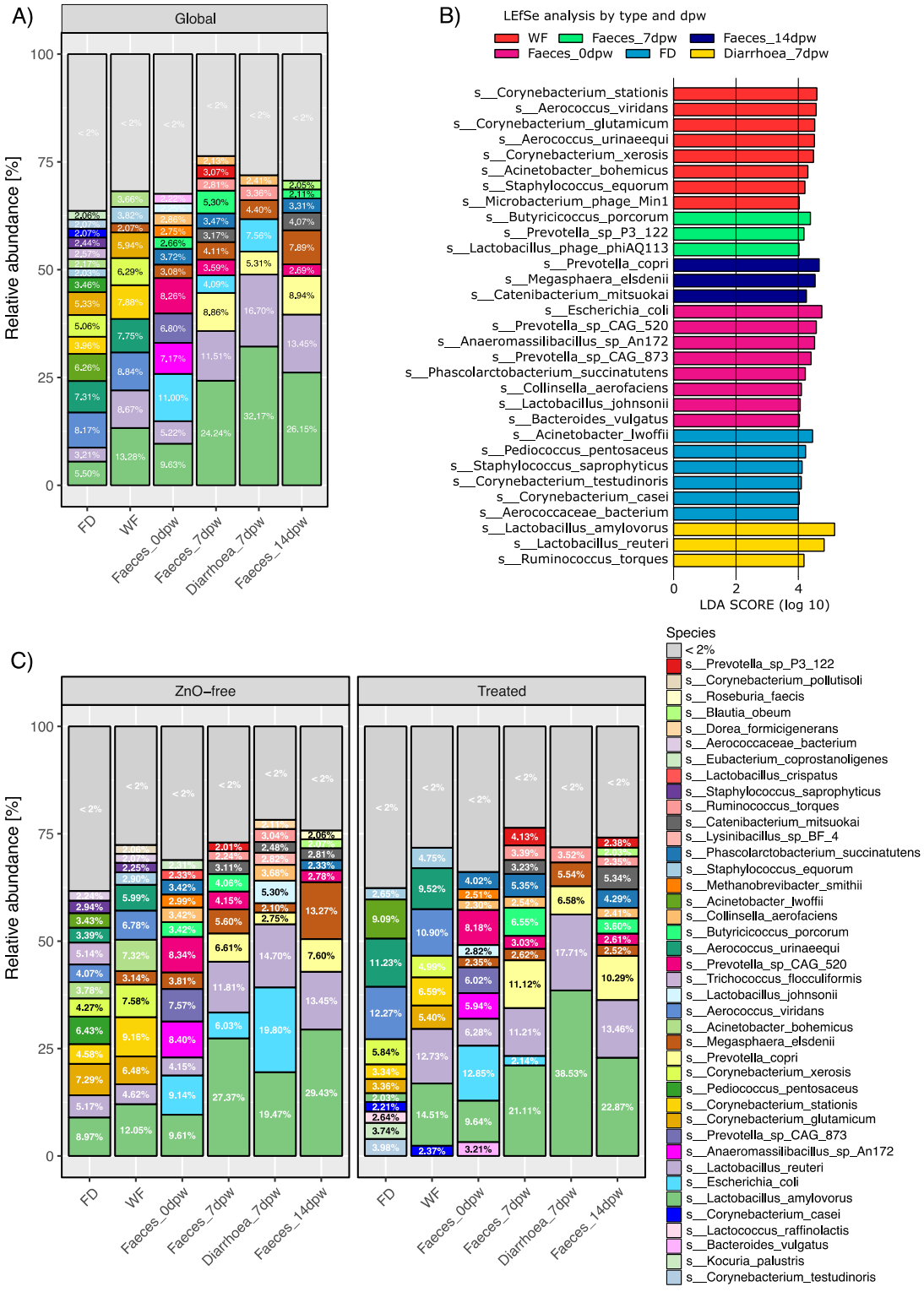


Chapter 3. Study II. Figure 2. Stacked bar plot of the relative abundance of the main genera in each sample analysed from 10 commercial farms. Profiles of samples are ordered by Ward clustering of the squared Weighted Unifrac distances between samples. Cluster dendrogram represents the similarity between samples regarding its microbial composition. Variables information in each sample (from lower to upper level: Farm, Treatment, Type-dpw) are indicated in the coloured squares below the bars. Taxonomic identification of sequences was performed using Metaphlan3.

Piglets' microbiome composition at weaning differs from the environmental microbiome and evolves such that particular species dominate

Figure 3A summarises the mean relative abundance of the most representative species in each sample type collected in the study. Environmental samples and faeces collected on the same day (0dpw) showed lower dominace of species than Faeces 7dpw (including Diarrhoea 7dpw) and Faeces at 14dpw. Focusing on faecal results, within the first 14 days after weaning, we

observed an increase in abundace of the dominant species, shifts in species from the same genus and the rise of anaerobes. Thus, *Lactobacillus amylovorus* and *Lactobacillus reuteri*, the two dominant species in faecal samples, increased in abundance ($P < 0.05$) through the post-weaning period (Supplementary table 3). Faeces 0dpw showed an even species composition, with the exclusive presence of *Prevotella* sp. CAG 873 and *Anaeromassilibacilus sp. An172* (both with relative abundance values over 2%) and higher abundance of *Prevotella* sp. CAG 520 (Figure 3B). Other species associated with 0dpw faeces were *Escherichia coli, Phascolarctobacterium succinatutens, Collinsella aerofaciens, Lactobacillus johnsonii* and *Bacteroides vulgatus*. In subsequent samplings, 7dpw and 14dpw, the abundance of *Prevotella* sp. CAG 873 decreased, while the abundance of two different species of *Prevotella* increased, *Prevotella* sp. P3 122 at 7dpw and *Prevotella copri* at 14dpw (Supplementary table 3). Two species exhibited higher abundance by linear discriminant analyses at 7dpw; *Butyricicoccus porcorum* and the virus Lactobacillus phage phiAQ133 and *Catenibacterium mitsuokai* at 14dpw. *Megasphaera elsdenii* increased in abundance towards the weaning period with higher abundance at 14dpw (Figure 3B), sampling time at which we also observed higher abundance of *C. mitsuokai*. While *M. elsdenii* relative abundance in piglets faecal microbiome was 3.08% at 0dpw, the value reached 7.89% at 14dpw. In contrast, we observed the opposite trend for *E. coli,* from 11% at 0dpw to an abundance below 2% at 14dpw. Diarrhoea 7dpw samples showed a pattern which resembled those of 7dpw faeces samples, with increased abundance of *E. coli*, and higher abundance of already dominant species such as *L. amylovorus*, *L. reuteri* and *Ruminococcus torques*. Both environmental samples showed similar patterns of abundance, with a high presence of aerobic species as *Aerococcus spp.* (*A. viridans, A. urinaeequi*) and *Corynebacterium spp* (*C. stationis, C. xerosis, C. glutamicum*).



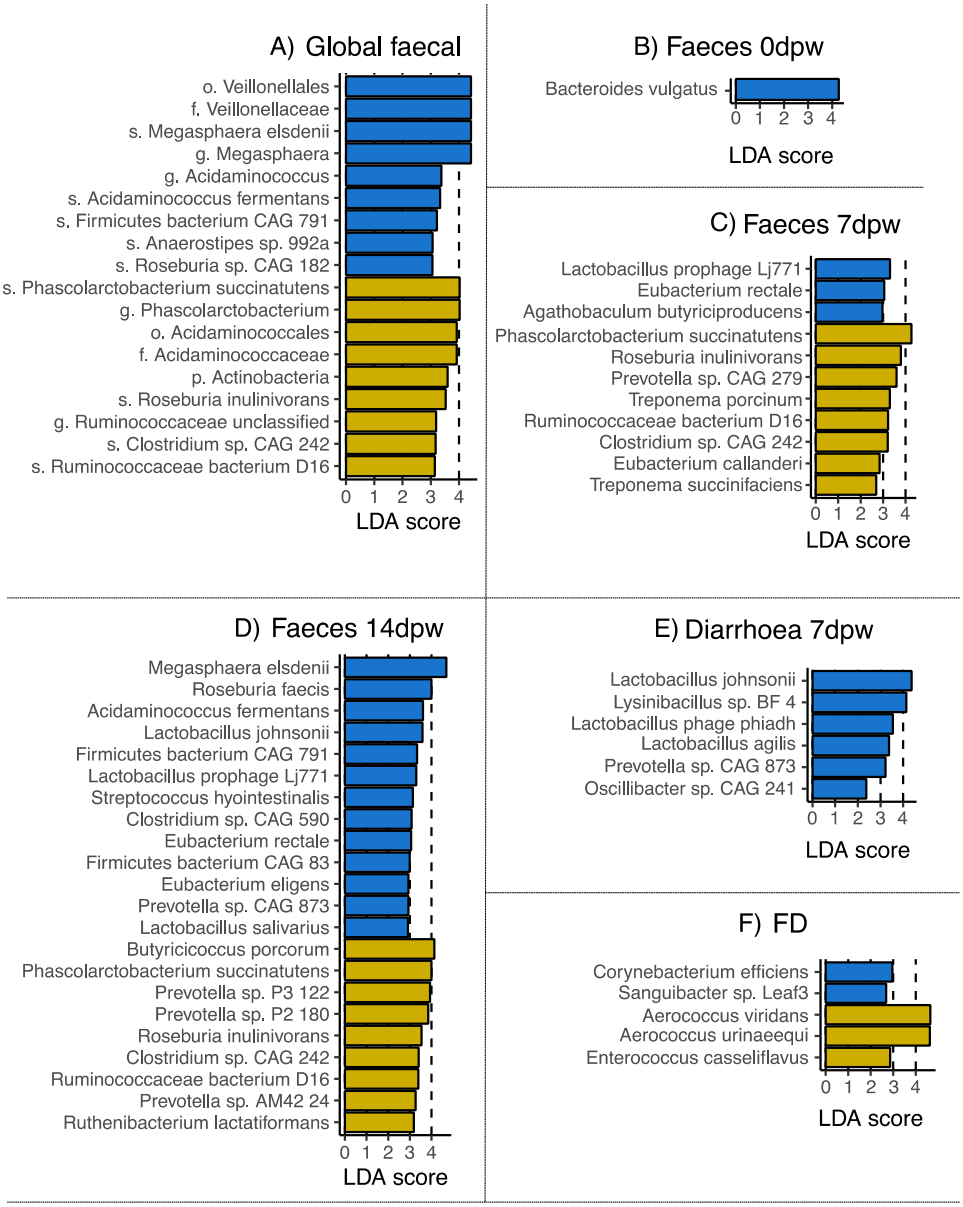
Chapter 3. Study II. Figure 3. Microbiome composition in environmental and faecal samples from weaning pigs from 10 commercial farms. (A). Mean relative abundance of the most representative species in each type-dpw group. (B) Differences in species abundance, returned by LEfSe analysis, most likely explaining the differences among type and day post-weaning variables. (C) Mean relative abundance of the main species in each type-dpw group splitted by treatment. * "Others", refers to those species accounting for less than 2 percent of abundance. Taxonomic identification of sequences was performed using Metaphlan3.

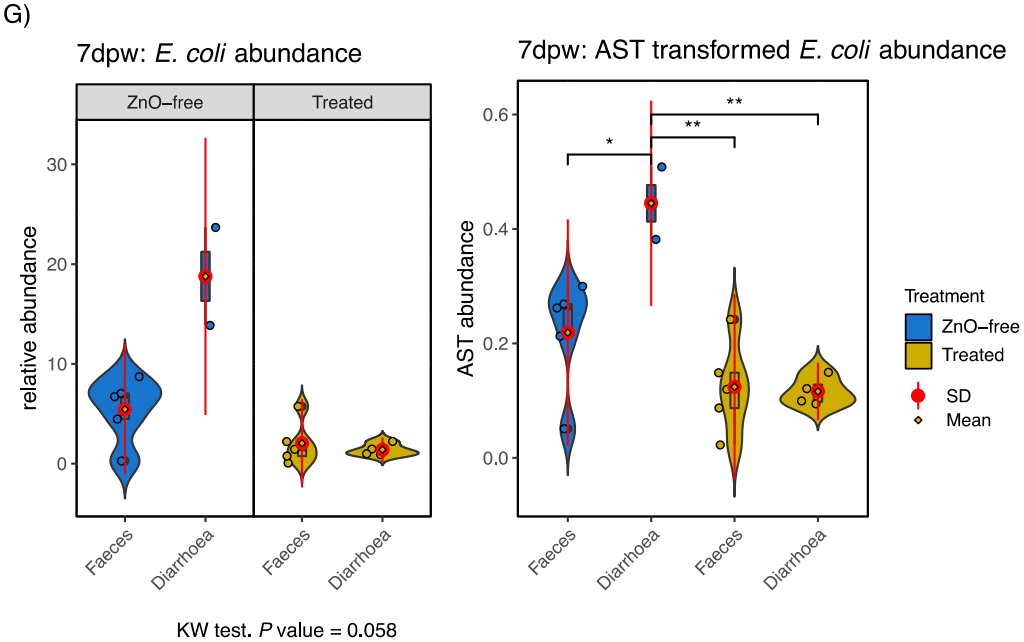
In-feed antibiotic and zinc oxide use impacts taxonomic microbial composition both in environments and animals

Taxonomic profiles at species level were compared according to treatment levels to compare their patterns of relative abundance (Figure 3C) and differential abundance analysis run by factors type and dpw (type-dpw) using LEfSe, revealed different species associated with treatment (Figure 4A). Overall, dominance of *Lactobacillus* spp. was not affected by the treatment and global analysis of species abundance by LDA LEfSe confirmed these differences among farms for *M. elsdenii* and *P. succinatutens* abundance. Analysis by dpw variable (figures 4B to 4D) showed that *B. vulgatus* was the unique species found to be more abundant in faeces from farms using in-feed antibiotics and ZnO at weaning, i.e., before the treatment began (Figure 4B). The species *P. succinatutens*, *Roseburia inulinivorans*, *Ruminococcaceae bacterium D16* and *Clostridium* sp. CAG 242 were also higher in abundance in faeces from treatment farms both at 7dpw and 14dpw (Figures 4C and 4D), whereas different species of *Prevotella spp.* were associated with faecal microbiomes in treated farms at different time points. In contrast, species more abundant in ZnO-free farms were *Eubacterium rectale* along with the virus Lactobacillus phage Lj711 in both faecal samples at 7dpw and 14dpw (Figure 4C and 4D); and *L. johnsonii* and *Prevotella* sp. CAG 873 in both Faeces 14dpw and Diarrhoea 7dpw (Figure 4D and 4E), along with *L. salivarius* and *L. agilis* in Faeces 14dpw and Diarrhoea 7dpw samples, respectively. Other species enriched in faeces from ZnO-free farms at 14dpw were *Eubacterium eligens*, *Clostridium* sp. CAG 590, *Streptococcus hyointestinalis*, *Acidaminococcus fermentans*, *Roseburia faecis* and *M. elsdenii.*

Analysis of *E. coli* abundance revealed a statistical tendency and a high within-group variability (Figure 4G). Analysis of variance stabilizing AST transformed *E. coli* abundance revealed greater *E. coli* abundance in Diarrhoea 7dpw samples from ZnO-free farms (Figure 4G). We observed a notable change in dominance of environmental species by treatment variable. Species associated to the treatment in FD microbiomes were *A. viridans* and *A. urinaeequii* and

Enterococcus casseliflavus, whereas *Sanguibacter sp Leaf3* and *Corynebacterium efficiens* were linked to ZnO-free farms FD (Figure 4F).


A) Global faecal
o. Veillonellales
f. Veillonellaceae
s. Megasphaera elsdenii
g. Megasphaera
g. Acidaminococcus
s. Acidaminococcus fermentans
s. Firmicutes bacterium CAG 791
s. Anaerostipes sp. 992a
s. Roseburia sp. CAG 182
s. Phascolarctobacterium succinatutens
g. Phascolarctobacterium
o. Acidaminococcales
f. Acidaminococcaceae
p. Actinobacteria
s. Roseburia inulinivorans
g. Ruminococcaceae unclassified
s. Clostridium sp. CAG 242
s. Ruminococcaceae bacterium D16
0 1 2 3 4
LDA score
B) Faeces 0dpw
Bacteroides vulgatus
0 1 2 3 4
LDA score
C) Faeces 7dpw
Lactobacillus prophage Lj771
Eubacterium rectale
Agathobaculum butyriciproducens
Phascolarctobacterium succinatutens
Roseburia inulinivorans
Prevotella sp. CAG 279
Treponema porcinum
Ruminococcaceae bacterium D16
Clostridium sp. CAG 242
Eubacterium callanderi
Treponema succinifaciens
0 1 2 3 4
LDA score
D) Faeces 14dpw
Megasphaera elsdenii
Roseburia faecis
Acidaminococcus fermentans
Lactobacillus johnsonii
Firmicutes bacterium CAG 791
Lactobacillus prophage Lj771
Streptococcus hyointestinalis
Clostridium sp. CAG 590
Eubacterium rectale
Firmicutes bacterium CAG 83
Eubacterium eligens
Prevotella sp. CAG 873
Lactobacillus salivarius
Butyricicoccus porcorum
Phascolarctobacterium succinatutens
Prevotella sp. P3 122
Prevotella sp. P2 180
Roseburia inulinivorans
Clostridium sp. CAG 242
Ruminococcaceae bacterium D16
Prevotella sp. AM42 24
Ruthenibacterium lactatiformans
0 1 2 3 4
LDA score
E) Diarrhoea 7dpw
Lactobacillus johnsonii
Lysinibacillus sp. BF 4
Lactobacillus phage phiadh
Lactobacillus agilis
Prevotella sp. CAG 873
Oscillibacter sp. CAG 241
0 1 2 3 4
LDA score
F) FD
Corynebacterium efficiens
Sanguibacter sp. Leaf3
Aerococcus viridans
Aerococcus urinaeequi
Enterococcus casseliflavus
0 1 2 3 4
LDA score


G)
7dpw: E. coli abundance
ZnO–free
Treated
relative abundance
0
10
20
30
Faeces
Diarrhoea
Faeces
Diarrhoea
KW test. P value = 0.058
7dpw: AST transformed E. coli abundance
AST abundance
0.0
0.2
0.4
0.6
*
**
**
Faeces
Diarrhoea
Faeces
Diarrhoea
Treatment
ZnO–free
Treated
SD
Mean

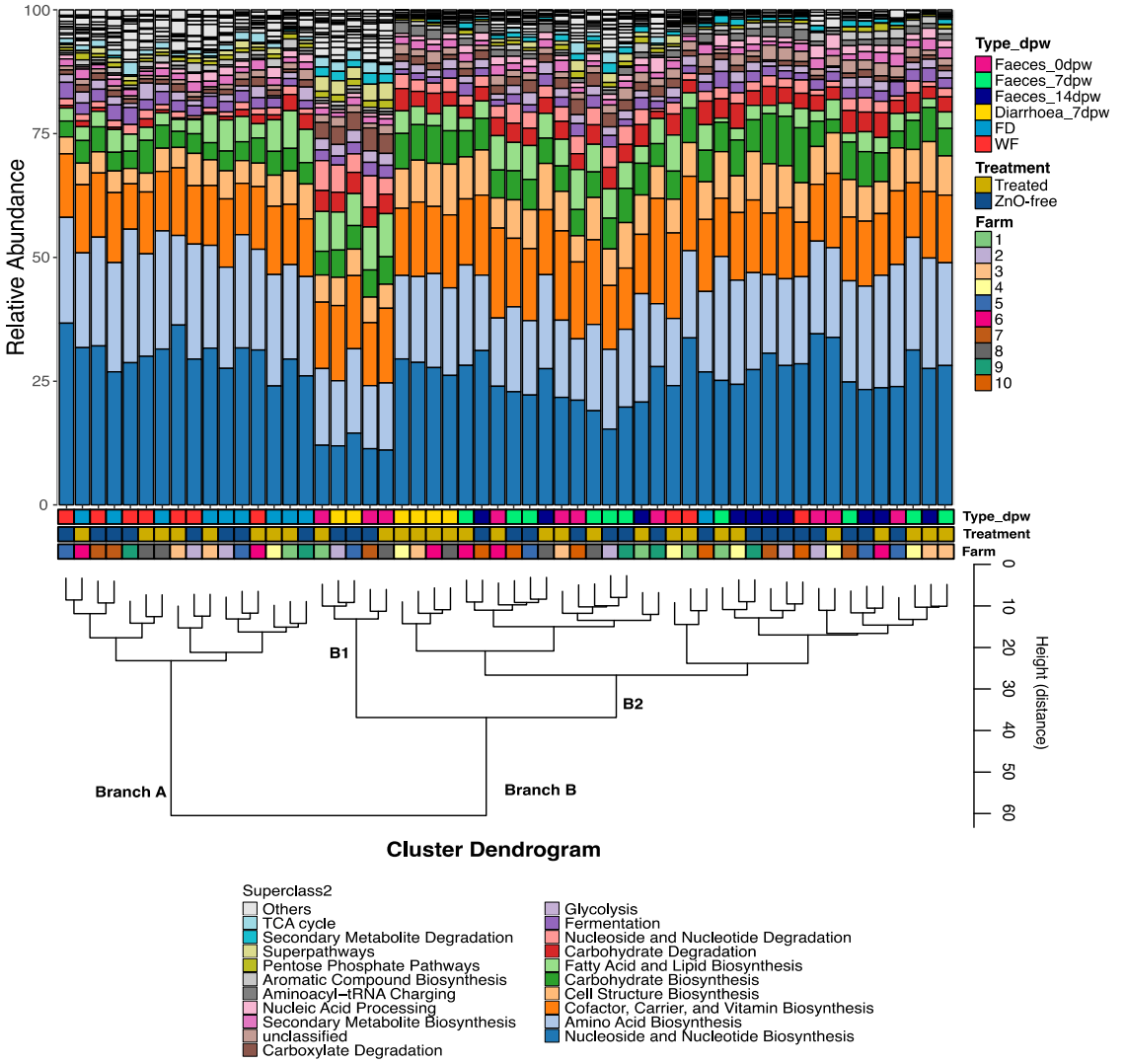
Chapter 3. Study II. Figure 4. Differences in species abundance, returned by LEfSe analysis, most likely explaining the differences among dietary treatments in each sample type and day post-weaning. (A) Global differences in species abundance returned by LEfSe by treatment. (B) Faeces 0dpw. (C) Faeces 7dpw. (D) Faeces 14dpw. (E) Diarrhoea 7dpw. (F) FD. Horizontal bars are coloured according to dietary treatment. (G) Relative abundance and Arcsine square root transfromed abundance of *Escherichia coli* in samples of faeces and diarrhoea coloured by treatment. Points represent samples' relative abundances values. Probability density curves at each values of abundance for each type of samples are depicted accross each range of abundance values of each type of sample. Boxplots summarizing relative abundance values are included within the density curves. The lower, medium, and upper horizontal box lines correspond to the first, second and third quartiles (the 25th, 50th and 75th percentiles). Upper and lower whiskers include the range of the upper and lower points within the 1.5 interquartile range. The standard deviation (SD) and mean value of each type of sample are depicted as a red line and an orange diamond. *P < 0.05, **P < 0.01, and ***P < 0.001, respectively. Taxonomic identification of sequences was performed using Metaphlan3.

In-feed antibiotics and zinc oxide treatment modifies the species contribution to functional profiles and dysbiosis status in diarrhoea samples from ZnO-free farms by aerobic processes triggered by *E. coli*

Analysis of theelative abundance of functional genes, grouped by superclass 2 level of MetaCyc metabolic pathways database, revealed the dominance of three functional superclasses, which accounted for more than 50% of the functional relative abundance in the faecal microbiome: (i) nucleoside and nucleotide byosynthesis, (ii) amino acid biosynthesis, and iii) cofactor, carrier and vitamin biosynthesis (Figure 5). The microbiome functional profiles, shown in Figure 5, are ordered according to Ward clustering using Aitchison distance. Most of the samples analysed were clustered by their environmental or faecal origin (branches A and B, respectively). We observed that in Faeces 0dpw or Diarrhoea 7dpw samples, functional abundance was ascribed to a lower number of species; whereas microbiome functions in Faeces 7dpw and Faeces 14dpw were built by a higher number of species (Supplementary Figure S2). Regarding the species contribution to each Superclass, the functional profile pattern was similar to the observed in species abundance (Supplementary Figure S2).

Supplementary figure S3 shows species significantly contributing to each superclass2 grouped metacyc pathways associated to the three factors under study, sample type, dpw and treatment. As already mentioned, the dominant functions were associated with different species to reflect

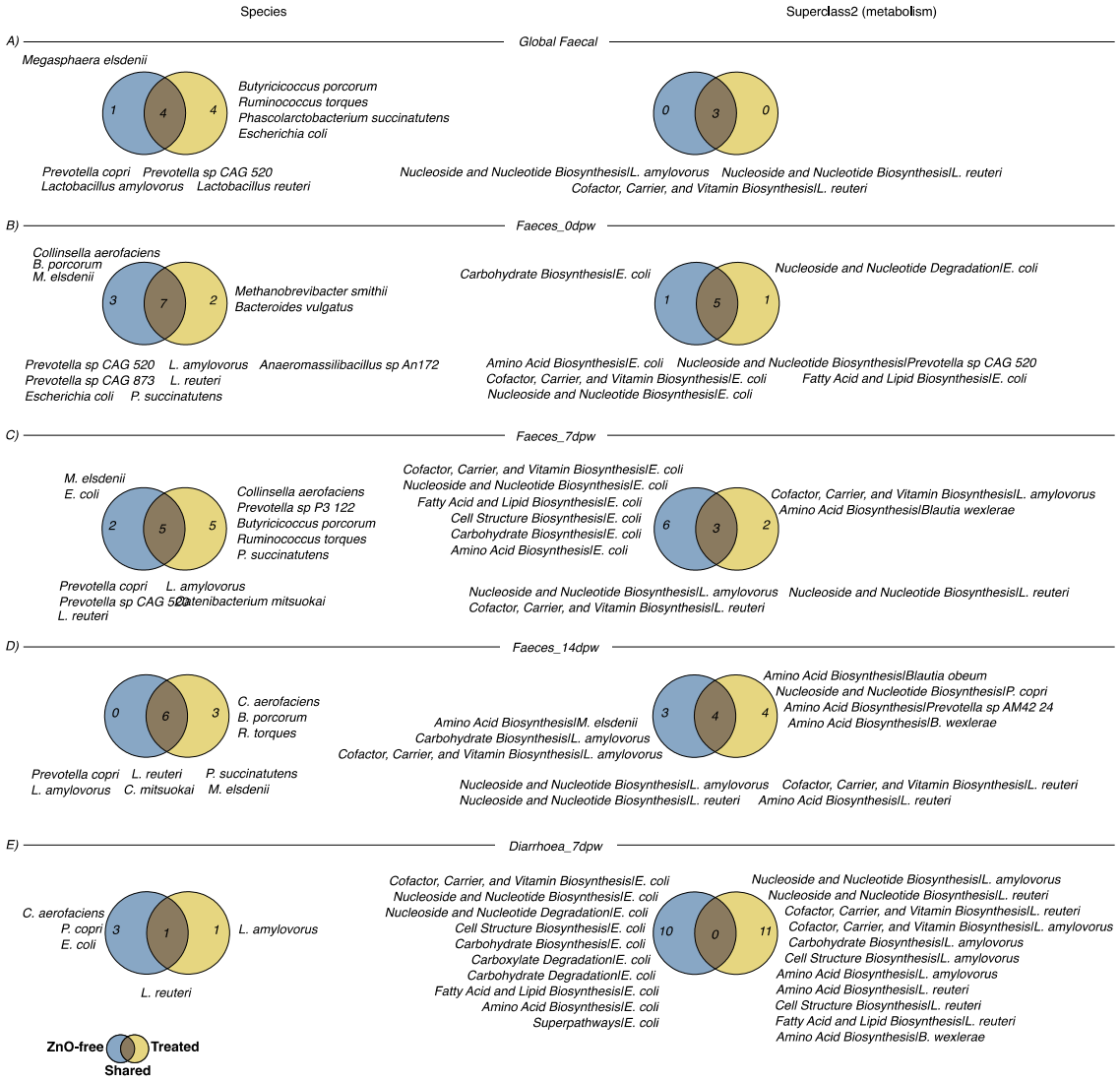
sample type, dpw and treatment. Among the results obtained, it is important to highlight that Diarrhoea 7dpw samples from ZnO-free farms were enriched in aerobic processes encoded by *E. coli*.



Chapter 3. Study II. Figure 5. Stacked bar plot of the relative abundance of the main metacyc superclass2 grouped pathways in environmental and faecal samples from weaned pigs. Samples are clustered by their functional abundance profile using Ward clustering and the squared Aitchison distances between samples. Cluster dendrogram represents the similarity between samples regarding its functional and species composition. Variables information in each sample (from lower to upper level: Farm, Treatment, Type and day post-weaning) are indicated in the coloured squares below the bars. Metabolic profiling was performed using HUMAnN3.

M. elsdenii and _E. coli_ are dominant contributors with respect to core composition and functionality on ZnO-free farms while treated farms exhibit a larger core taxa composition

The core microbiome species and core microbiome functions for Treated and ZnO-free farms was calculated in samples collected at different sampling time-points and also in diarrhoea samples. Unique and shared species and functions are depicted in Figure 6. Both microbial species and functional core microbiomes were largely impacted by in-feed antibiotic and ZnO inclusion. More specifically, global analysis revealed *M. elsdenii* as a species uniquely detect in ZnO-free animals, whereas those found only in treated animals were *Butyricicoccus porcorum*, *R. torques*, *P. succinatutens* and *E. coli*. Shared species between those corresponding to the *Prevotella* and *Lactobacillus* genera. When looking at each dpw, the presence and contribution of species to each function was highly influenced by treatment. The main species consistently found in ZnO-free farms were *M. elsdenii* and *E. coli*, particularly in Faeces 7dpw and Diarrhoea 7dpw, which functional cores clearly influenced by *E. coli*. In contrast, the cores species in samples from Treated farms at 7dpw were *C. aerofaciens*, *Prevotella sp. P3 122*, *R. torques*, and *P. succinatutens*; and functions were linked to *L. amylovorus* and *Blautia wexlerae*. At 14dpw, *P. succinatutens* and *M. eldenii* moved to the shared set between the Treated and ZnO-free. On this sampling day, shared functions were all performed by *Lactobacillus spp*, whereas functional core of ZnO-free treated animals included *M. elsdenii* (Amino acid biosynthesis) and two functions associated to *L. amylovorus*. Treated functional-core set included functions pertained by *Blautia spp* and *Prevotella spp*.



Chapter 3. Study II. Figure 6. Shared species and grouped metabolic pathways core microbiomes with a minimum abundance of 2% in the 50% of samples of each treatmet, sample type and day post-weaning. (A) Species and functional core of Global faecal dataset. (B) Species and functional core of faecal samples collected at weaning (Faeces 0dpw). (C) Species and functional core of faecal samples collected 7 days post-weaning (Faeces 7dpw). (D) Species and functional core of faecal samples collected 14 days post-weaning (Faeces 14dpw). Taxonomic and functional profiling of sequences was performed using Metaphlan3 and HUManN3 respectively.

Microbial environment weakly contributes to piglets microbiome composition early after weaning

Figure 7 shows the evolution of the presence of species at a prevalence of 60% in each type of sample and dpw, from the starting room environment to 14dpw, split by treatment while FD and WF data were joined together in a new variable called “environmental”. Room environment weakly contributed to the pigs’ microbiome composition and evolution. *Dorea formicigenerans*

was the species present in the room environment from ZnO-free farms detected in the following days post-weaning; and *C. glutamicum*, *A. urinaeequi*, *A. viridans*, *Eubacterium hallii*, *Dorea longicatena* and *C. mitsuokai* in environments from farms using ZnO. The rest of the species that were shared between the environment and faeces 0dpw and subsequently found in the following days were 8 for ZnO-free farms and 11 for Treated farms were *Methanobrevibacter smithii*, *C. aerofaciens*, *L. amylovorus*, *L. reuteri*, *Butyricicoccus porcorum*, *Blautia obeum*, and *M. elsdenii*, regardless of ZnO usage; as well as *C. mitsuokai* in ZnO-free farms, and *P. copri*, *R. torques*, *D. formicigenerans* and *P. succinatutens* in ZnO using farms.

Shared species between Environment and piglets microbiota at 0dpw and mantained across weaning

Methanobrevibacter smithii
Collinsella aerofaciens
Lactobacillus amylovorus
Lactobacillus reuteri
Butyricicoccus porcorum
Blautia obeum
Catenibacterium mitsuokai
Megasphaera elsdenii

Unique species seen in room environment microbiomes and present at the following sets

Dorea formicigenerans

Shared species between Environment and piglets microbiota at 0dpw and mantained across weaning

Methanobrevibacter smithii
Collinsella aerofaciens
Prevotella copri
Lactobacillus amylovorus
Lactobacillus reuteri
Butyricicoccus porcorum
Blautia obeum
Ruminococcus torques
Dorea formicigenerans
Phascolarctobacterium succinatutens
Megasphaera elsdenii

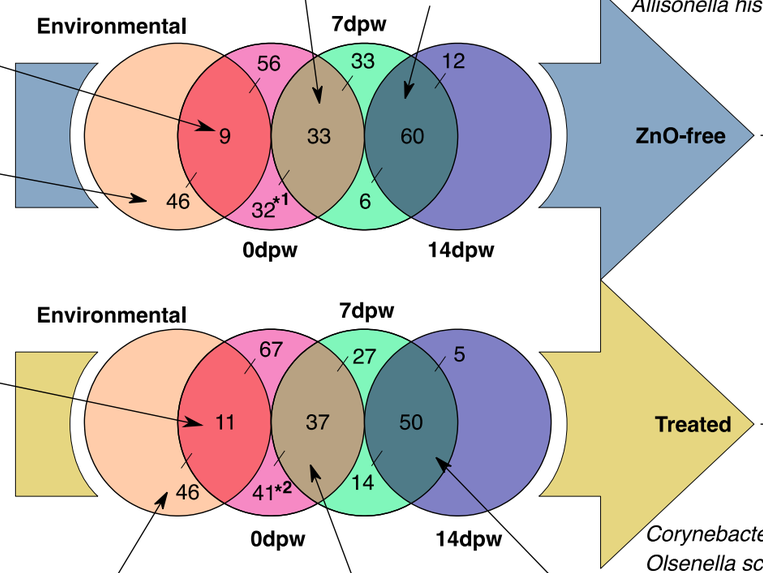
Shared Prevotella spp. between 0dpw and 7dpw

Prevotella copri
Prevotella sp CAG 485
Prevotella sp CAG 520
Prevotella sp CAG 873
Prevotella sp P2 180
Prevotella sp P3 122
Prevotella sp P5 92
Prevotella stercorea

Shared Prevotella spp. between 7dpw and 14dpw

Prevotella copri
Prevotella sp AM42 24
Prevotella sp CAG 1092
Prevotella sp CAG 279
Prevotella sp CAG 520
Prevotella sp CAG 873
Prevotella sp P2 180
Prevotella sp P3 122
Prevotella sp P5 92
Prevotella stercorea

Slackia isoflavoniconvertens
Lactobacillus mucosae
Lactobacillus salivarius
Streptococcus hyointestinalis
Clostridium sp CAG 632
Bacteroides pectinophilus
Coprococcus comes
Ruminococcus bicirculans
Ruminococcus sp CAG 488
Firmicutes bacterium CAG 110
Firmicutes bacterium CAG 238
Allisonella histaminiformans



Corynebacterium glutamicum
Olsenella scatoligenes
Chlamydia suis
Eubacterium limosum
Firmicutes bacterium CAG 83

Unique species seen in room environment microbiomes and present at the following sets

Corynebacterium glutamicum
Aerococcus urinaeequi
Aerococcus viridans
Eubacterium hallii
Dorea longicatena
Catenibacterium mitsuokai

Shared Prevotella spp. between 0dpw and 7dpw

Prevotella copri
Prevotella sp AM42 24
Prevotella sp CAG 520
Prevotella sp CAG 873
Prevotella sp P2 180
Prevotella sp P3 122
Prevotella sp P5 92
Prevotella stercorea

Shared Prevotella spp. between 7dpw and 14dpw

Prevotella copri
Prevotella sp AM42 24
Prevotella sp CAG 1092
Prevotella sp CAG 279
Prevotella sp CAG 520
Prevotella sp P2 180
Prevotella sp P3 122
Prevotella sp P5 92
Prevotella stercorea

[*1] Unique species at 0dpw (compared to 7dpw)

Bacteroides fragilis
Bacteroides massiliensis
Bacteroides uniformis
Bacteroides vulgatus
Parabacteroides distasonis
Parabacteroides merdae
Lactobacillus crispatus
Lactobacillus delbrueckii
Lactobacillus mucosae
Lactobacillus salivarius
Lactobacillus vaginalis
Anaeromassilibacillus sp An172

[*2] Unique species at 0dpw (compared to 7dpw)

Bacteroides fluxus
Bacteroides fragilis
Bacteroides massiliensis
Bacteroides plebeius
Bacteroides uniformis
Bacteroides vulgatus
Bacteroides thetaiotaomicron
Butyricimonas virosa
Prevotella sp CAG 485
Parabacteroides distasonis
Parabacteroides merdae
Lactobacillus crispatus
Lactobacillus delbrueckii
Lactobacillus johnsonii
Lactobacillus mucosae
Lactobacillus vaginalis
Streptococcus gallolyticus

Chapter 3. Study II. Figure 7. Evolution of species presence from room environment at day of weaning towards day 14dpw. Pairwise comparison of the presence of species with a prevalence of 60% across types of sample and day post-weaning splited by treatment. Data from environmental samples (FD and WF) were grouped in a variable called "environmental". Circular sets are arranged in a "longitudinal" fashion, thus comparing two levels at a time, from Environmental samples to day 14dpw, showing the presence of each species at each type of sample and day post-weaning with a prevalence of 60% in each group. Number of species compared between two stages are joined by two diagonal lines readed from the bottomright side of the left set circle to the upper side of the next set. Species seen only at the last set (day 14dpw) and not in day 7dpw are depicted at the end of each arrow. Taxonomic identification of sequences was performed using Metaphlan3.

Discussion

Weaning is a critical moment in the life of piglets, where barriers built against pathogens during lactation are suddenly broken down. The immune factors supplied by colostrum and milk are lost and the competitive exclusion exerted by the microbes colonising the intestine is weakened. This intestinal imbalance has been managed by the use of in-feed prophylactic antibiotics and therapeutic ZnO in pig farms until recently (Gresse et al., 2017). Several farms have removed these treatments before their ban, providing an excellent opportunity to compare how their use or removal affects these aspects, particularly the establishment and evolution of the gut microbiome after weaning. Studying the changes occurring in microbiome due to antibiotic and ZnO treatment and investigating possible associated biomarkers may open new opportunities to intervene during post-weaning period without antibiotic and ZnO use. Therefore, in this study we sequenced the environmental microbiome of clean weaning rooms and faecal microbiomes of piglets in the first two weeks post-weaning on farms still regularly using in-feed antibiotics and ZnO relative to those that do not.

In this study, we wanted to study if the background microbiome of the rooms where piglets are allocated has any influence in the faecal microbiome of piglets. Both samples collected, feeder-drinkers and wall-floor pen surfaces, exhibited high microbial richness and evenness, represented mostly by aerobic microorganisms. No significant variations in these environmental bacteria were observed among farms using or not using antibiotics and ZnO. The analysis of association between farm environment and faecal microbiomes early after weaning revealed a

weak influence of the pen environment on the faecal microbiome of piglets. Despite the potential impact of the environment on the new-born human and piglet microbiome (Merrifield et al., 2016; Chen et al., 2018, Law et al., 2021), there is a need for more studies evaluating the potential role of the weaning environment in this regard. Based on our results, feed composition and feed-treatments seem to have a larger impact than the microbiome colonising the environment at this stage, although further studies are required.

Piglets at weaning exhibited a microbiome clearly differentiated from 7 and 14dpw, possibly explained by a greater species and functional evenness, and the presence of some species not seen as such abundance in the following time points as *Prevotella sp. CAG 873* and *Anaeromassilibacillus sp. An172*. During subsequent time points, microbiome composition shifted towards the dominance of *L. amylovorus*, *L. reuteri*, *P. copri* or *M. elsdenii*. A positive evolution towards the colonisation of bacteria such as *P. copri* and *M. elsdenii* has been reported previously (Wang et al., 2019). This temporal shift was clearly demonstrated by the ordination plots which grouped faecal microbiomes on the basis of (i) Faeces 0dpw, (ii) Faeces 7dpw and 14dpw, and (iii) Diarrhoea 7dpw. This difference was more subtle at the functional level where functionality was enriched in contributing species towards time points. Interestingly, we found differences in species abundance within the same genus. Either in the analysis of treatment associated species or the profile of relative abundance, this within-genus species succession was apparent through the 2 weeks of the study. Thus, we found different species of *Prevotella* spp. shifting across weaning period. Previous studies of pig microbiome using 16S rRNA sequencing have reported a microbial shift from a milk-oriented microbiome in piglets (composed mainly of *Lactobacillus* and *Bacteroides*), to a more complex dietary fibre and carbohydrate adapted microbiome (dominated mainly by genus *Prevotella*) (Frese et al., 2015). One of the main advantages of shotgun metagenome sequencing is the ability to reach species level resolution, enabling the exploration of species succession even in a short-term period of two weeks post-weaning. In this sense, treated animals exhibited a more varied diversity of *Prevotella* species,

with the exception of *Prevotella sp. CAG 873*, which was enriched in ZnO-free piglets' microbiomes in Diarrhoea 7dpw and Faeces 14dpw samples, and was more associated with Faeces 0dpw microbiomes (Figure 3A and 3C).

Diarrhoea is the final disease outcome of the intestinal dysfunction at weaning. It can be the consequence of malabsorption, linked to atrophy of intestinal villi, or a secretory diarrhoea when enterotoxigenic *E. coli* is present (Luppi, 2017). Here, we evaluated the composition in diarrhoeic faeces collected at 7dpw. Microbiome structural and compositional analyses revealed that Diarrhoea 7dpw samples from ZnO-free farms resembled the composition of the microbiome at weaning (0dpw), while on farms using antibiotics and ZnO, Diarrhoea 7dpw samples structure was closer to non-diarrhoeic samples collected at 7dpw and 14dpw. This information, together with the microbial evolution observed at weaning, shows how antibiotic and ZnO use favours the early transition and maturation of the gut microbiome, even in animals with enteric problems. Further Ward clustering analyses of species and pathways by Weighted Unifrac and Aitchison distances pointed to a different composition of these samples. Indeed, fitting of pathways onto ordination as well as pathways relative abundance pattern analysis revealed different metabolic profiles in these samples, with greater representation of metabolic processes linked to *E. coli*. Although the abundance of *E. coli* did not reach significant differences among Treated and ZnO-free farms, the analyses performed revealed a higher abundance of *E. coli* in ZnO-free samples either in faeces or diarrhoea at day 7 post-weaning. Limited sample size and large within group variability in *E. coli* abundance may explain the lack of significance. Further analyses with AST, a variance-stabilizing transformation, revealed significant differences when *E. coli* abundance data was AST transformed (Figure 4G).

Species associated with antibiotic and ZnO treatment within each type-dpw microbiomes were representatives of the orders Eubacteriales, and Bacteroidales (*Prevotella* spp), and, to a lesser extent to Spirochaetales and Acidaminococcales. *P. succinatutens,* belonging to the Acidaminococcales order, was a common species enriched in Treated animals both at 7 and

14dpw. This species is a succinate utilizing anaerobic species that has been reported to increase in abundance as a result of antibiotic treatment, and was part of the core microbiome at 7dpw (Yan et al., 2020). Similarly, based on results in the current and previous studies, *Prevotella* seems to be a genus within which many representatives are resistant to ZnO, which becomes favoured by antimicrobial treatments (Soler et al., 2018; Li et al., 2020).

Microbiomes from ZnO-free farms were enriched in *Lactobacillus spp* and other species within the class Bacilli. In this study, the dominance of *L. amylovorus* and *L. reuteri* could be observed across the sampling time points, whereas other lactobacilli as *L. johnsonii*, *L. salivarius* and *L. agilis* were affected by antibiotic and ZnO treatment. *Eubacterium* is a genus found to be an important member of the human core microbiome, described as a butyrate-producing bacteria. In this study, we found two of the major species of interest within this genus, *E. rectale* and *E. eligens,* to be present at higher abundance on ZnO-free farms (Mukherjee et al., 2020). Another two species linked to ZnO-free herds were *M. elsdenii* and *Acidaminococcus fermentans*, with *M. elsdenii* being the dominant species associated to the core microbiome in ZnO-free herds. Previous studies have also reported the negative impact of ZnO on the abundance of this species (Pieper et al., 2020; da Silva et al., 2021), which is capable of produce butyrate from lactate (Sarmikasoglou and Faciola., 2022, Tsukahara et al., 2006). Both groups of species are common members of the adult pig microbiome, which increase in the intestine as the animal grows, and are species capable of utilizing complex carbohydrates from plant derived ingredients and produce short chain fatty acids (Wang and Schaffner, 2011; Li et al., 2020). Considering the characteristics of the microorganisms mentioned, the taxonomic variations observed likely reflect the ability of certain microorganisms to colonise or outcompete bacteria with similar broad roles in the presence of antibiotics and ZnO and *vice versa*.

These taxonomic changes were reflected as well in the metabolic activity of the microbiomes, which were associated with some of the aforementioned species. The most remarkable differences were found in Faeces 14dpw microbiomes, with *M. elsdenii* associated with

pathways of ZnO-free farms, while microbiome functions in Diarrhoea 7dpw samples in ZnO-free farms were dominated by functions linked to *E. coli* with higher abundance of aerobic-related pathways as TCA cycle or glycolysis. Impairment of anaerobic population and thriving of facultative anaerobic species as a consequence of intestinal inflammation has already been described as a strategy of some opportunistic enteric pathogens, such as *Salmonella enterica* or *E. coli* (Baümler and Sperandio, 2016; Gresse et al., 2017). Aerobic environments generated by the immune response provides an advantage to these facultative anaerobes, such as *E. coli*, in microbiomes from diarrhoeic ZnO-free treated samples.

In this study we show the transition and maturation of the microbiome early after weaning, which is remarkably consistent among farms and is more impacted by feed and feed treatments than the environmental microbiome. The use of antibiotics and ZnO altered the taxonomy and functionality in faecal microbiomes where bacteria such as *Prevotella* spp., *Phascolarctobacterium succinatutens, M. elsdenii* and to a lower extent *E. coli,* colonised the intestinal niche in response to this dietary treatment. The taxonomical and functional analysis of faeces with diarrhoea evidenced that antibiotic and ZnO treatment favoured the microbial transition observed in healthy animals. These results demonstrate the link between this treatment and microbiome composition/functionality and provide interesting insights to study potential strategies to replace antibiotics and ZnO based on microbiome studies.

Material and Methods

Sampling

This study was licensed by Teagasc Animal Ethics Committee and was carried out in commercial farms. Ten farms were selected, 5 farms with regular use of in-feed prophylactic antibiotics and therapeutic ZnO (3,000 ppm) in the first two weeks post-weaning (Treated, n = 5) and 5 farms not using them for the last 3 years (ZnO-free, n = 5). The antibiotics used in the farms were amoxicillin (Stabox, Virbac, France) and sulphadiazine-trimethoprim (Sulfoprim, Univet Limited, Ireland) at the dose indicated by the manufacturer. In each farm, two pens from two different rooms were sampled. Environmental sampling was performed in empty clean pens immediately before the pigs were moved into the rooms on weaning day (0 day post-weaning, dpw) using sponge swabs (3M^{TM} Sponge-Stick in sample bag with 10 mL D/E neutralising broth, 3M Deutschland GmbH, Neuss, Germany). One swab was used to sample the feeders and the drinkers in the pen (FD) and another swab was used to sample two sections of 50 cm2 of the walls and the floor of the pen (WF). Pigs faecal samples were collected at 0, 7 and 14dpw. In addition, a diarrhoea sample was collected at 7dpw when available. For the faecal sampling, one random freshly voided faecal sample from one pig per pen was collected and transferred to 1.5mL microcentrifuge tube. The samples were collected using a 140x7mm conical steel spatula avoiding the part in direct contact with the floor and a new sterile spatula was used for each sample. Samples were transported to the laboratory under cooling conditions where swabs were processed extracting the sampled material from the swabs using 5 mL of sterile Phosphate Saline Buffer 1X (PBS). 8 mL and 16 mL approximately were recovered from each WF and FD swab, respectively, and transferred to a 20mL centrifuge tube. These tubes were centrifuged at 3000 x g for 15 min at 4° C, the supernatant was discarded, and the pellet was suspended in 1mL of PBS and transferred to a 1.5 mL tube, which was stored together with the faecal samples at -80° C until DNA extraction.

DNA extraction and library preparation

The DNA was extracted using the QIAamp PowerFecal Pro DNA Kit (Qiagen, Crawley, West Sussex, UK) following the manufacturer's instructions, using 200 ± 50 mg of faecal content from samples. Environmental samples were previously thawed on ice, centrifuged at 15.000 rpm for 1 min at 4° C, the supernatant was discarded and pellet was used for DNA extraction. A Qubit fluorometer (Qubit 3, BioSciences, Dublin, Ireland) was used to determine the total DNA concentration. The 2 samples from the different rooms for each type and time point were pooled by adding 5µL of each sample at a concentration of 1ng/µL. Paired-end sequencing libraries were prepared from the extracted DNA using the Illumina Nextera XT Library Preparation Kit (Illumina Inc., San Diego, CA) followed by sequencing on the Illumina NextSeq 500 platform using high-output chemistry (2 × 150 bp) according to the manufacturer's instructions. Libraries size from each sample was assessed on an Agilent Technology 21000 Bioanalyzer using a High Sensitivity DNA chip.

Bioinformatic analysis

Raw reads were filtered using trimmomatic v0.38 (Bolger et al., 2014). An average quality threshold score of 15 in a sliding window of 4 base pairs was used to trim reads below the threshold. A minimum length of 40 base pairs was ensured for all reads. Bowtie2 v2.4.4 (Langmead and Salzberg, 2012) was used to map the reads against host and human reference genomes, keeping the unmapped reads for the downstream analysis. Reference genomes were downloaded from Illumina iGenomes (https://support.illumina.com/sequencing/sequencing_software/igenome.html). Read duplicates were removed using a bbmap 38.22 tool called clumpify.sh (Bushnell, 2014). Analysis of microbial composition was carried out using Metaphlan v3.0 (Beghini et al., 2021). Functional profiles were assigned using HUMAnN v3.0 (Beghini et al., 2021).

Statistical Analysis

All analyses were carried out in R v4.0.2 with alpha level for significance of 0.05 and trend between 0.05 and 0.10 unless otherwise indicated. The fixed factors to be studied were the type of sample (FD, WF, Faeces or Diarrhoea), treatment with in-feed ZnO and antibiotics (Treated or ZnO-free) and day post-weaning (0dpw, 7dpw or 14dpw). The type of sample and day post-weaning were merged into a unique factor named "type-dpw", having 6 different levels for this factor: Faeces 0dpw, Faeces 7dpw, Faeces 14dpw, Diarrhoea 7dpw, FD and WF. For the analysis of the effect of the environment on the gut microbiome, FD and WF samples were merged into a new variable called "environmental". The farm was included in all the clustering analyses.

The effect of the treatment on the microbiome were studied between and within each type-dpw level. Alpha and beta diversities were both computed at the species and functional level using the R package Vegan v2.5-7 (Oksanen et al., 2020). For alpha diversity estimation, Species richness, Inverse Simpson, and Shannon and Pielou evenness indices of diversity were calculated. Statistical differences in alpha diversity indexes were tested, after checking their normal distribution, with ANOVA and pairwise compared with Tukey (car v3.0.10 ; Fox and Weisberg, 2019; multcompView v0.1.8, Graves, 2019) and lsmeans v2.30.0 (Lenth, 2016) R packages or otherwise by Kruskal-Wallis and pairwise tested with Wilcoxon test (stats v4.0.2,R Core Team, 2020) R package. Beta diversity and ordination of samples were performed by non-metric multidimensional scaling (NMDS) of previously calculated Weighted Unifrac and Aitchison distances between samples of Species and functional abundance data, respectively. Weighted Unifrac distances on the species abundance table were calculated using the utility R script calculate_unifrac.R from Metaphlan. Aitchison distances were computed calculating the Euclidean distances of the CLR transformed pathways. Separation between groups was tested with PERMANOVA (*adonis2* and *pairwise adonis,* Martinez Arbizu, 2020). Factors and species influencing the ordination were assessed by linear models fitting on the ordination results (*envfit* function in Vegan R package). All p-values were adjusted by Benjamini-Hochberj (BH) procedure.

For fitting species in ordination space, taxa and pathways were filtered, keeping the top 15 species and functions with the highest mean abundance across samples.

Metacyc pathways obtained using HUMAnN were regrouped into metacyc superclasses using humann2meco function from microeco R v0.11.0 package (Liu et al., 2021). Bacteria and function abundance analyses among type, dpw and treatment were performed using Linear Discriminant Analysis Effect Size (LDA LEfSE, Segata et al., 2011). Data grouped in variables type_dpw, treatment, or type_dpw_treatment were used as classes selecting an alpha cut-off of 0.05 and a LDA threshold of 4 for type_dpw species composition analysis and type_dpw_treatment metacyc grouped superclass2 analysis, and 2 for species composition comparison between treatments. Species and functions explaining differences between classes were determined by LEfSE using Kruskal-Wallis test ($P<0.05$) followed by linear discriminant analysis. Core microbiomes of each dietary treatment group were calculated for a minimum threshold of the abundance of 2% in at least 50% of samples of each group using the phyloseq and microbiome R packages (McMurdie and Holmes, 2013; Lahti and Shetty, 2019). Venn diagrams were built using “venn” and “get.venn.partitions” functions from gplots v3.1.1 and VennDiagram v1.6.20 packages, respectively (Warnes et al., 2020; Chen et al., 2018). Plots were built using ggplot2 v3.3.3 and pheatmap v1.0.12 (Wickham, 2016; Kolde, 2019). Figures were produced in R and subsequently arranged using inkscape software v1.0.2 (Inkscape Project, 2020).

Chapter 4. Study III: Sequencing the microbiome of post-weaning pigs in commercial farms that failed to remove in-feed antibiotics and zinc oxide

Sequencing the microbiome of post-weaning pigs in commercial farms that failed to remove in-feed antibiotics and zinc oxide

Juan M. Ortiz Sanjuán[a,b], Héctor Argüello[c] , Raúl Cabrera-Rubio[d,e], Fiona Crispie[d,e], Paul D. Cotter[d,e,f], Juan J. Garrido[b], Daniel Ekhlas[a,g,h], Catherine M. Burgess, Edgar G. Manzanilla[a,h].

INSTITUTIONS

[a]Pig Development Department, Teagasc Grassland Research and Innovation Centre, Moorepark, Fermoy, Co. Cork, Ireland.
[b]Grupo de Genómica y Mejora Animal, Departamento de Genética, Facultad de Veterinaria, Universidad de Córdoba, Córdoba, Spain.
[c]Animal Health Department, Veterinary Faculty, Universidad de León, León, Spain.
[d]Teagasc Food Research Centre, Moorepark, Fermoy, Co. Cork, Ireland.
[e]APC Microbiome Institute, University College Cork, Co. Cork, Ireland.
[f]VistaMilk SFI Research Centre, Fermoy, Co. Cork, Ireland.
[g]Department of Food Safety, Teagasc Food Research Centre, Ashtown, Dublin, Ireland.
[h]School of Veterinary Medicine, University College Dublin, Dublin, Ireland.

#Address correspondence to Juan M. Ortiz Sanjuán, jmos11.1993@gmail.com

Sequencing the microbiome of post-weaning pigs in commercial farms that failed to remove in-feed antibiotics and zinc oxide

ABSTRACT

Post-weaning diarrhoea is a disease that causes economic problems and piglet mortality at weaning. Current restrictions limit the use of in-feed antibiotics (Ab) and therapeutic in-feed zinc oxide (ZnO) use was forbidden recently. Microbiome changes occurring at weaning comprise both a risk factor and a consequence of PWD. In this study, shotgun metagenome sequencing was used to characterize these changes at the first two weeks post-weaning, as wll as those triggered by Ab and ZnO withdrawal in farms regularly using antimicrobials in the post-weaning period. Diversity was mainly affected by day post-weaning (dpw) as well as by treatment and diarrhoea. Microbiome composition evolved towards the dominance of groups of species such as *Prevotella* spp. at day 14dpw. ZnO maintained gut microbiome stability inhibiting *E. coli* overgrowth. Pigs receiving in-feed ZnO had higher abundance of species within the family Bacteroidaceae and decreased *Megasphaera* spp., whereas this genus was not affected by Ab treatments. Functions associated to Ct diet were related to virulence at day 7dpw in faecal and diarrhoea samples. Microbiome functions of pigs treated with ZnO were linked to functions related to sulfur and DNA metabolism, as well as mechanisms of antimicrobial and heavy metal resistance.

Keywords: Antimicrobial use, diarrhea, piglet, shotgun sequencing, swine

Introduction

High throughput sequencing methods have revolutionised the knowledge about microbial communities, particularly in the gut (Cullin et al., 2021). The role of the microbiota in inflammatory or metabolic diseases, cancer, neurologic disorders or even behaviour are just a few examples of the influence these microbes can have (Dominguez-Bello et al., 2019; Cullin et al., 2021) .

Animals, and particularly livestock, are also joining this trend and currently there is detailed information about the microbiota in different species (Xiao et al., 2016; Li et al., 2020; Feng et al., 2021). Research in pigs has described in detail the evolution of the microbiome, at genus and species level, along the productive cycle of pigs (Mach et al., 2015, Xiao et al., 2018, Wang et al 2019b). These and other studies detail the sudden disruption of the microbiota in piglets weaned under intensive commercial condition (Gresse et al., 2017; Guevarra et al., 2019). Weaning in intensive farms usually takes place between the third and the fifth week of age. Piglets are removed from the lactation facilities, separated from the sow, allocated with piglets from other litters into new pens and fed a solid diet. This abrupt change including environmental, social and nutritional changes impacts severely the composition of the microbiota, as stated above. Indeed, the microbiota disbalance occurring at weaning is one of the major factors prompting post-weaning diarrhoea (PWD), the most frequent health problem in commercial pig farms and a major reason for antimicrobial use (Luppi et al., 2018). PWD is usually associated to *Escherichia coli*, enterotoxigenic *E. coli* (ETEC) strains mostly, but also other pathogens such as *Salmonella enterica* or Rotavirus.

For more than five decades, the control of PDW has been based on the systematic use of antibiotics and heavy metals, particularly, zinc oxide (Gresse et al., 2017; Luppi, 2017; Rhouma et al., 2017; Fairbrother and Nadeau, 2019. These compounds provide an antimicrobial effect on targeted pathogens (e.g., ETEC) but probably also allowed a competitive exclusion effect through microorganisms resistant or adapted to these antimicrobials. There are a few studies

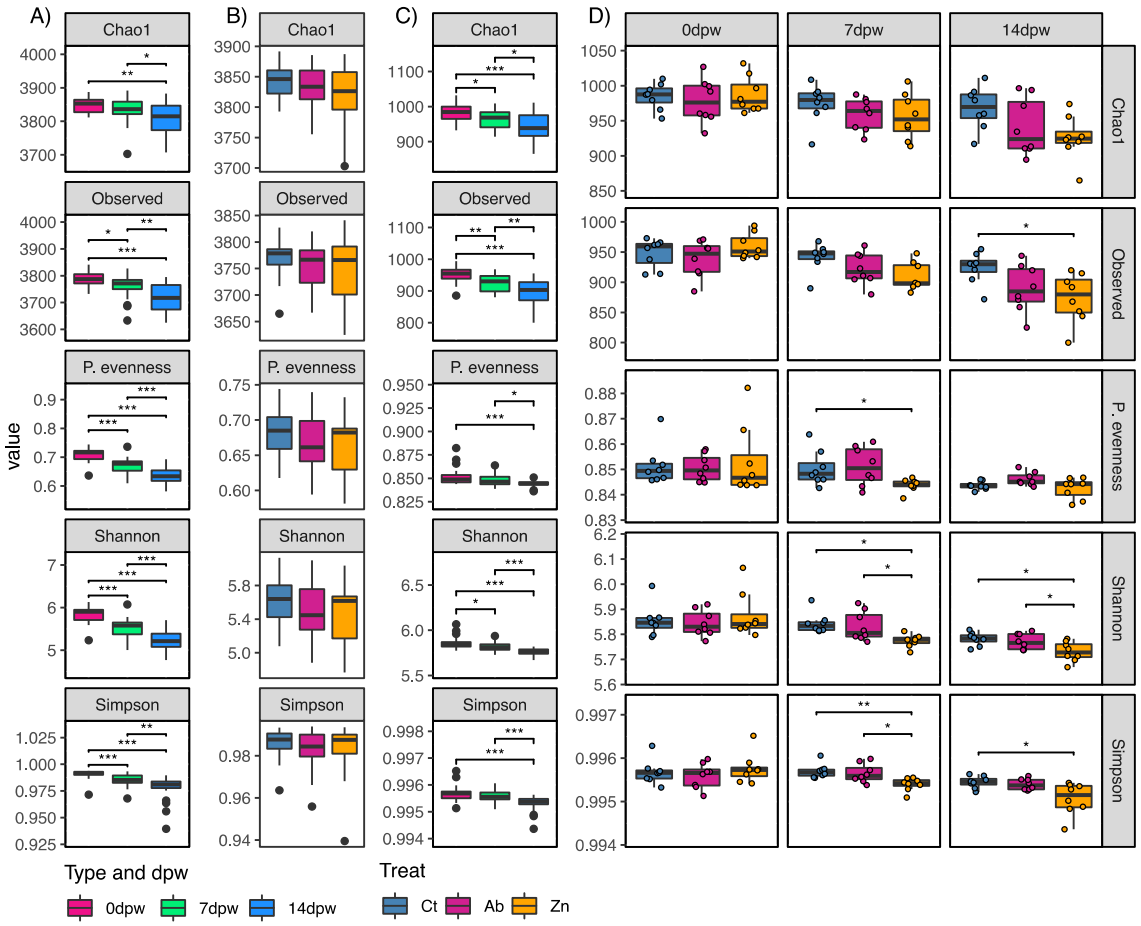
which already demonstrate the role of the microbiota in PWD competitive exclusion under controlled or field conditions (Dou et al., 2017; Argüello et al., 2019; Karasova et al., 2021) . The impact of the use of in-feed antibiotics or large doses of zinc oxide in the microbiota of piglets at weaning is already described in previous studies (Kociova et al., 2020; Pieper et al., 2020; Wei et al., 2020; Ortiz Sanjuán et al., 2022; Sun et al., 2022b). In experimental conditions, different authors demonstrate that feed supplementation is the main driver of the microbiota composition after weaning over environmental microbiota (Ortiz Sanjuán et al, 2022). However, there is also important variability in the changes observed between these studies. To date, it has been difficult to find common changes in the microbiota across studies probably due to differences in methodology, different farm management, different diets or background microbiota in the experimental farm. The information gathered in studies in experimental facilities needs to be validated in field trials accounting for variability under commercial conditions with different health status, different managements or different background use of antimicrobials that may have strong influence on the microbiota.

The present study was designed to compare the effects of in-feed antibiotics and high doses of zinc oxide in commercial conditions accounting for the variability among farms. The hypothesis was that accounting for the variability among farms in the design would help clarify the changes that are common across farms in the effects of in-feed antibiotics and zinc oxide. For this we repeated the same design in 4 different farms and analysed the effects of antibiotics and zinc on the microbiota of piglets accounting for background variability among the 4 farms.

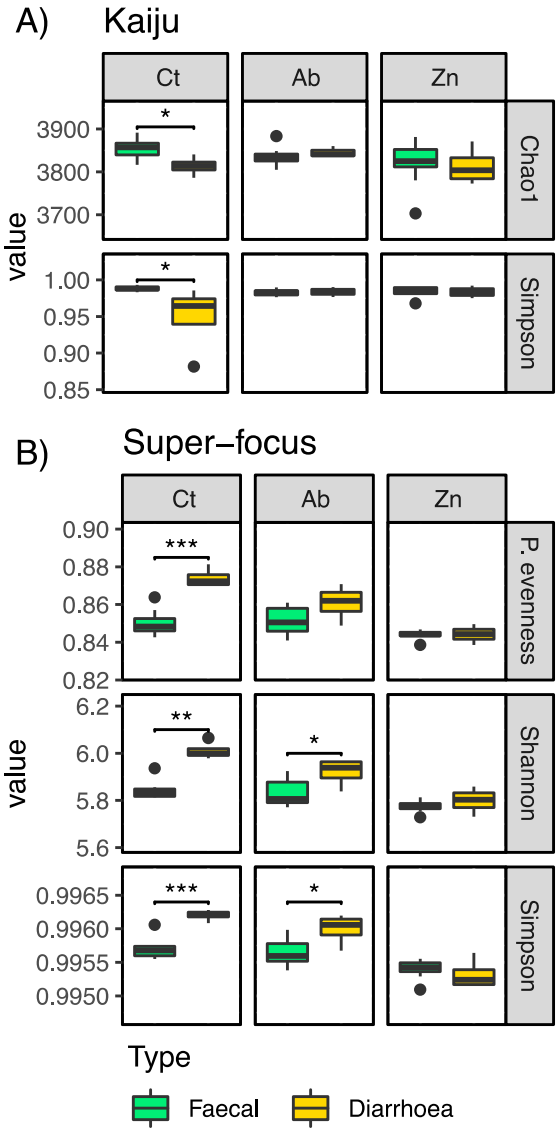
Results

Microbial diversity and richness are defined mostly by day post-weaning but is also affected by treatment and diarrhoea occurrence

The analysis of alpha diversity was performed by estimation of richness and evenness (Observed, Chao1, Simpson, Shannon, Pielou's evenness). Differences between treatments and along days post-weaning were analysed for all the computed indexes either at species or functional level (Figure 1). Diversity decreased with dpw for all indexes at species (Figure 1A) and functional level (Figure 1C). The treatment did not affect diversity at species level (Figure 1B) but it affected diversity at a functional level on days 7 and 14 (Figure 1D) when animals fed Zn showed lower diversity for some of the indexes. As expected, no differences were found on 0dpw between treatments. At 7dpw, Ct animals had greater Pielou evenness than Zn, and also Ct and Ab groups exhibited higher in Shannon and Simpson values than Zn group. At 14dpw, Ct animals had greater Observed and Simpson diversity than Zn, and Ct and Ab also exhibited higher values than Zn in Shannon index. Finally, we compared the alpha diversity between faecal samples of animals showing diarrhoea or not at 7dpw. At species level, diarrhoeic faeces showed lower diversity for Chao1 and Simpson indexes only in the Ct group (Figure 2). At a functional level, diarrheic samples of the control showed a clear increase in diversity in all the indexes compared to non-diarrheic pigs. Diarrheic samples of the antibiotic also showed higher diversity than non-diarrheic but only for Shannon and Simpson and in a much smaller magnitude. Detailed information about alpha diversity values is also available in supplementary file 1.

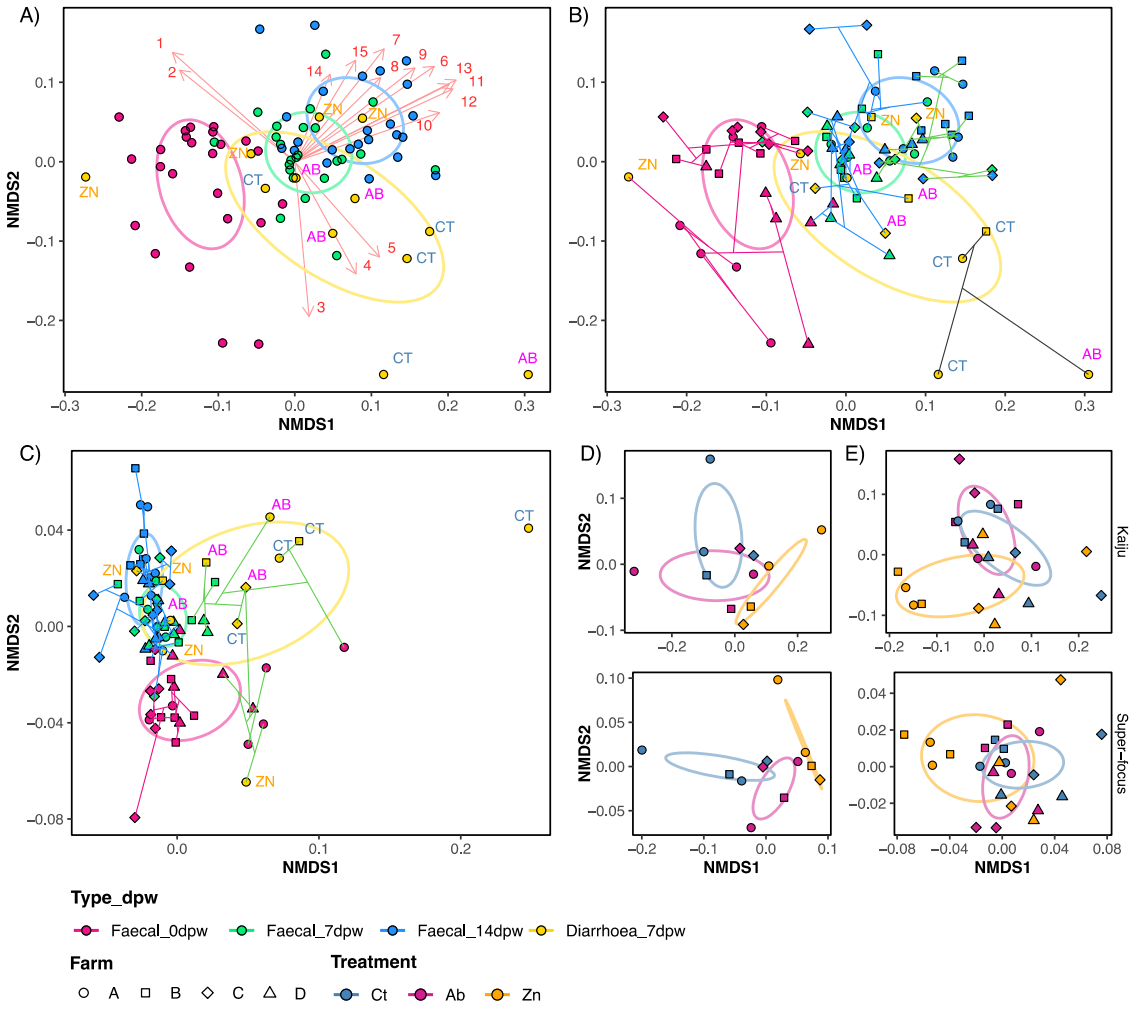


Chapter 4. Study III. Figure 1. Analysis of microbiome α-diversity at species (A-B) and functional levels (C-D): Chao1, Observed richness, Pielou evenness, Shannon and Simpson diversity indices, by day post-weaning (dpw) and treatment. A) Results of α-diversity at species level, by treatment. B) Alpha diversity values by day post-weaning factor, at species level. C) Results of α-diversity, at functional level (Super-focus level 3 category). D) Results of α-diversity analysis by treatment within each day post-weaning, performed at functional level (Super-focus level 3 category). *P < 0.05, **P < 0.01, and ***P < 0.001. Sequences were taxonomically and functionally assigned using Kaiju and Super-focus, respectively.



Chapter 4. Study III. Figure 2. Results of α-diversity analysis showing significant differences in diversity indexes of day 7dpw data, comparing by type factor (faecal and diarrhoea) and performed at species (A) and functional data grouped in super-focus level 3 category (B) in each dietary treatment group.

Ordination of samples by Bray-Curtis distance into an NMDS revealed differences in microbiome composition by treatment, day post-weaning, as well as by farm and type factors, both at species and functional level (Figure 3A, B and C, Table 1 and 2).



Chapter 4. Study III. Figure 3. Analysis of microbiome β-diversity by type of sample, day post-weaning and treatment. A) Samples ordination using Bray-Curtis distances at species level ploted in a NMDS, samples are coloured according type and dpw factor (type_dpw). Arrows display the top 15 species with the highest mean abundance returned by "envfit" model, influencing the ordination of samples (Arrows showing BH p.adjusted significant species; Arrows length shows the strength of each specie influencing the ordination of samples). Species fitted onto ordination are indicated as numbers, ordered according to its NMDS coordinates. 1: *Oscillibacter* sp. PEA192, 2: *Clostridiales* bacterium CCNA10, 3: *Escherichia coli*, 4: *Megasphaera elsdenii*, 5: *Lactobacillus reuteri*, 6: *Eubacterium hallii*, 7: *Faecalibacterium prausnitzii*, 8: *Roseburia hominis*, 9: *Prevotella ruminicola*, 10: *Eubacterium rectale*, 11: *Prevotella dentalis*, 12: *Prevotella enoeca*, 13: *Prevotella oris*, 14: *Blautia* sp. YL58, 15: *Lachnospiraceae* bacterium GAM79. B) Species level ordination of samples showing Ward Clustering of Bray Curtis distances ploted onto a NMDS. Dendrogram was split into 4 branches and coloured according to the samples arrangement within the NMDS space. Samples are coloured according type and dpw factor (type_dpw) C) Ordination of samples using Bray-Curtis distances at functional level of Super-focus level 3 category, samples are coloured according type and dpw factor (type_dpw). D) Ordination of diarrhoea samples of day 7dpw, using Bray-Curtis distances, onto a NMDS, coloured by treatment both at species (top) and functional (bottom) level. E) Ordination of faecal samples of day 14dpw, using Bray-Curtis distances, onto a NMDS, coloured by treatment both at species (top) and functional (bottom) level. Ellipses represent each factor level: type and dpw in figures A-C; and treatment in figures D-E, with their shape being defined by the covariance within each group. Treatments to which each sample belonged to is indicated for

diarrhoea samples in figures A-C as CT(Ct), AB (Ab) and ZN (Zn). Farms factor is indicated by the shape of the points in figures B-E, coloured according to type and dpw (B-C) or treatment (D-E). Taxonomic and functional profiling of sequences was performed using Kaiju and Super-focus, respectively.

Chapter 4. Study III. Table 1. P-value(R^2) results returned by *envfit* function of vegan R package.

Factor	Global	0dpw	7dpw	14dpw
Species				
Treatment	0.139(0.041)	0.948(0.017)	0.352(0.098)	0.057(0.195)
Dpw	0.001***(0.407)			
Treat:dpw	0.001***(0.484)			
Functional				
Treatment	0.329(0.028)	0.924(0.021)	0.054(0.193)	0.146(0.148)
Dpw	0.001***(0.252)			
Treat:dpw	0.001***(0.341)			

The analysis including treatment and dpw (Table 1) revealed influences in the ordination of both species and functional datasets. Pairwise level comparison confirmed differences in microbiota composition among all dpw but not for the treatments (Table 2).

Chapter 4. Study III. Table 2. PERMANOVA results on Global data. P. values are indicated for each factor and level. R^2 values are indicated within parenthesis.

Factor		Species	Functional
Treat		0.042*(0.038)	0.121(0.031)
	Ct vs Ab	0.62(0.015)	0.677(0.014)
	Ct vs Zn	0.24(0.039)	0.384(0.036)
	Zn vs Ab	0.24(0.031)	0.646(0.019)
Dpw		P < 0.001***(0.326)	P < 0.001***(0.294)
	0dpw vs 7dpw	0.002**(0.250)	0.002**(0.208)
	0dpw vs 14dpw	0.002**(0.384)	0.002**(0.336)
	7dpw vs 14dpw	0.002**(0.083)	0.002**(0.094)
treat:dpw		0.282(0.043)	0.527 (0.038)

Despite the lack of an interaction and based on the NMDS plots, further analyses were carried out for the treatment within each dpw level (Table 3 and Figure 3F). In this analysis, Zn showed changes in the taxonomic analysis on day 14 compared to both Ab and Ct (Table 3, Figure F).

Chapter 4. Study III. Table 3. P.value(R^2) results of PERMANOVA analysis performed within each dpw level.

Factor	0dpw	7dpw	14dpw
Species			
Treat	0.961(0.043)	0.042*(0.142)	0.008**(0.193)
Ct vs Ab	0.995(0.045)	0.205(0.096)	0.561(0.053)
Ct vs Zn	0.995(0.014)	0.084(0.132)	0.022*(0.205)
Zn vs Ab	0.995(0.043)	0.173(0.104)	0.022*(0.170)
Functional			
Treat	0.972(0.034)	0.022*(0.152)	0.047*(0.161)
Ct vs Ab	0.958(0.029)	0.407(0.066)	0.589(0.052)
Ct vs Zn	0.958(0.020)	0.012*(0.166)	0.132(0.172)
Zn vs Ab	0.958(0.030)	0.104(0.121)	0.132(0.126)

In the analysis of diarrheic vs non-diarrhoeic samples for dpw 7, treatment showed differences in the taxonomic and functional analysis, as well as in the ordination of both datasets, but no significant differences were found among treatment levels (Figure 3E; Table 4). Finally, the farm showed no effect on the taxonomic of functional analysis.

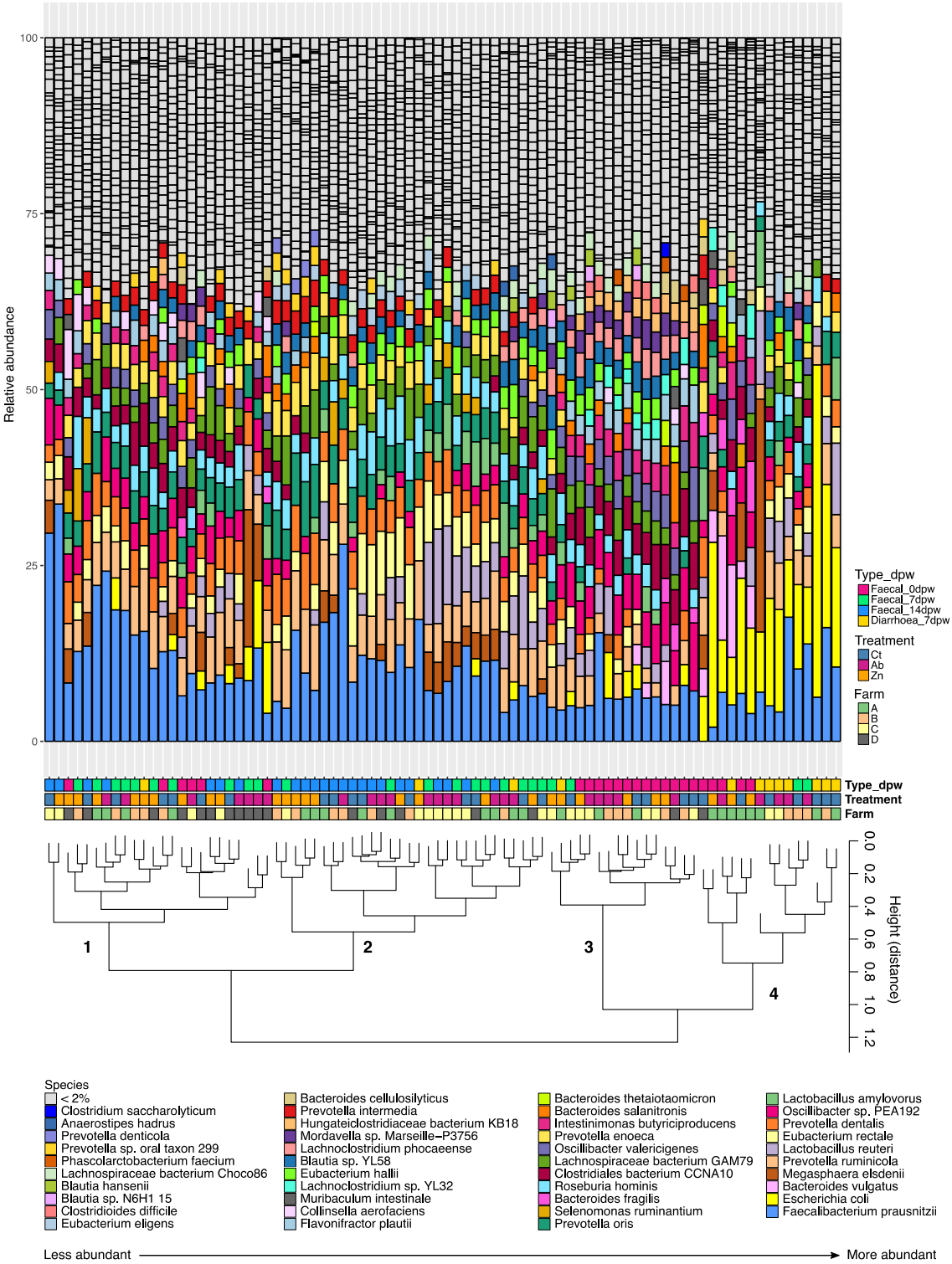
Chapter 4. Study III. Table 4. P.value(R^2) results of PERMANOVA and envfit analysis performed separately in diarrhoea samples of day 7dpw.

PERMANOVA		
Factor	**Species**	**Functional**
Treatment	0.035*(0.295)	0.004**(0.447)
Ct vs Zn	0.087.(0.316)	0.076.(0.478)
Ct vs Ab	0.297 (0.159)	0.076.(0.314)
Zn vs Ab	0.087.(0.236)	0.076.(0.263)
envfit	**Species**	**Functional**
Treat	0.108(0.339)	0.0047**(0.508)

Weaning and diarrhoea occurrence define the main bacterial clusters

Further analysis of samples clustering and indicator species across samples was performed by Ward clustering of Bray-Curtis distance, which arranged the samples in 4 main clusters (Figure 4). Cluster 1 included samples from all dpw and treatments. Cluster 2 included only samples from dpw 7 and 14. Cluster 3 included almost exclusively samples of day 0dpw (13 out of 16 samples). Cluster 4 included most of the Diarrhoea_7dpw samples (8), 2 samples from 7dpw and 5 from 0dpw. Species associated to the four clusters defined by Ward clustering are shown in

Figure 4 too. Samples in cluter 1 were linked to *Faecalibacterium prausnitzii* and *Collinsella aerofaciens*. Cluster 2 included species of *Prevotella spp*., *Eubacterium spp*., *Anaerostipes hadrus*, *Roseburia hominis*, and *Lachnospiraceae bacterium GAM79*. Patterns in cluster 3 were defined by several species of *Clostridium spp*., *Ruminococcus spp*., *Blautia spp*., *Oscillibacter spp.*, *Lachnoclostridium spp., Intestinimonas butyriciproducens, Flavonifractor plautii, Mordavella* sp. Marseille−P3756*, Hungateiclostridiaceae bacterium KB18, Lachnospiraceae bacterium Choco86, Bacteroides cellulosilyticus* and *Phascolarctobacterium faecium*. Cluster 4 was associated with *Escherichia coli*, *Bacteroides vulgatus* and *Bacteroides fragilis*.



Species associated to each Ward Clustering defined group

1 *Collinsella aerofaciens*
Faecalibacterium prausnitzii

4 *Bacteroides fragilis*
Bacteroides vulgatus
Escherichia coli

2 *Anaerostipes hadrus*
Eubacterium eligens
Eubacterium hallii
Eubacterium rectale
Lachnospiraceae bacterium GAM79
Prevotella dentalis
Prevotella denticola
Prevotella enoeca
Prevotella intermedia
Prevotella melaninogenica
Prevotella oris
Prevotella ruminicola
Prevotella sp. oral taxon 299
Roseburia hominis

3 *Bacteroides cellulosilyticus*
Blautia hansenii
Blautia sp. N6H1 15
Blautia sp. YL58
Clostridiales bacterium CCNA10
Clostridioides difficile
Clostridium saccharolyticum
Clostridium sp. SY8519
Flavonifractor plautii
Hungateiclostridiaceae bacterium KB18
Intestinimonas butyriciproducens
Lachnoclostridium phocaeense
Lachnoclostridium sp. YL32
Lachnospiraceae bacterium Choco86
Mordavella sp. Marseille−P3756
Oscillibacter sp. PEA192
Oscillibacter valericigenes
Phascolarctobacterium faecium
Ruminococcus albus
Ruminococcus bicirculans

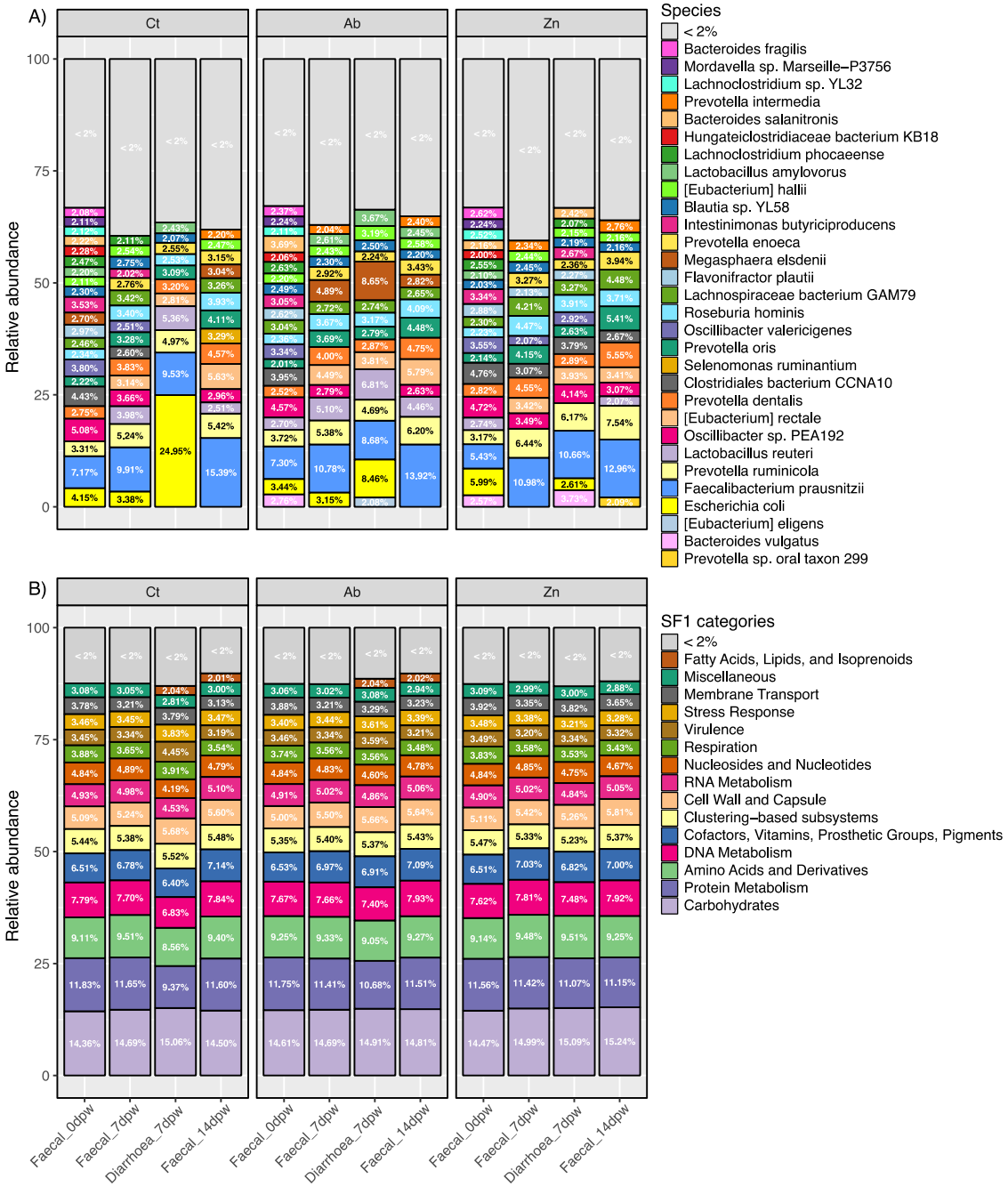
Chapter 4. Study III. Figure 4. Stacked bar plot of the relative abundance of the main species in each sample. Profiles of samples are ordered by Ward clustering of the squared Bray-Curtis distances between samples. Cluster dendrogram represents the similarity between samples regarding its microbial composition. Variables information in each sample (from lower to upper level: Farm, Treatment, Type-dpw) are indicated in the coloured squares below the bars. Numbers besides each branch refers to the main groups defined by Ward clustering, for which indicator species of each group determined by '*multipatt*' are showed at the bottom part of the figure. Taxonomic identification of sequences was performed using Kaiju.

Microbial composition evolves rapidly post-weaning and is affected by treatments and diarrhoea occurrence

Next, to compare the microbiota evolution across the two weeks post-weaning in each treatment, the mean relative abundance species profiles of each type-dpw level were separately investigated (Figure 5A). *F. prausnitzii* was the dominant species regardless the type-dpw except in the case of diarrheic samples. Samples of day 0dpw revealed a notable similar pattern between treatments with, as seen is alpha diversity analyses, an even distribution of low-abundant species and few dominant species such as *E. coli*, *F. prausnitzii*, *Oscillibacter spp.*, and *Clostridiales bacterium CCNA10*. Species relative abundance of some groups were affected after one week post-weaning (Faecal_7dpw). Increased abundance was observed for species such as *Prevotella spp.*, *Lachnospiraceae bacterium GAM79* or *L. reuteri*; while decreased abundance was detected in *Intestinimonas butyriciproducens* and *Oscillibacter* spp in Ct (Figure 5A). Some other species such as *E. rectale* or *P. intermedia* were detected for first time in Ab and Zn treated groups. In addition, an increase in *Megasphaera elsdenii* abundance was observed in Ab treatment group at 7 and 14 dpw. The relative abundance of *Lactobacillus* spp. (*L. reuteri*, *L. amylovorus*) was affected by Zn treatment, with low abundance or even absence within the 2 weeks of study. A clear effect seen among treatments was the lower abundance or absence of *E. coli* in Ab and Zn groups in samples from 7 and 14 dpw. In animals with diarrhea 7 dpw, *E. coli* reached a relative abundance of 24.95% in the Ct group with much lower levels in Ab and Zn treated animals (8.46 and 2.61%, respectively). Other bacteria affected by treatments in diarrhoea samples were *L. bacterium GAM79*, which was absent in Ct samples and maintained

a similar relative abundance in Ab and Zn treatment groups. Species seen only in Zn diarrhoea samples were *C. bacterium CCNA10* and *B. vulgatus* (3.80 and 3.70%, respectively). Finally, Faecal_14dpw microbiota was dominated by *Prevotella* spp. (*P. ruminicola, P. dentalis, P. enoeca* and *P. oris*), with the presence of other species at lower relative abundance, such as *M. elsdenii*, observed in Ct and Ab while absent in the Zn group, *Selenomonas ruminatum* was only found in Ct animals, and *C. bacterium CCNA10* and *P. oral taxon 299* in the Zn treated group (2.66 and 2.01%, respectively).

Functional data grouped in SF level 1 categories (Figure 5B) revealed that the most abundant categories in all type-dpw samples reaching together a 50% approximately were: (i) Carbohydrates, (ii) Protein Metabolism, (iii) Amino acids and derivatives, (iv) DNA metabolism, and (v) Cofactors, vitamins, prosthetic groups, and pigments. These functions reached less than 50% in diarrhoea 7dpw samples of Ct diet, in which the category Virulence accounted for 4.45% compared to a range of 3.19 to 3.59% in the rest of the samples.



Chapter 4. Study III. Figure 5. Microbiome composition in samples from weaning pigs at days 0, 7 and 14 post-weaning (dpw) separated by treatments. (A). Mean relative abundance of the most representative species in each type-dpw group. (B) Mean relative abundance of the most representative functional categories of super-focus level 1 in each type-dpw group. Taxonomic and functional profiling of sequences was performed using Kaiju and Super-focus, respectively.

Species differential abundance

Analysis of global dataset (Figure 6A y B) and separated by type and dpw factors (Figure 6C, D and E) revealed different results in each subset. Global analysis revealed several species within

Selenomonadaceae (*Selenomonas* spp., *Megamonas hypermegale*) and Sporomusaceae familiae (*Methylomusa anaerophila*, *Pelosinus fermentans*) associated to animals fed the Ct diet, as well as *Diallister sp Marseille P5638* and two species from *Megasphaera* spp. (*M. hexagonica* and *M. stationii*), whereas *M. elsdenii* abundance was higher in Ab treated animals. Zn treated animals had higher relative abundance of *Bacteroides* spp., *Flavonifractor plautii*, *Phascolarctobacterium faecium*, *Candidatus Methanomethylophilus alvus, Tannerella sp oral taxon HOT 286* and *Parabacteroides spp.* (Figure 6A).

Analyses by dpw revealed that at 0dpw, no differences, except for *Bacteroides salanitronis* enriched in microbiota of Ab group, were found between dietary treatments (Supplementary figure S1A). At day 7dpw (Figure 6C, Supplementary figure S1B), faecal samples of Ct animals showed higher abundance of *Desulfovibrio piger*, species associated with the family Hungateiclostridiaceae (*Hungateiclostridium clariflavum*, *Hungateiclostridium saccincola*, *Pseudoclostridium thermosuccinogenes)*, *Ruminococcus albus*, *Ruminocuccus bicirculans*, *Caproiciproducens sp NJN 50*, *Clostridium sp BNL1100*, *Alkaliphilus oremlandii*, *Akkermansia muciniphila*, *Dehalococcoides mccartyi*, *Olsenella sp oral taxon 807*, *Murdochiella vaginallis*, *Enterococcus faecium* and *Streptococcus suis,* as well as *Christensenella massiliensis, Dehalobacterium formicoaceticum,* and several species within the family Clostridiales Family XIII Incertae Sedis (*Aminipila sp JN 39*, *Eubacterium sulci*, *Mogibacterium diversum*). Ab treated animals were associated to higher abundance of *Selenomonas sputigena* and *D. sp Marseille P5638*. Finally, Zn treated animals exhibited higher relative abundance of *Selenomonas ruminantium, Selenomonas sp oral taxon 920, Clostridioides difficile*, *Bacteroides vulgatus, Bacteroides dorei*, *Eubacterium callanderi* and *Eubacterium limosum*.

Faecal samples collected at 14 dpw (Figure 6D, Supplementary figure S1C) revealed the strong association of *Megasphaera* spp. (*M. elsdenii*, *M. hexagonica*, *M. stationii*), several species within the Selenomonadaceae (*S. ruminantium*, *S. sp oral taxon 920*, *Selenomonas sputigena*, and *M. hypermegale*) and Sporomusaceae familiae *(Methylomusa anaerophila, Pelosinus*

fermentans), as well as *Diallister* sp Marseille P5638*, Acidaminococcus intestinii*, *Lactobacillus delbrueckii, E. coli*, *Desulfovibrio fairfieldensis* and *D. piger.* to Ct diet. Samples from Ab treated animals were associated with *Acidaminococcus fermentans*, whereas samples from Zn treated animals exhibited higher abundance of species belonging to *Tanerellaceae* (*Tannerella forsythia*, *Tannerella sp oral taxon HOT 286*, *Parabacteroides sp CT06*, *Parabacteroides distasonis*) and *Bacteroidaceae* families (*B. vulgatus*, *B. thetaiotaomicron*, *B. salanitronis*, *B. heparinolyticus*, *B. helcogenes*, *B. fragilis*, *B. cellulosilyticus*, *B. caecimuris* and *B. caccae*), as well as *Phascolarctobacterium faecium, Clostridioides difficile*, *Ornithobacterium rhinotracheale*, *Draconibacterium orientale*, *Paludibacter propionicigenes*, *Proteiniphilum saccharofermentans*, *Odoribacter splanchnicus*, *Butyricimonas faecalis*, *Mucinivorans hirudinis*, and *Candidatus Methanomethylophilus alvus*.

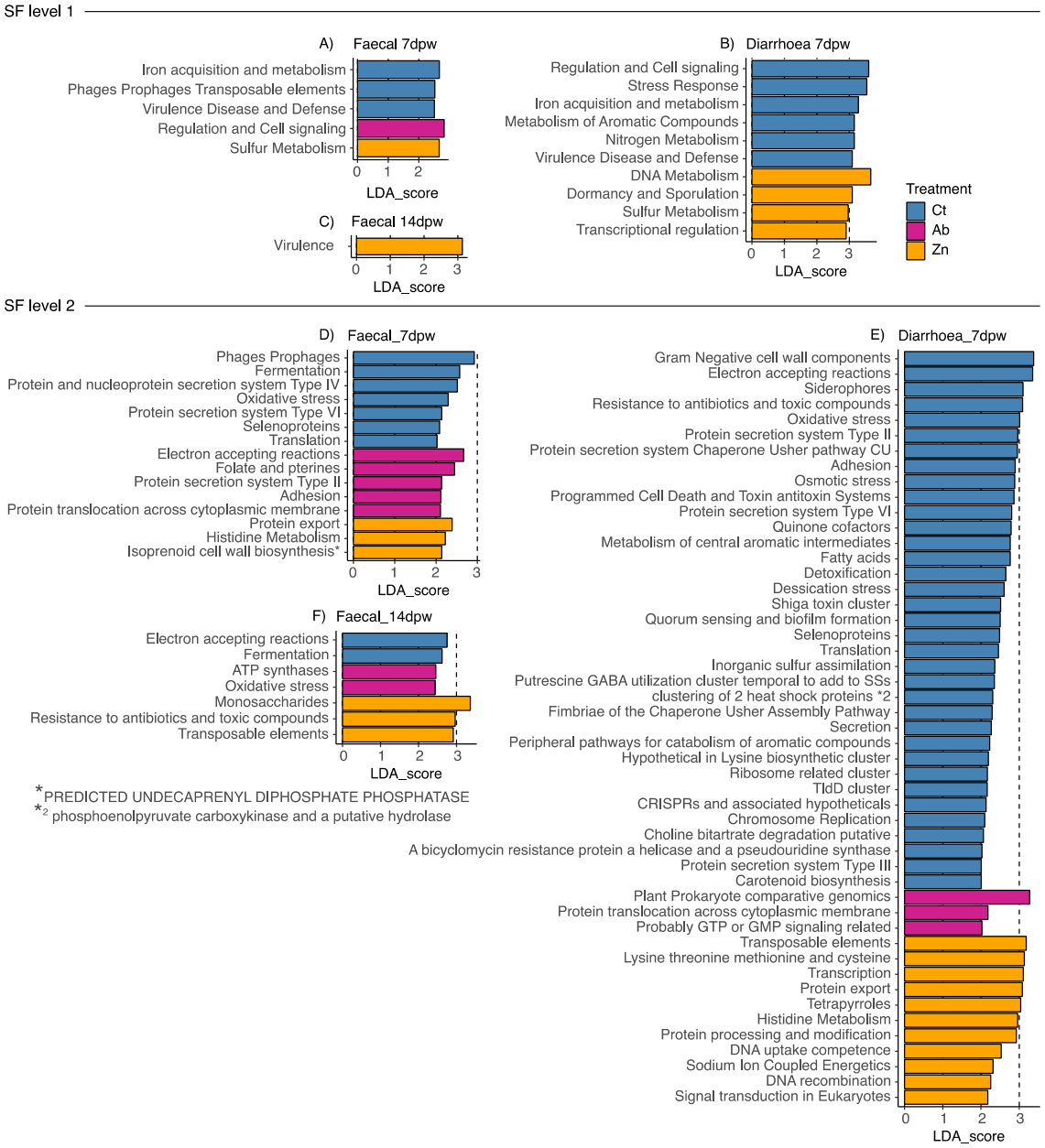
Diarrhoea_7dpw samples (Figure 6E, Supplementary figure S1C)had higher abundance of *E. coli* and *Suterella megalosphaeroides* in Ct group, *Bacteroides* spp., *F. plautii and Mageeibacillus indolicus* in Zn group and *L. reuteri* in Ab group.

Chapter 4. Study III. Figure 6. Differences in species abundance, returned by LEfSe (Linear discriminant analysis Effect Size), most likely explaining the differences among dietary treatments. A) Species associated with each dietary treatment in the analysis of global species data. B) Taxa associated with each dietary treatment in the analysis of global species data.

Significant species are coloured according to the treatment to which they are associated to, and are annotated in the cladogram as letters, which can be identified bellow. C) Species associated to each treatment at day 7 post-weaning (7dpw). D) Species associated to each treatment at day 14 post-weaning (14dpw). E) Species associated to each treatment, in diarrhoea samples of day 7 post-weaning (7dpw).

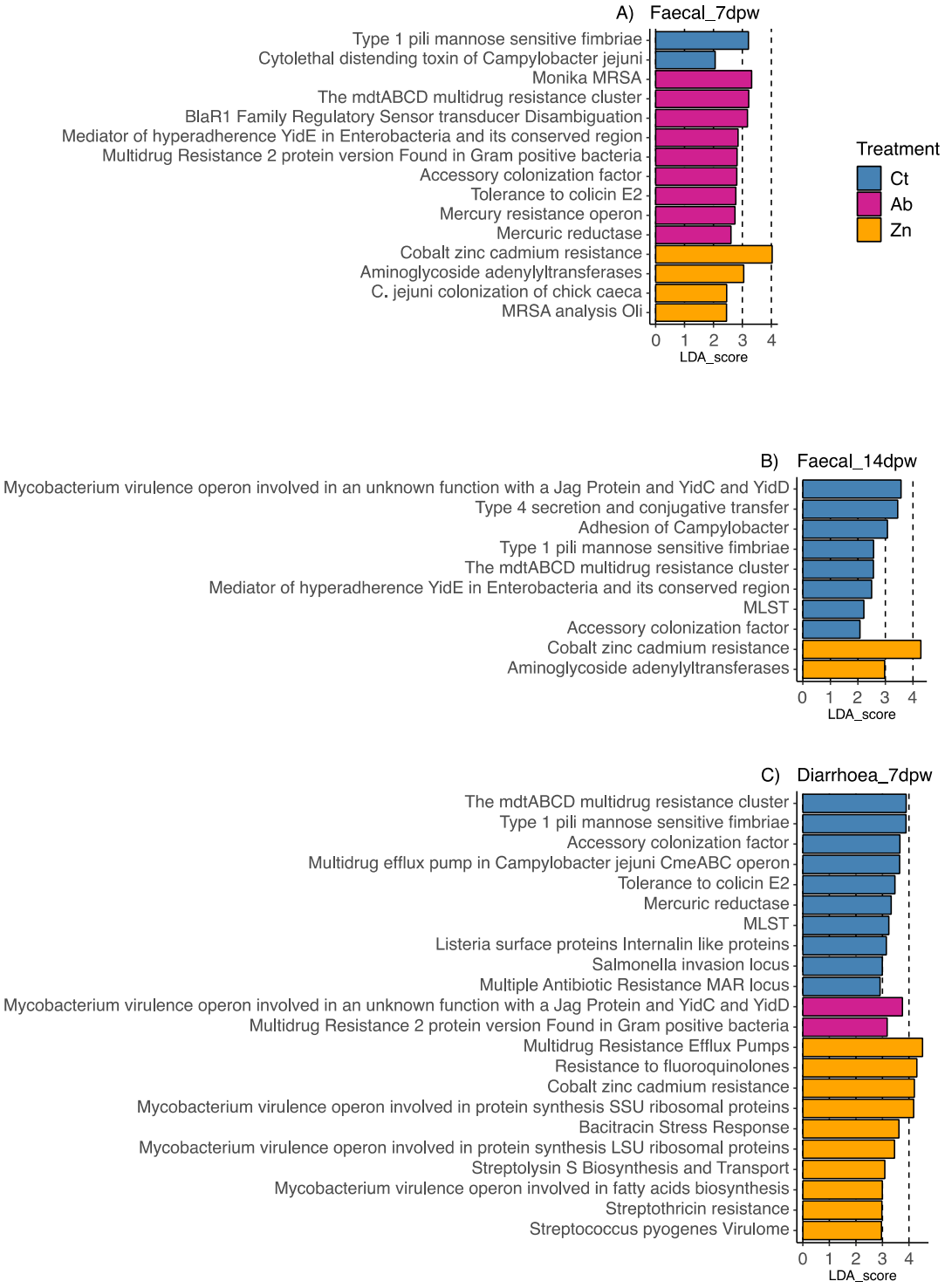
Functional differential abundance

The effect of each treatment in SF1 and SF2 grouped functional categories as well as SF3 categories related to virulence, disease and defence was separately investigated by type-dpw levels (Figure 7 and Figure 8). LEfSe (Linear discriminant analysis Effect Size) analyses revealed no associations of any category to any of the treatments of 0dpw. Effects of treatment in SF1 categories at days 7dpw (Faecal and Diarrhoea) and 14dpw are showed in Figures 7A, 7B and 7C, respectilvely. In Faecal_7dpw samples, the categories Phages-Prophages, Fermentation, Protein and nucleoprotein secretion system type IV, Protein secretion system type VI, Oxidative stress, Selenoproteins and Translation were associated to microbiomes of animals fed the Ct diet, whereas categories of Electron accepting reaction, Folate and Pterines, Protein secretion system Type II, Adhesion and Protein translocation across the cytoplasmic membrane were associated to microbiomes from Ab treated pigs. The functions of Protein export, Histidine metabolism, and Isoprenoid cell wall biosynthesis were associated to Zn (Figure 7D). Faecal 14dpw samples from Ct group were enriched in Electron accepting reactions and Fermentation functions, Ab group in ATP synthases and Oxidative stress while Zn group exhibited higher abundance of functions linked to Monosaccharides, Resistance to antibiotics and toxic compounds and Transposable elements (Figure 7F). Analysis in Diarrhoea_7dpw samples revealed a high number of categories linked to Virulence in Ct samples, Protein translocation across cytoplasmatic membrane and GTM or GMP signalling associated to Ab, and functions related to aminoacids metabolism (Lysine, threonine, methionine, and cysteine, and histidine metabolism), Protein export, processing and modification, DNA uptake, competence, and recombination, and Transposable elements related to Zn (Figure 7E).



Chapter 4. Study III. Figure 7. Differences in Super-focus (SF) functional levels 1 (A-C) and 2 (D-F), returned by LEfSe (Linear discriminant analysis Effect Size), most likely explaining the differences among dietary treatments. A) Functional SF1 categories associated with each dietary treatment in the analysis of Faecal 7dpw. B) Functional SF1 categories associated with each dietary treatment in the analysis of Diarrhoea 7dpw. C) Functional SF1 categories associated with each dietary treatment in the analysis of Faecal 14dpw. D) Functional SF2 categories associated with each dietary treatment in the analysis of Faecal 7dpw. E) Functional SF2 categories associated with each dietary treatment in the analysis of Diarrhoea 7dpw. F) Functional SF2 categories associated with each dietary treatment in the analysis of Faecal 14dpw. Significant functional categories are coloured according to the treatment to which they are associated to.

Analysis of level SF3 related to Virulence, disease, and defence category of SF1 (Figure 8) revealed antimicrobial resistance mechanisms and cobalt, zinc and cadmium resistance associated to Zn treatment in all type-dpw levels. Functions associated to Ct were related to Gram-negative mechanisms of virulence, adhesion, and colonization, among others, as well as other mechanisms related to adhesion and multidrug resistance in Ab treated animals.



Chapter 4. Study III. Figure 8. Differences in Super-focus (SF) functional level 3 with functions related to Virulence, Disease and Defense of SF1, returned by LEfSe (Linear discriminant analysis Effect Size), most likely explaining the differences among dietary treatments. A) Functional Viruelnce-related SF3 categories associated with each dietary treatment in the analysis of Faecal 7dpw. B) Functional Viruelnce-related SF3 categories associated with each dietary treatment in the analysis of Diarrhoea 7dpw. C) Functional Viruelnce-related SF3 categories associated with each dietary treatment in the analysis of Faecal 14dpw. Significant functional categories are coloured according to the treatment to which they are associated to.

Discussion

In-feed Ab and ZnO have been widely used to prevent and control PWD, a disease affecting piglets in one of the most critical moments of the productive cycle. Environmental soil pollution and antimicrobial resistance have raised concerns about the regular use of these products and new approaches to control PWD are needed. Understanding how antibiotics and zinc oxide control diarrhoea is key to develop effective approaches to control PWD. The role of microbiome composition and functionality in this context is gaining attention (Gresse et al., 2017). Studying changes occurring in microbiome ecology as a consequence of weaning, antibiotics or ZnO treatment, and diarrhoea will provide knowledge to create a more resilient microbiome at this critical stage by finding taxonomic and functional traits linked to PWD resistance/onset.

Research in this area is generally carried out in experimental farms, normally using one farm per study, by using experimental infections or different types of challenges (Kwon et al., 2014; Kim et al., 2015). While this approach helps control the background variability and standardise results, it is also less representative of the commercial conditions, where challenges are more severe and diverse, and of the variability among farms. The background microbiome of a farm will define the effects observed when using Ab and Zn. This becomes especially relevant when using analysis that are expensive and result in big databases, like sequencing, where the limitations in sample size are evident. In this study, we used shotgun metagenomic sequencing to explore the effect of in-feed Ab or Zn on piglet microbiome composition and functionality by replicating the same design in different commercial farms that had tried and failed to remove this products from the diets of the piglets. The effect of the farms was not of interest but the objective was to capture the variability among farms in the design to find what is that Ab and Zn do in all farms.

Broadly, we found that the microbiome of the piglet dramatically adapts to weaning, rapidly shifting during the 2 weeks after weaning, and that diarrhoea markedly modifies this adaptation. Furthermore, Ab and Zn impact the evolution of species and functional richness, diversity and

composition of the microbiome inhibiting *E. coli* overgrowth and favouring or impairing bacteria within Veillonelaceae family in Ab or ZnO, respectively, as well as give advantage to other within Tannerellaceae and Bacteroidaceae in the case of Zn. This effect of Ab and Zn is much more evident when looking at animal with clinical diarrhoea, which had not been described until now.

At taxonomic level, the treatments did not affect alpha diversity, however, at functional level, Zn reduced diversity compared to Ct and Ab at 7 and 14dpw. Ct pigs also had higher richness levels than Zn treated animals at 14dpw. Alpha diversity has been reported to decrease in Zn treated animals (Xia et al., 2017; Yu et al., 2017b; Ortiz Sanjuán et al., 2022) at species level, but up to date and to our knowledge, there is no information about functional diversity in Zn treated pigs. Weaning has been reported to increase richness and diversity as the animal grows and is fed a more complex diets (Frese et al., 2015; Guevarra et al., 2019). In our study, we observed a shift in microbial dominance towards the 14dpw (P. evenness, Shannon, Simpson) that was similar in the three treatments. This shift was different at functional richness and diversity, exhibiting Zn treated animals a rapid decrease in richness and diversity. These and the obtained results comparing faeces and diarrhoea at 7dpw demonstrate the impact of Ab and Zn in the development of the microbiota.

Ordination of samples produced a clear separation of each dpw group both at species and functional level, revealing a quick adaptation of microbiome soon after weaning and exhibiting a drastic separation between samples of 0dpw and samples of 7dpw and 14dpw. Previous studies addressed this rapid adaptation of piglet microbiome to solid feed (Frese et al., 2015; Guevarra et al., 2019). Ordination of samples also revealed a remarkable difference between diarrhoea samples and all the other groups. Interestingly, 3 of 4 diarrhoea Ct samples were located far apart from the rest of the samples, whereas 3 of 4 samples of Ab were located closer to faecal samples, and Zn samples seemed to be scattered within the rest of the faecal groups. Indeed, representation of Ward Clustering of Bray Curtis distances onto NMDS ordination space revealed more complex relationships between these samples, 4 diarrhoea samples (of which 3

controls and 1 Ab) forming an isolated group and the rest of diarrhoea samples pertaining to other groups of the dendrogram. Other relationships were observed at a functional level, where 6 samples (5 faecal 0dpw and 1 diarrhoea Zn) belonged to the same clustering group of some faecal 7dpw and diarrhoea samples. These results seen in diarrhoea ordination may indicate Ab and specially Zn, reverting microbiome composition and functionality to its normal state.

Ward clustering of squared Bray Curtis distances produced 2 main branches: one formed by clusters 1 and 2 (mostly faecal 7dpw and 14dpw) and the other formed by clusters 3 and 4, composed of faecal 0dpw and diarrhoea samples, respectively. Interestingly, each one of the groups defined by the clustering exhibited a very specific pattern of relative abundance of species. Cluster 2 consisted in a majority of 14 and 7 dpw faecal samples, cluster 3 was defined by samples of 0 dpw with species associated to immature milk-oriented microbiome, and cluster 4 by diarrhoea, an unbalanced microbiome dominated by *E. coli*. Cluster 1 was the most heterogeneous one composed of a mix of 0, 7 and 14 dpw faecal samples. The presence of groups of species in lower abundance as *Prevotella* spp and *Eubacterium rectale*, and *Roseburia hominis* from cluster 2 in cluster 1, as well as some species seen in cluster 3 as *Clostridiales bacterium CCNA10* and *Oscillibacter* spp in cluster 1, may indicate an interface-like stage of species microbiome composition between 0 dpw (cluster 3) and 7-14 dpw (cluster 2) from weaning to 2 weeks post-weaning. The clustering also revealed certain sub-clusters of samples composed of samples from the same farm or treatment, such as samples of farm D (4 of which were from Ab treated pigs) in cluster 1, samples of farm C in cluster 2, 4 of which were from Ab treated animals in cluster 2, and samples of farm A (4 of 0 dpw and 1 of diarrhoea) in cluster 4. This sub-clusters reveal the influence of treatments and farms at a lower hierarchical order that dpw and diarrhoea occurrence.

Evolution of microbial composition along days post-weaning followed a different path depending on the dietary treatment, maintaining some species with a similar relative abundance across days and treatments such as *F. prausnitzii*, *L. reuteri*, *P. ruminicola*, *P. denticola*, *E. rectale*,

and some characteristic species regarding each treatment at each day post-weaning. Starting at the day of weaning (0dpw), before any treatments were applied, species relative abundance was virtually the same in all treatments, and the three reflected the high evenness seen in alpha diversity analysis. The relative stability of microbiome across the 2 weeks post-weaning was broken in Ct, and to a lesser extent in Ab, diarrhoeic samples where there was a sudden shift towards other species as *E. coli* (in Ct 24.95% and Ab 8.46%) and *M. elsdenii* in Ab treated animals (8.65%). Diarrhoea samples of Zn treated pigs exhibited a very similar pattern to faecal 0dpw and 7dpw samples. Both, Ab and Zn prevented *E. coli* overgrowth in healthy and diarrhoeic animals at 7dpw. This finding can explain why, in the absence of Ab and Zn, PWD can go out of control and result in significant mortality and loss of performance and why Ab and Zn help control PWD.

Microbiome stability was reflected again in relative abundance patterns of super-focus 2 categories, where all categories were stable throughout the 2 weeks of the study, with an abnormal pattern in diarrhoea samples of Ct pigs. Once more, these results evidence the microbiome as an ecological community able to adapt and maintain a necessary pool of functions despite the species succession and abundance fluctuation seen across time-points, and other perturbations. These concepts are known as resilience and functional redundancy (Sommer et al., 2017; Fassarella et al., 2021).

Regarding differential species associated to each dietary treatment, species exhibiting consistency across days post-weaning and treatment were those ascribed to phylum Proteobacteria in Ct animals (*D. piger* in 7 and 14 dpw, *E. coli* in diarrhoea 7 and faecal 7 dpw), and species belonging to family Bacteroidaceae (*Bacteroides* spp) and *Clostridioides difficile* in faecal 7 and 14dpw in Zn treated animals. The effect exerted by ZnO in those bacteria belonging to phylum Proteobacteria (*E. coli*, *Desulfovibrio* spp) is in accordance with other studies (Rattigan et al., 2020; Ortiz Sanjuán et al., 2022). The association of species within Bacteroidaceae such as *Bacteroides* spp and *Parabacteroides* spp to Zn medication has also been reported (Pieper et al.,

2020; Rattigan et al., 2020; Ortiz Sanjuán et al., 2022). Indeed, these species as well as others within this family as *Prevotella* spp seem to be tolerant to Zn and Ab (Pieper et al., 2020; Tunsagool et al., 2021; Ortiz Sanjuán et al., 2022). We observed a high diversity of *Bacteroides* spp associated to this treatment. As a common member of pig microbiome, *Bacteroides* spp are Gram negative bacteria associated to a less mature microbiota, such as lactating animals (Frese et al., 2015; Guevarra et al., 2019; Cremonesi et al., 2022), while in humans, these bacteria are linked to diets with high content of proteins and fat (van de Wouw et al., 2017). On the other hand, the relationship of mutualism between *Bacteroides* spp and human host has been extensively described. Some species within this genus exhibit cross-feeding abilities, providing simpler nutrients to host and other microorganism within the gut, and are able to modulate immune response (Wexler, 2007). Several studies using in-feed have ZnO reported anti-inflammatory effects through overexpression of IL-10 mRNA levels (Hu et al., 2013; Shen et al., 2014). Despite the reported shift towards a more *Prevotella*-dominated gut, several studies report the high adaptability of *Bacteroides* spp in the intestinal environment (Wexler, 2007). Shotgun sequencing approach enables microbiome characterization at species level. Notably, here we found a high diverse group of *Bacteroides* spp associated with Zn treatment. The majority of the studies are focused on dominant species and there is still a question about how important non dominant species in a microbial community are. Future studies assessing the influence of small changes on microbial composition and functionality on host whole host homeostasis would fill these knowledge gaps.

Other species associated to each treatment included species within class Clostridia, order Eubacteriales, *Streptococcus suis*, *Enterococcus faecium* and *Akkermansia muciniphila* in Ct animals of faecal 7 dpw; whereas species explaining differences among groups at 14 dpw in Ct animals shifted to species within Negativicutes class (families Acidaminococcaceae, Selenomonadaceae, Sporomusaceae and Veillonellaceae); as well as *Clostridioides difficile* in Zn treated pigs 7 and 14 dpw. In this study, ZnO and Ab controlled *E. coli* overgrowth, as well as *S.*

suis, another known pathogen of pigs and zoonotic agent (Haas and Grenier, 2018). *A. muciniphila* is a mucosa-dwelling bacterium which has been associated with mucosa damage in association with *Salmonella* Typhimurium infection (Argüello et al., 2018). At 14 dpw, Ct animals had higher abundance of *E. coli* as well as *Megasphaera* spp, whereas species associated to Ab diet shifted across days post-weaning. For instance, while *M. elsdenii* was linked to Ab diet in global data, as well as *Dialister sp Marseille P5638* and *Selenomonas sputigena* to Ab at 7 dpw, these species were associated to Ct animals at 14 dpw. *M. elsdenii* is a common member of the pig's microbiome described in adult pigs microbiome, which gains presence in the microbial community as the animals consume feed (Wang et al., 2019b), and which has been reported to arise in abundance in animals fed Ab diets (Stanton and Humphrey, 2011; Stanton et al., 2011; Ortiz Sanjuán et al., 2022). Species from the genus *Megasphaera* and *Diallister* spp, all from Veillonellaceae family, exhibited a higher abundance in Ct pids 14 dpw, and decreased in abundance in Zn treated animals in all dpw. Some of these mentioned members appear to be susceptible to ZnO or the conditions originated by it in the gut (Pieper et al., 2020; da Silva et al., 2021; Ortiz Sanjuán et al., 2022).

Regarding functional data, broad categories of functions seen in level 1 of super-focus were maintained throughout the study, although the presence of other functions, particularly those related to virulence, exerted a great influence in species composition and host homeostasis, manifesting the clinical sign of diarrhoea as a final outcome, even though these functional categories are kept within the community. Results seen on functional data may indicate signs of an unstable state in faecal 7 dpw in Ct animals (Iron acquisition and metabolism, Prophages, transposable elements, Virulence, disease and defence) and its continuation and exacerbation in diarrhoea 7 dpw. Sulfur metabolism was enriched in pigs medicated with Zn in faecal 7 dpw and diarrhoea 7 dpw, as well as DNA metabolism, transcriptional regulation, and Dormancy and Sporulation in diarrhoea 7 dpw. Higher abundance of this last category may indicate groups of bacteria adaptation to the unfavourable conditions generated either by the state of intestinal

dysbiosis, the ZnO effects at the gut, or both. Zn animals were linked to functions associated to virulence at 14 dpw, mostly related to metals (zinc, cobalt and cadmium resistance) and antimicrobial resistance. At higher levels of description, functions associated to Ct pigs, particularly in diarrhoea samples, were related to adhesion and invasion. Indeed, previous studies performed in vitro reported a reduced bacterial adhesion and invasion of ETEC in human Caco-2 enterocytes, and a mild inhibition of biofilm formation in cultured *E. coli* (Roselli et al., 2003; Wu et al., 2013). Similar results were obtained in a previous study in animals receiving a Ct diet at the 7th day post-weaning (Ortiz Sanjuán et al., 2022). These results can only demonstrate the potential of *E. coli* as a causative agent of a dysbiosis state within the gut. Future studies focused on microbiome activity such as meta-transcriptomics or meta-proteomics will help to confirm these findings.

In this study, we used shotgun metagenome sequencing to characterize microbiome of pigs across farms during two weeks post-weaning, as well as the effects of Ab and Zn in species and broad functional composition of pigs microbiome. Results show that the microbiome of the piglet evolves quickly in the first weeks post-weaning and pigs suffering diarrhoea have a different microbial composition from the rest of the animals. Effects of Ab and Zn may denote a microbiome restoration/reversion to more stable community compositions or the prevention of microbial disbalance and *E. coli* overgrowth. These results will be useful to find alternatives to antimicrobial compounds to keep or restore the microbial equilibrium at the most critical days at the beginning of the post-weaning period.

Materials and Methods

Experimental design. In this study, we assessed the effect of 3 different dietary treatments, antibiotic (Ab), therapeutic zinc oxide (Zn) or negative control (Ct), across 4 farms where antibiotics and Zn were used regularly at post-weaning period. The four farms had recently tried to remove Ab and Zn for the feed but it resulted in outbreaks of diarrhoea and reductions in performance. Farms A, B and C used the control diet + 250mg/Kg of Sulphadiazine and 50mg/Kg of Trimethoprim as Sulfoprim 15% (Univet Limited, Ireland), while in farm D control diet was medicated with 400mg/Kg of Amoxicillin as Stabox 5% (Virbac, France). The Zn group was fed with the control diet + 2500 mg/Kg of Zinc Oxide as Pigzin (DSM Animal nutrition, United Kingdom). The dietary treatments were administered in the 2 weeks post-weaning and samplings were scheduled at days 0, 7 and 14 post-weaning (0dpw, 7dpw, and 14dpw. respectively). The same experiment was replicated twice in each of the four farms (A, B, C, D). Piglets were weaned at 4 weeks of age and moved to the weaning rooms, where the groups were balanced by weight. Pigs were fed a pelleted starter diet in dry form fulfilling nutritional requirements (NRC, 2012). Feed was provided manually in bags and intake was recorded at pen level by weighing the bags weekly.

Sample collection, DNA extraction and library preparation. At sampling days 0, 7 and 14dpw, three random freshly voided faecal sample from three pigs per treatment pen were collected and pooled into a 100mL cup where they were mixed and homogenised using a sterile 210x11mm sampling spatula, and then transferred to 1.5mL microcentrifuge tube using a sterile 140x7mm conical steel spatula. On day 7dpw, diarrhoea samples were collected from all pens and treatments where fresh diarrhoea was observed on the walls of the pen. Using a sterile sampling spatula, diarrhoea samples were collected into a 1.5mL microcentrifuge tube. Samples were immediately stored on dry-ice upon collection and transported to the research facilities where they were stored at -80° C until processed. The samples were collected avoiding the part of faeces in direct contact with the floor. The DNA from the faecal samples was extracted using

the QIAamp PowerFecal Pro DNA Kit (Qiagen, Crawley, West Sussex, UK) following the manufacturer's instructions, using 200 ± 50 mg of faecal content. A Qubit fluorimeter (Qubit 3, Invitrogen) was used to determine the total DNA concentration. Paired-end sequencing libraries were prepared from the extracted DNA using the Illumina Nextera XT Library Preparation Kit (Illumina Inc., San Diego, CA) followed by sequencing on the Illumina NextSeq 500 platform using high-output chemistry (2 × 150 bp) according to the manufacturer's instructions. Library size from each sample was assessed on an Agilent Technology 21000 Bioanalyzer using a High Sensitivity DNA chip.

Bioinformatic analysis

Raw reads were filtered using trimmomatic v0.38 (Bolger et al., 2014). An average quality threshold score of 25 in a sliding window of 10 base pairs was used to trim reads below the threshold. A minimum length of 150 base pairs was ensured for all reads. Bowtie2 v2.4.4 (Langmead and Salzberg, 2012) was used to map the reads against host and human reference genomes, keeping the unmapped reads for the downstream analysis. Reference genomes were downloaded from Bowtie2 website. Read duplicates were removed using a bbmap 38.22 tool called clumpify.sh (Bushnell, 2014). Analysis of microbial composition was carried out using Kaiju v1.7.4 (Menzel et al., 2016). Functional profiles were assigned using SUPER-FOCUS v0.0.0 (Silva et al., 2016).

Statistical Analysis

Analyses were carried out in R v4.2.1 (R Core Team., 2022), studying differences for variable day post-weaning (0, 7 or 14 dpw), treatment (Ct, Ab or Zn), farm (A, B, C, D) and type (diarrhoea, non-diarrhoea). In-feed dietary treatments effects on microbiome were studied globally and within each type and dpw level. For first ordination and clustering, and differential abundance analysis within each type and dpw, type and dpw variables were merged into a new variable called "type-dpw": Faecal_0dpw, Faecal_7dpw, Diarrhoea_7dpw, and Faecal_14dpw. To ensure a balanced statistical arrangement, diarrhoea samples were not included as part of the global

analysis nor when studying differences among sampling time points either. Alpha and beta diversities were both computed at the species and functional level using the phyloseq R package v1.40.0 and vegan R package v2.6-2, respectively (McMurdie and Holmes., 2013; Oksanen et al., 2020). The estimation of alpha diversity in taxonomic and functional data, included an initial analysis of alpha diversity indexes computed using raw reads. Potential differences in diversity between sequencing runs associated to variations in sequencing depth was checked using Kruskal Wallis test or ANOVA depending on their data distribution. When significant differences were found for any of the computed indexes, data was normalized using rarefaction. Raw reads classified by kaiju were normalized using rarefaction by the minimum total number of sequences using the function *rarefy_even_depth* from phyloseq R package. Samples that did not reach the richness plateau in the rarefaction curve were removed from the analysis of alpha diversity. Alpha diversity was estimated by Observed richness, Chao1, Simpson, and Shannon and Pielou evenness indexes. Statistical differences in alpha diversity indexes were tested, after testing their distribution (Shapiro Wilk test, $p < 0.05$), with ANOVA followed by pairwise comparison with Tukey (car v3.0.10, Fox and Weisberg, 2019), multcompView v0.1.8 (Graves, 2019) and lsmeans v2.30.0 (Lenth., 2016) R packages) or by Kruskal-Wallis followed by pairwise Wilcoxon test (stats v4.0.2 R package) (R Core Team, 2022), when data did not follow a normal distribution. *Wilcox.exact* from exactRankTests v0.8-35 R pakage function was used for exact p-value estimation of data with ties (Hothorn., 2022). Beta diversity and ordination of samples were performed in relative abundance transformed data, by NMDS of previously calculated Bray Curtis distances between samples of species and functional abundance data, using vegan package in R. Distance between groups centroids was tested with PERMANOVA (*adonis2* and *pairwise adonis* (Martinez Arbizu, 2020)). Factors and species influencing the ordination were assessed by linear models fitting on the ordination results (*envfit* function in Vegan R package). All p-values were adjusted by Benjamini-Hochberj (BH) approach. For fitting species in

ordination space, taxa and pathways were filtered, keeping the top 15 species and functions with the highest mean abundance across samples.

Taxa and function abundance analyses among treatments analysed globally and within each type_dpw level, were performed using Linear Discriminant Analysis Effect Size (LEfSe (Segata et al., 2011)). Treatment factor was used as class selecting an alpha cut-off of 0.05 and a LDA threshold of 2. Species and functions explaining differences between treatments were determined by LEfSE using Kruskal-Wallis test ($P<0.05$) followed by linear discriminant analysis. Figures were produced with R and subsequently arranged using inkscape software v1.0.2 (Inkscape Project, 2020).

Chapter 5. General Discussion

Recent changes in European legislation regarding the prophylactic and metaphylactic use of antimicrobials and therapeutic use of heavy metals, mostly Zn, are impacting the way post-weaning diarrhoea (PWD) is tackled on farm. The vast research in PWD and its main causative agent, enterotoxigenic *Escherichia coli*, contrast to the scarce information about a relevant factor in PWD outcome: the gut microbiota.

In the last decade, the new high-throughput sequencing (HTS) strategies are providing an interesting resource to fill this data gap. Recent studies supported by HTS have enabled metagenomic cataloguing of pig intestinal samples, thereby providing insights into the microbial species present within the porcine intestinal tract (Xiao et al., 2016; Chen et al., 2021). A number of these studies are focusing either in the evolution of the microbiota through the different production stages in pig's life (Mach et al., 2015; Ramayo-Caldas et al., 2016; Wang et al., 2019b) or in particular production stages, such as weaning (Frese et al., 2015; Wang et al., 2019b; Saladrigas-García et al., 2021; Gaio et al., 2021; Gaio et al., 2022).

Factors influencing microbiome results

The present thesis focuses on weaning in pig intensive pig farming and post-weaning diarrhoea with particular emphasis in the use of therapeutic zinc and antibiotics (Abs) in its control, within their impact in the intestinal microbiota, both taxonomically and functionally, by the information gathered in three studies. The first study was performed in Teagasc Pig Research Unit and the subsequent studies on commercial farms. Other than the effect antibiotics and ZnO, each study involved other factors in the design. For instance, the first study not only evaluated the impact of Antibiotics and ZnO on the microbiome but also the potential impact of different cleaning approaches in weaning rooms. Further influence of the environment in the microbiome composition was evaluated in the second study by including the analysis of the environmental microbiota of walls, floors, feeders and drinkers from empty pens of clean weaner rooms.

Finally, in the third study where the same treatments were applied on different farms to evaluate, among others, the role of the farm in the microbiota development at weaning.

The results evidenced a similar pattern of relative abundance in the three studies, regardless the idiosyncrasy of each experiment. The first two studies were characterized by the dominance of *Lactobacillus* spp. In the first and third study, samples were dominated by *F. prausnitzii*. In the first study, the abundance of *E. coli* and *Megasphaera* spp and *Prevotella* spp, *Bacteroides* spp, and *Eubacterium* were driving the differences between microbiomes. In the second study there was more representation and diversity of *Prevotella* spp, and differences in clustering were driven by the abundance of *E. coli* in samples of 0dpw faeces and 7dpw diarrhoea. In the third study, samples *Prevotella* spp and to a lower extent *Lactobacillus* spp and *E. coli* were the most representative bacteria (in 0dpw faeces and 7dpw diarrhoea samples). Although comparison of results between studies must be done with caution, these results exhibit the relevance of these species in the gut microbiome of the pig, and that excessive growth of *E. coli* is involved in the dysbiosis that clearly splits the microbiome of diseased animals from those that remain healthy during weaning.

It is also interesting to mention that despite the differences in study design, farms used, etc, results were consistent among the three studies. The results from these studies demonstrate that neither the type of cleaning, nor the pen environment (feeders and drinkers) or the farm of origin had a strong influence in the microbiota establishment and development in the first weeks after weaning. Indeed, ordination analyses confirmed in the three studies the strong influence of antibiotic and ZnO treatment in the composition and functionality of the microbiota of the piglets. Despite the lack of strong association to the factors mentioned above, there were some interesting insights. For instance, feeders and drinkers microbiome composition was similar to aerobic bacteria present in the faeces of the piglets, so it can contribute to the composition of the intestinal microbiota to a certain extent. In addition, the farm impact on microbiome composition was more evident in samples collected both at weaning and two weeks after

weaning than in analyses performed a week after weaning, moment at which the impact of PWD may hinder the influence of other factors.

Different factors such as sample collection, storage, processing or sequencing, including bioinformatic analyses and statistical approach are shown to influence microbiome outputs (Wu et al., 2019; Galloway-Peña and Hanson, 2020; Poulsen et al., 2021). Different collection and storage protocols were used in this thesis. In the first study, samples were kept at 4° C for a few hours, around 4 hours, until the sampling was finished and subsequently frozen at -80° C once all samples were collected. In the second study, in commercial farms, samples were transported under cooling after collection during approximately 1 hour and further stored at -80° C. In the third study, samples were placed in dry ice as soon as they were collected, and transported to the research facilities where they were stored at - 80° C. Despite these variations in sample preservation strategy, we did not observe notable differences among study results. Previous studies assessing the influence of sample handling and storage in DNA quality and integrity of the microbiome composition did not revealed differences associated to the lapse time between collection and storage (Fouthy et al., 2015, Marques Ribeiro et al., 2018).

Another factor that could have an influence in the final microbiota output is DNA handling. We chose different approaches to prepare DNA samples across the studies included in this thesis. In the first study, sequenced DNA was extracted from a single faecal sample per library. In contrast, in the second study, libraries were prepared from a pool of two concentration-normalized DNA samples per faecal sample, reducing the potential laboratory variability in DNA extraction. Aiming at capturing pen variability, a pool of three faecal samples of the same pen per treatment and farm was prepared to extract the DNA and build the libraries in the third study. Ordination of samples results in studies 2 and 3 were similar, exhibiting a similar grouping distance patterns, despite the different initial approaches in sample preparation mentioned. Thus, it can be concluded that the impact of these factors in the final output was minimal.

Different classifiers were also used to assign taxonomy to the sequenced data (Figure 3). In the first study, we used in parallel Kraken2 as well as Metaphlan2 selecting finally Kraken2 data for the results output (Figure 3A). Notably, although both datasets had similar dominant species composition, we observed a higher number of species detected in the dataset identified with Kraken2 (most of them were either filtered or fell within the section "Others"), and a smaller number of detected species in Metaphlan2 data.

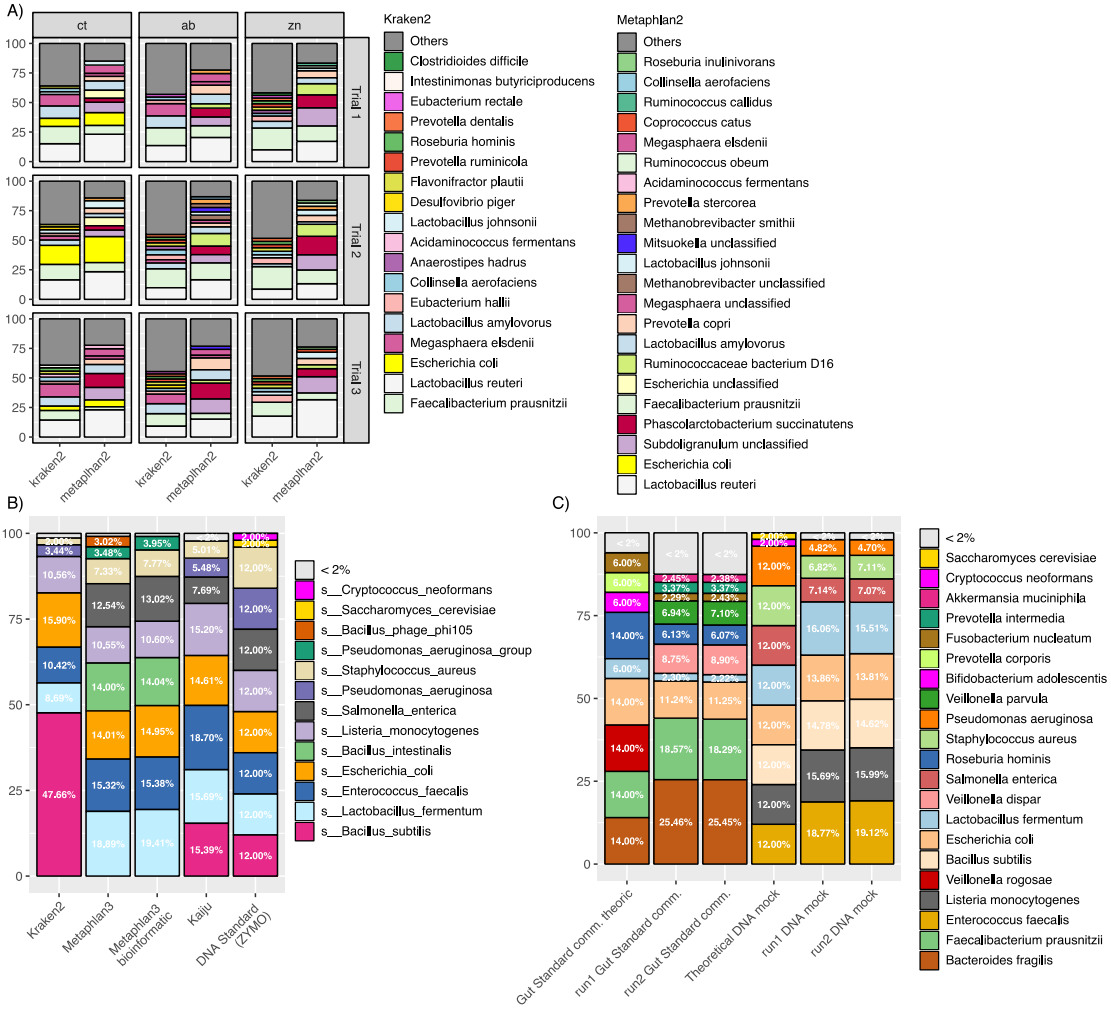


Figure 3. Taxonomic assignation of sequencing data performed throughout the three studies. A) Mean relative abundance values of species data of the first study separated by in-feed treatment, trial, and assigner (Kraken2 or Metaphlan). "Others" refers to a value of relative abundance of less than 2% of the total relative abundance per sample, Kraken2 data was previously filtered, removing taxa nor present in at least 30% of samples, with a relative abundance lower than a 0.05% per sample. B) Relative abundance species profiles of the same control library built using the DNA standard community classified with different assigners, from left to right: Kraken2, Metaphlan3 and Kaiju, sequenced along with the samples of the second study of the thesis. C) Relative abundance species profiles of different positive controls used in

the third study, from left to right: Theoretical relative abundances of the microbial gut standard community used as a positive control for DNA extraction (Gut Standard comm. theoric), libraries prepared with the microbial community sequenced in two different runs (run1 and run2), Theoretical relative abundance of the DNA standard community used in the library preparation as positive control of library preparation (Theoretical DNA mock), libraries prepared using the DNA standard community, sequenced in two different runs (Run1 and run2).

In the second study, Kraken2, Metaphlan3, and Kaiju were the chosen computational tools to profile the microbiome in piglets and farm environments (Figure 3B). In this study, a microbial human DNA standard mock community (ZymoBIOMICS™ Microbial Community DNA Standard, Zymo Research®) was used to compare the efficiency of libraries preparation and sample sequencing. Data was finally released with Metaphlan 3 classifier as it offered the most reliable microbial pattern with minor changes in *Bacillus* and *Pseudomonas* species and contrasting to Kraken2 with a bias in *Bacillus subtilis* abundance and Kaiju which identified the species with high fidelity, although with differences in their relative abundance. The same approach was followed in the third study, where Kaiju (Figure 3C) provided the most reliable results. Results were similar to those described in the second study, with mock libraries results showed nearly all the species present in the DNA standard. DNA extraction controls showed a similar pattern to the theoretical standard, exhibiting poorer performance in extracting tough-to-lyse Gram-positive bacteria (i.e., *Roseburia hominis* or *Bifidobacterium adolescentis*) and some misclassified species within the same genus (*Veillonella* spp). Therefore, an appropriate DNA extraction of all microorganisms that composes the sample and the ability of the software to classify its sequences is crucial. Previous studies have assessed the capabilities of different classifiers (Mas LLoret et al., 2020, Clooney et al., 2016). As observed in these studies, these results must be interpreted with caution and, if possible, doubtful results must be checked with other techniques such as qPCR. Our results demonstrate the usefulness of including mock communities in the analysis to evaluate the most reliable classifier for sequence analysis.

Effects of Antibiotics and ZnO in faecal microbiome

As stated at the beginning of this general discussion, the main goal of this thesis was to increase the knowledge at the most critical point of the microbiome of the pig, weaning, as well as to clarify the changes occurring in microbiome in response to ZnO and antibiotics. The exact mechanism by which ZnO effectively improves pig's health is not well understood yet. A direct antimicrobial activity, exerted locally in the gut lumen, against *E. coli* and other enterobacteria, seems to be the most plausible effect of its use (Boneti et al., 2021; Wei et al., 2021). Indeed, that mechanism of action overlaps with the other tool used to deal with PWD: antibiotics. The most commonly antibiotics used in PWD control are apramycin, amoxicillin, ceftiofur, colistin, enrofloxacin, gentamicin, neomycin and trimethoprim/sulphonamide (Fairbrother et al., 2019). Thus, we used antibiotics frequently used to treat post-weaning colibacillosis. In the first study, we used in-feed apramycin. In the second study, the Treated farms used amoxicillin 5% (Stabox) and sulphadiazine-trimethoprim (Sulfoprim 15%); whereas in the third study, we used sulphadiazine/trimethoprim (Sulfoprim) on farms A, B, and C, and amoxicillin (Stabox) on farm D. In the first study were included different treatment groups (a group treated with apramycin, another with ZnO and a third control group with no treatment); in the second study we selected farms using both ZnO and antibiotics (Treated) and compared them with farms not using in-feed Ab and ZnO; whereas in the third study we used the antibiotic that was already being used on the farm, including the same three treatment groups mentioned above. The results found in both studies showed that the three antibiotics were efficient in controlling *E. coli* diarrhoea and had a clear impact in the intestinal microbiome composition. Previous studies assessing the impact of antibiotics in pig microbiome reveal either inconsistent results, weak effects or different changes possibly linked to each antibiotic used (Poulsen et al., 2018). Indeed, major changes are reported about an increase of diverse of antimicrobial resistant genes regardless of the antibiotic used (Looft et al., 2012; Looft et al., 2014a), but this topic falls beyond the scope of this phD thesis. Our results add valuable information about how the antimicrobial use shapes

the gut microbiota. Inhibition of *E. coli* overgrowth was clear by our results, which also showed a change in the microbial population, although there was certain inconsistence among trials, the abundance of a few species was associated to Ab use, such as *Megasphaera elsdenii* and, in less extent, *Acidaminococcus fermentum* in both studies.

In the studies performed in this thesis, we observed how ZnO prevents microbiome disbalance and *E. coli* overgrowth within pig's intestinal lumen. In fact, the dominant species in diarrhoea samples collected at 7dpw from pigs medicated with ZnO was practically the same as the observed in samples from "healthy animals" from the control group, except in *Megasphaera* spp., abundance that was impaired by ZnO. From these results, we can hypothesize that ZnO helps to maintain a microbial balance within the community, maintaining the stability of the microbiome, even in animals suffering diarrhoea. The antimicrobial activity of ZnO against microbial groups cannot be entirely addressed in this thesis due to the limitations in information about sensitivity to ZnO in non-culturable bacteria. Although there is information in literature about in-vitro antimicrobial effects against some groups of bacteria, especially Gram-positive bacteria, we cannot conjecture about antimicrobial effects of ZnO within the intestine.

Non-antimicrobial effects of ZnO in the intestinal microbiome

The intestine is an ecosystem for a microbial community composed of a myriad of microorganisms which are interconnected and interact with the host as well, thereby influencing and cooperating in physiological functions such as the digestion or immune response, thus contributing to animal health and general homeostasis (Tremaroli and Bäckhed., 2012). In the same way, ZnO improves pigs productive performance, intestinal barrier integrity, and local immune response in the intestine (Kwon et al., 2014; Grilli et al., 2015; Li et al., 2018, Li et al., 2021; Bonetti et al., 2021; Wei et al., 2021; Dowley et al., 2022; Sun et al., 2022b; Tang et al., 2022; Venardou et al., 2022). Whether these beneficial effects are triggered directly in the animal or via interaction of ZnO-modified microbiota will require further research. Apart from

the direct antimicrobial effect, ZnO targets different functions performed locally at gut lumen (Bonetti et al., 2021; Sales., 2013), and from there to the rest of the organism. In this regard, intestine may be seen as a matrix from which all functions affected by ZnO are inter-connected, and due to this multi-target effect, the elucidation of its whole mechanism of action in the organism is more complex. This mechanism might not be ascribed to a single effect but rather to a set of processes and/or structures which get modified in the intestine, or on the other hand, its effect in just a part of the processes which are affected, triggering a cascade of linked effects. A well stablished/functional community microbiome is known to perform many beneficial or even essential functions for the host (Tremaroli and Backhed., 2012; Brestoff and Artis; 2013), that can become a problem if it gets unbalanced (Gresse et al., 2017, Fassarella et al., 2018). There are several problems occurring at weaning leading to PDW. One of these factors considered in PWD onset is the microbiome composition. The alteration of this community is caused by other factors such as the first anorexia and the gut inflammation state occurring in the first 24-48 hours post-weaning (Gresse et al., 2017; Lallès et al., 2007). Results obtained in this thesis suggest that ZnO contributes to maintain microbial stability during the first 2 weeks post-weaning. Indeed, several authors suggested that ZnO may promote this effect on certain group of bacteria such as coliform (Katouli et al., 1999; Vahjen, Pieper and Zentek, 2011), yet the inhibiting effect of ZnO in enterobacteria in the studies of this thesis is clear, both in faecal and diarrhoea samples. Microbial stabilization effect might be caused by some of the other effects of ZnO in the inter-connected grid of described effects for it in the intestine and subsequent effects in the rest of the organism, as well as its direct effect in the microbial community, such as inhibition of metabolic processes, adhesion, biofilm formation, metals receptor blockade, bile secretion, bacteria competition and cooperation, improved digestibility (Hedemann, Jensen and Poulsen, 2006; Klemm, Vejborg and Hancock, 2010; Gielda and DiRita, 2012; Wu et al., 2013; Grilli et al., 2015; Ye et al., 2020; Bonetti et al., 2021). Deeper studies combining microbiome, immune response, digestibility, and performance exploration will be

needed to disclose the mechanism of action of ZnO in order to emulate its protective effects at weaning.

Functional microbiome characterization insights in PWD

Based on the results obtained in this thesis, in PWD onset *E. coli* takes advantage of initial unstable state of the microbiota and thrives in this hostile environment to impair other bacteria. Under these favourable conditions, the pathogen is able to multiply and cause disease. The functional data derived from the studies 1 and 3 revealed genes associated with virulence of *E. coli*. In the first study, the differences on the presence of *E. coli* virulence factors among groups were confirmed directly by screening assembled reads in contigs (using Abricate, Seemann T, Abricate, Github https://github.com/tseemann/abricate) and unassembled reads using SUPER-FOCUS. In study 3, the genes associated to virulence functions were identified using a read approach, and could be linked to the higher abundance of *E. coli* in 7dpw diarrhoea samples from the control group (which levels of abundance were higher). Although the involvement of these virulence factors in diarrhoea outcome cannot demonstrate by these analyses, their presence in higher abundance might be, at least, interpreted as a risk or as a biomarker of intestinal sickness. Among the observed functional results, most of the consistently virulence associated categories observed in control group samples belonged to adhesion, biofilms and siderophores. In this respect, Roselli et al., (2003) demonstrated ZnO inhibited ETEC adhesion and invasion to Caco2 cells, and Wu et al., (2013) revealed that ZnO inhibits biofilm formation of *E. coli* and *Salmonella* among others. Indeed, these three categories are linked traits (Klemm et al., 2010). Biofilm formation is an important step on adherence to organic and inorganic surfaces, which is important to exert pathogenesis. Siderophores inhibition by Zn has been described previously, by means of receptors metal interference of other metals by Zn, which binds with higher affinity to receptor ligands of other metals such as Fe, Mn and Mg (Hancock et al., 2010; Klemm et al., 2010; McDevitt et al., 2011; Wątły et al., 2016; Sheldon and Skaar., 2019). To our knowledge, these are two of the first studies characterizing virulence factors and

virulence related functional categories in PWD using whole metagenome shotgun sequencing. Boeckman et al., (2022) characterized the virulence factors of a pathogenic strain of *E. coli* isolated from a pig exhibiting clinical signs of oedema disease and subsequently challenged a group of pigs with this strain. In the studies conducted in this thesis, we identified virulence related traits directly from samples obtained in field conditions.

Microbiota establishment and development at weaning

Shotgun metagenome sequencing enables accurate taxonomic identification at species level, as well as functional profiling of microbiome communities (Quince et al., 2015). In this thesis, we observed the dynamics of pigs' gut microbial populations in the first two weeks post-weaning, meaningful insights in microbiome ecology of piglets treated with ZnO or antibiotics, as well as in situations of dysbiosis. In this sense, the three studies carried out in this thesis also offer a valuable information in the characterization of the microbiome at weaning. Furthermore, the analysis of diarrhoea samples allow to determine differences in microbiota composition between normal and diarrhoea samples within PWD context. We also provided a glance in functional microbiome profiling of weaned pigs, focusing on the most problematic days of this period. Among the most interesting findings, it is worthy to mention the observed balance in the gut microbiota at weaning, where the dominance of groups that metabolize milk oligosaccharides, mostly *Bacteroides* (Chen et al., 2018; 2019; Wang et al., 2019b) was not observed anymore after weaning. Instead, an increase of *E. coli* was observed. In this first two weeks post-weaning there seems to be a transition of microorganisms, from an evenly distributed milk-oriented microbiome at 0dpw, to a complex-carbohydrate adapted microbiome at 14dpw, being the first week post-weaning the transition between these two stages and the most critical period for PWD outbreaks. Indeed, on farms studied in the third study, we were not able to collect enough diarrhoea samples at day 14dpw (Figure 4), suggesting the first week post-weaning as a period of microbiome instability which can be exploited by *E. coli*. Compared to previous studies using 16S rRNA sequencing, our results are able to fine tune the microbiota

composition up to species level. This taxonomic characterization demonstrates, for instance, that different *Prevotella* and *Lactobacillus* species are involved in the microbiota transition at weaning and that strict anaerobes such as *Megasphaera elsdenii* or *Faecalibacterium praunitzii* require that other colonizers are established before (Wang et al., 2019b). In addition, the characterization of the faecal microbiome in diarrhoea samples a week after weaning, allowed us to observe that their composition resembled faeces after weaning with an *E. coli* dominance in non-treated animals. Thus, PWD not only impairs intestinal function, nutrient and water absorption, but the maturation of the intestinal microbiota, which may affect piglets growth later on. By the results obtained in these three studies and, in line with previous research on this topic (Wei et al., 2020; Juház et al., 2022; Tang et al., 2022; Zhang et al., 2022; Sun et al., 2022b; Pieper et al., 2020; Xu et al., 2022; da Silva et al., 2021; Li et al., 2021), we conclude that antibiotics and ZnO treatment exhibited similar effects with differences in the presence of particular taxa, either promoting species within the Bacteroidales order (ie., *Prevotella* spp; *Bacteroides* spp, *Parabacteroides* spp), or reducing *Megasphaera* and Veillonellaceae abundance in pigs treated with ZnO. This reduction of abundance in species within Veillonellaceae was an effect specifically linked to ZnO treatment in the three performed studies.

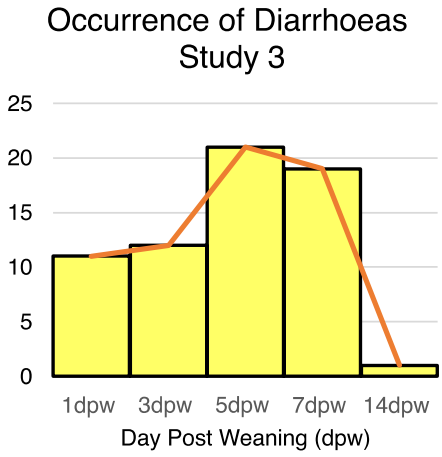


Figure 4. Histogram showing occurrence of diarrhoea samples collected in the third study of this phD.

As complex as they can be the effects triggered by ZnO, another possible effects may be stimulation of feed intake (Sales, 2013). Stress generated during weaning is followed by a short period of anorexia that leads to gut inflammation, that opens a window of opportunity to enteric pathogens to invade the gut. This anorexia and lack of nutrients in the intestine, along with the associated inflammation is the main perturbance occurring in microbiome at weaning, that can become unstable if pathogens take advantage of the situation, by overcoming the unfavourable conditions in the gut caused by inflammation and blooming, displacing the general microbiota of their niches (Gressse et al., 2017). The early and cumulative feed consumption might be one of the causes of microbial stability maintenance, along with the effects of ZnO in the gut environment. In fact, studies conducted in zinc-deficient rats improved feed intake only when administered zinc sulphate orally, this feed intake was not improved when administered zinc sulphate intraperitoneally (Elise et al., 2010). Moreover, there is also evidence that weaned pigs may be subjected to a transient zinc deficiency at weaning (Davin et al., 2013). Other studies performed in pigs at weaning confirmed higher feed intake in in-feed ZnO medicated pigs (Hu et al., 2013; Sales, 2013). At the same time, several studies reported Zn effects in normal mood restoration in rodents (Elise et al., 2010).

Future studies should confirm and add knowledge to these findings, through the use of further sequencing resources, algorithms and meaningful analyses, in order to expand the insights observed here. One of the objectives of this thesis was to study which changes were occurring in pigs microbiome at weaning, when they were treated with ZnO, in order to try to seek alternatives to the use of it by emulating its effect in gut microbiome. Future alternatives that maintain the structure and functionality of microbiome as ZnO seems to do may serve as alternative to facilitate weaning transition and prevent PWD in the first weeks post-weaning, thus attempting to maintain piglets microbiome composition similar to those in the branches of the clusters with an adult-like resembling microbiome, ensuring a smooth transition between a milk-oriented microbiome and the adult-like microbiome.

Chapter 6. Conclusions

The conclusions raised from the results presented in this Ph.D., are:

1. Pigs' microbiome rapidly evolves from an evenly distributed group of milk-oriented-microbiome species towards complex carbohydrate utilizing taxa such as *Prevotella* spp. This shift of species occurs via succession of within-genus species as well. Slight differences in species abundance can be attributed to in-feed antibiotic and ZnO treatment with species within Bacteroidaceae family such as *Prevotella* spp or *Bacteroides* spp increased in abundance in ZnO treated pigs and *Megasphaera elsdenii* from the familiae veillonellaceae more abundant in antibiotic treated pigs.
2. Microbial community broad functions are maintained across the two weeks post-weaning, reflecting a high resilience and functional redundancy capabilities, and are slightly compromised in pigs with diarrhoea, particularly in non-medicated pigs, which show a higher number of virulence-related functions.
3. In-feed antibiotics and ZnO treatments prevent microbial community disbalance by inhibiting *E. coli* overgrowth during the first two weeks post-weaning, promoting the transition to a stable microbiome composition with biomarkers of an adult-like microbiome. These differences were also evident in diarrhoea samples a week after weaning, while *E. coli* dominated the abundance in non-treated animals, other species such as *Lactobacillus* were predominant in animals treated either with antibiotics or ZnO.
4. Weaning and diarrhoea occurrence showed the most marked effect on the microbiome, followed by antibiotic and ZnO treatments. The effects of farm of origin, the environmental microbiota analysed by feeders and drinkers microbiome and different cleaning protocols had a weaker influence in the microbiome of the piglets.

5. Microbiome analyses evidenced differences in weaned piglet's microbiome transition between farms that needed the use of in-feed antibiotics and ZnO and farms that did not, during the two weeks post-weaning, being particularly influenced by these in-feed treatments.
6. Diarrhoea of ZnO treated pigs microbiome composition at seven days post-weaning resembles to "healthy" pigs species pattern, compared to microbiome composition from non-medicated pigs, thereby suggesting a protection mechanism from the perturbances caused by weaning and/or *E. coli* overgrowth.

Conclusiones

Las conclusiones alcanzadas a partir de los resultados presentados en esta tesis doctoral son:

1. El microbioma de los cerdos evoluciona rápidamente de un grupo uniformemente distribuido de especies asociadas al microbioma de la leche hacia taxones capaces de utilizar carbohidratos complejos como *Prevotella* spp. Este cambio de especies también ocurre a través de la sucesión de especies dentro del mismo género. Las pequeñas diferencias observadas en la abundancia de especies pueden atribuirse a los tratamientos en el pienso, con especies dentro de la familia *Bacteroidaceae* como *Prevotella* spp o *Bacteroide*s spp, que aumentaron en abundancia en animales tratados con ZnO, y *Megasphaera elsdenii* de la familia Veillonellaceae más abundante en cerdos tratados con antibióticos.
2. Las funciones generales de la comunidad microbiana se mantienen a lo largo de las dos semanas posteriores al destete, lo que refleja una alta capacidad de resiliencia y redundancia funcional, y se ve ligeramente comprometida en animales con diarrea, particularmente en aquellos cerdos no medicados, en los que se detectó un microbioma con mayor número de funciones asociadas a virulencia.
3. La inclusión de ZnO y los tratamientos antimicrobianos en el pienso previenen el desequilibrio de la comunidad microbiana al inhibir el crecimiento excesivo de *E. coli* durante las dos primeras semanas pos-destete, promoviendo la transición hacia una composición del microbioma estable con biomarcadores de un microbioma similar a un adulto. Estas diferencias también fueron evidentes en las muestras de diarrea una semana después del destete, mientras que *E. coli* dominó la abundancia en los animales no tratados, otras especies como *Lactobacillus* predominaron en los animales tratados con antibióticos o ZnO.

4. El destete y la diarrea mostraron los efectos más pronunciados sobre el microbioma, seguido de los antibióticos y el ZnO. Los efectos de la granja de origen, el microbioma ambiental analizado a través del microbioma de los comederos y bebederos, así como los diferentes protocolos de limpieza tuvieron una menor influencia en el microbioma de los lechones.
5. Los análisis del microbioma evidenciaron diferencias en la transición del microbioma de los lechones destetados entre granjas que necesitaban incluir antibióticos y ZnO en el pienso y granjas que no lo necesitaban, durante las dos semanas pos-destete, siendo esta transición particularmente influenciada por estos tratamientos.
6. La composición del microbioma la diarrea de animales tratados con ZnO a los siete días pos-destete es similar a un patrón de especies observado en cerdos “sanos”, comparado con la composición del microbioma de cerdos no medicados, lo que sugiere un mecanismo de protección contra las perturbaciones causadas por el destete y/o el crecimiento excesivo de *E. coli*.

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Annex 1. Supplemental material

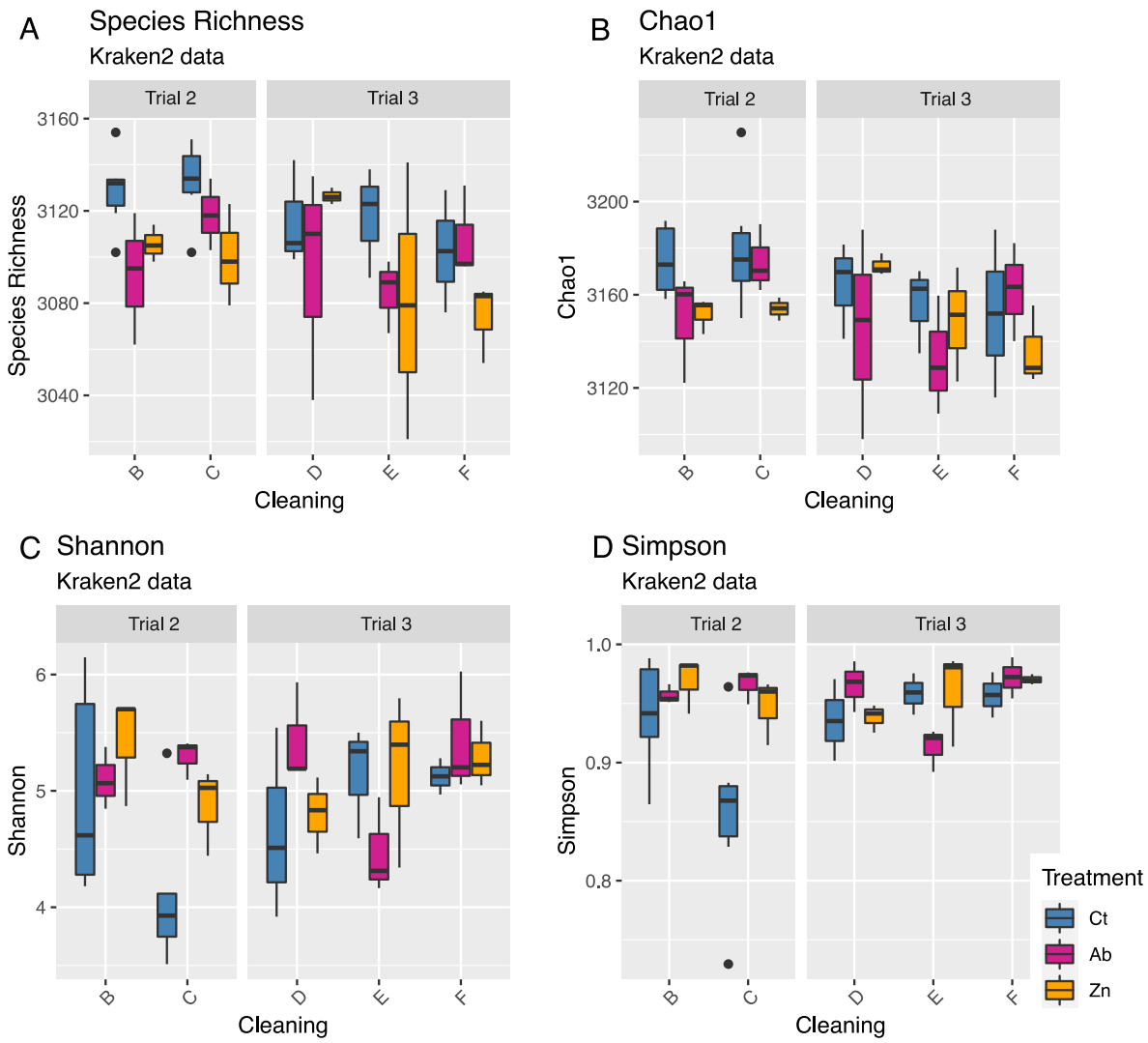
Chapter 2. Study I: Using Shotgun Sequencing to Describe the Changes Induced by In-Feed Zinc Oxide and Apramycin in the Microbiomes of Pigs One Week Postweaning

Chapter 2. Study I. Supplementary Table S1. Analysis (*envfit* function of Vegan) of the influence of different factors on the ordination of samples. Significance was established at α=0.05.

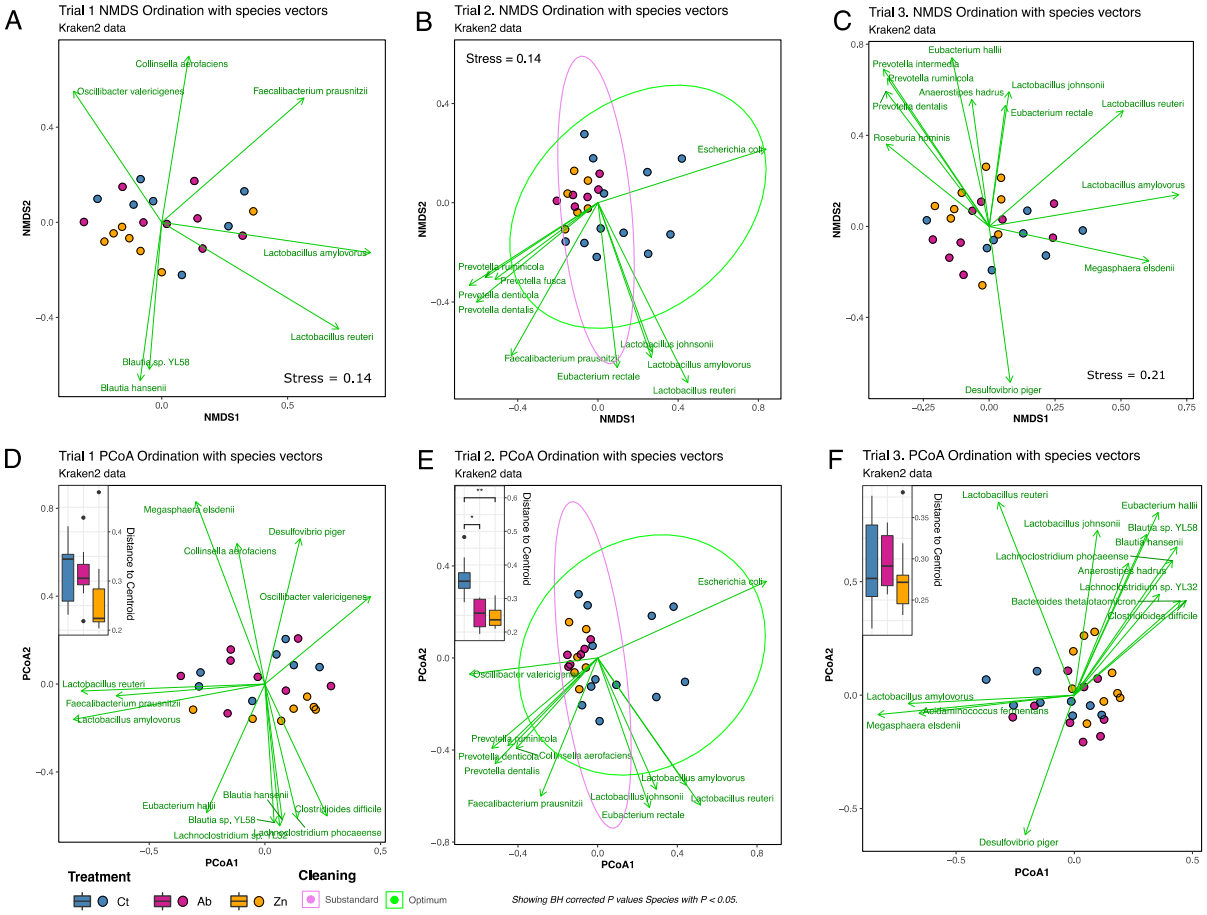
Data	Factor	Ordination Method P. value (R2) Kraken2	
		PCoA	**NMDS**
Global	Treatment	0.041 (0.071)	0.190 (0.043)
Trial 1	Treatment	0.111 (0.175)	0.444 (0.085)
Trial 2	Treatment	0.039 (0.209)	0.022 (0.221)
Trial 3	Treatment	0.007 (0.264)	0.104 (0.155)

Chapter 2. Study I. Supplementary Table S2. Results of the permutation multivariate ANOVA test performed in the ordination analysis.

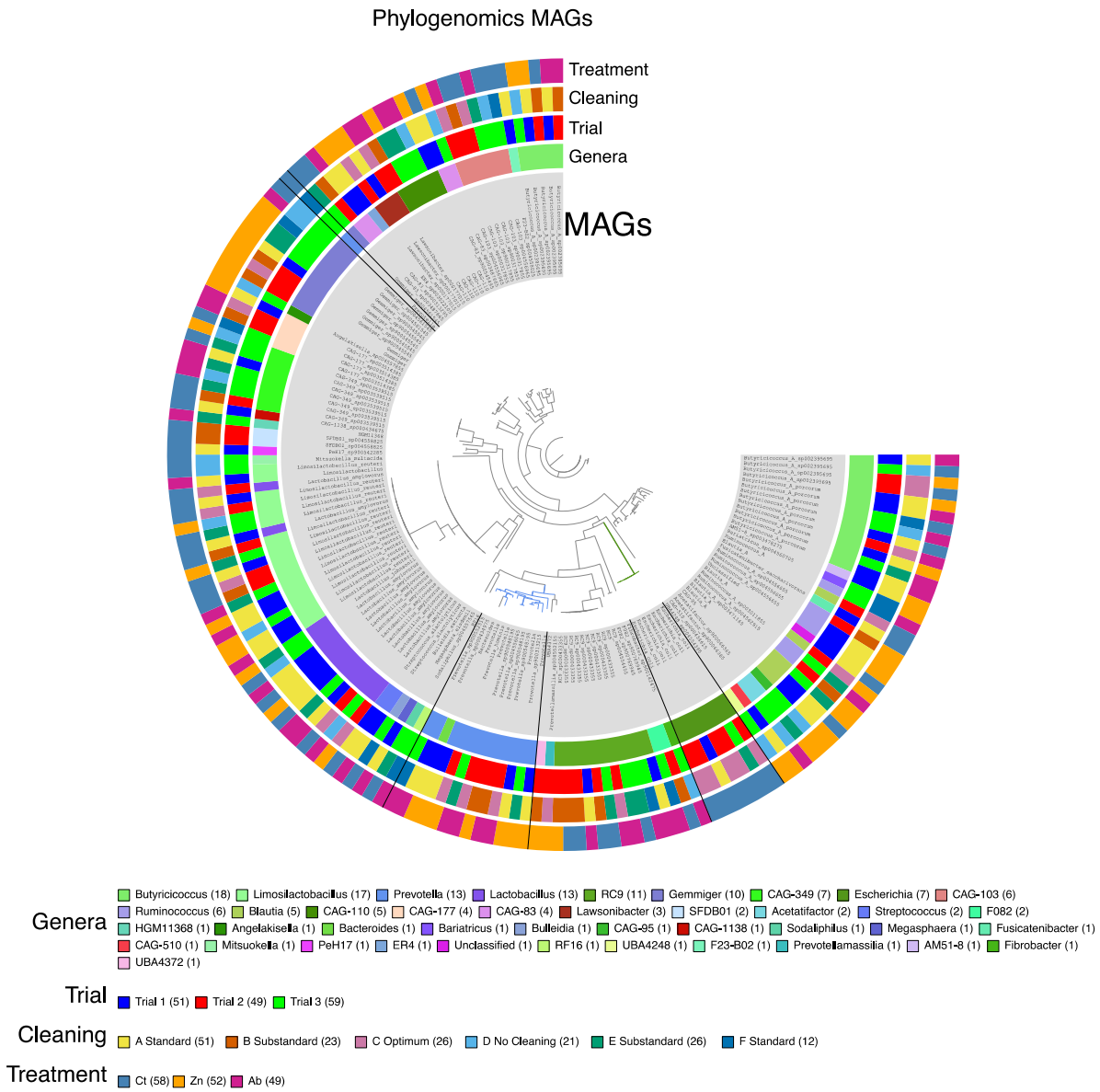
Data	Factor	P. value (R2)
Global analysis	Treatment	0.001 (0.109)
	Ct vs Ab	0.009 (0.054)
	Ct vs Zn	0.002 (0.121)
	Ab vs Zn	0.002 (0.074)
Trial 1	Treatment	0.068 (0.156)
	Ct vs Ab	0. 510 (0.059)
	Ct vs Zn	0.069 (0.181)
	Ab vs Zn	0.127 (0.133)
Trial 2	Cleaning	0.029 (0.088)
	Treatment	0.003 (0.169)
	Ct vs Ab	0.063 (0.118)
	Ct vs Zn	0.063 (0.139)
	Ab vs Zn	0.614 (0.076)
	Treatment:Cleaning	0.180 (0.095)
Trial 3	Cleaning	0.139 (0.097)
	Treatment	0.011 (0.148)
	Ct vs Ab	0.399 (0.064)
	Ct vs Zn	0.018 (0.168)
	Ab vs Zn	0.036 (0.121)
	Treatment:Cleaning	0.203 (0.167)



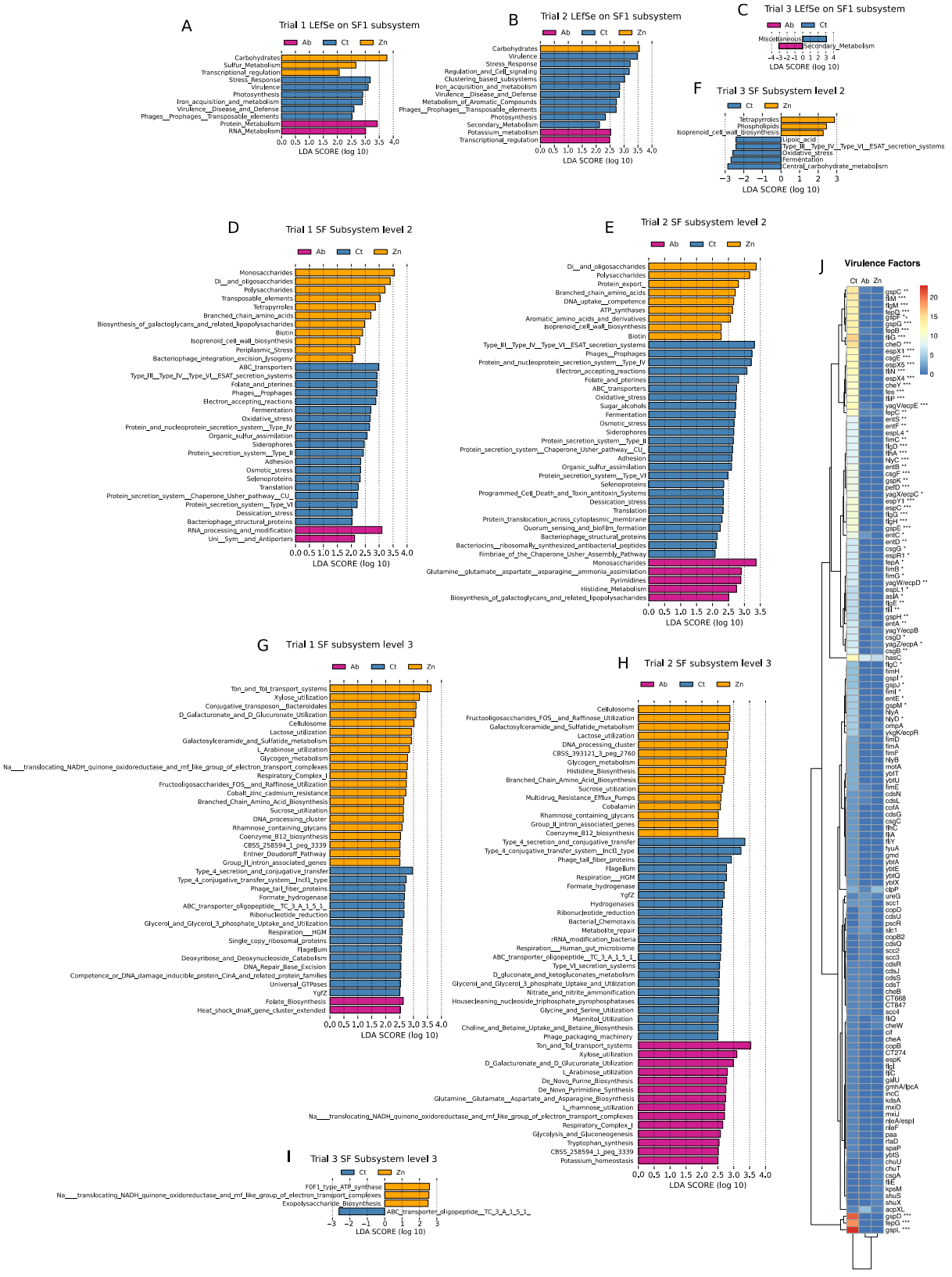
Supplementary Figure S1. Alpha diversity of different dietary treatments microbiomes, separated by cleaning procedures applied in trials 2 and 3, measured by Microbial richness (Species richness, Chao1 and Shannon) and evenness (Simpson index). The lower, medium, and upper horizontal box lines correspond to the first, second and third quartiles (the 25th, 50th and 75th percentiles). Upper and lower whiskers include the range of the upper and lower points within the 1.5 interquartile range. A) Species richness; B) Chao1 index; C) Shannon diversity index; D) Simpson's diversity index. *P < 0.05, **P < 0.01, and ***P < 0.001, respectively. Taxonomic identification of sequences was performed using Kraken2.



Supplementary figure S2. Ordination of microbiomes performed with NMDS, for trial 1, 2 and 3 (A, B, and C, respectively); and PCoA, for trials 1, 2 and 3 (D, E and F, respectively), with samples coloured by dietary treatment. Green arrows display the species returned by "envfit" model, influencing the ordination of samples (Arrows showing BH p.adjusted significant species; Arrows length shows the strength of each specie influencing the ordination of samples). Ellipses drawn on Figures B and E represent each cleaning group, with their shape being defined by the covariance within each group. Taxonomic identification of sequences was performed using Kraken2.



Supplementary Figure S3. Metagenome-Assembled genomes (MAGs) of samples using MEGAHIT. Seven genomes of *E. coli* found in Ct dietary treatment samples, as well as 12 genomes of *Prevotella* spp. in Ab and Zn, and 1 in Ct, are highlighted within 2 black bars going from inner to outer part of the circle.



Supplementary Figure S4. Microbial functions differentially abundant identified for each dietary treatment group using LEfSe (A-I). (A-C) Results for the analysis performed for trial 1(A), 2 (B) and 3 (C) in level 1 of Super-Focus data. (D-F) Results for the analysis performed for trial 1(D), 2 (E) and 3 (F) in level 2 of Super Focus data. (G-I) Results for the analysis performed for

trial 1(G), 2 (H) and 3 (I) in level 3 of Super Focus data. (J) Heatmap showing virulence factors identified for each dietary treatment. *P < 0.05, **P < 0.01, and ***P < 0.001, respectively.

Chapter 3. Study II: Comparing the microbiome of post-weaning pigs in farms using, or not using, in-feed zinc oxide and antibiotics

Chapter 3. Study II. Supplementary Table S1. Analysis (*envfit* function of Vegan) of the influence of different factors on the ordination of samples.

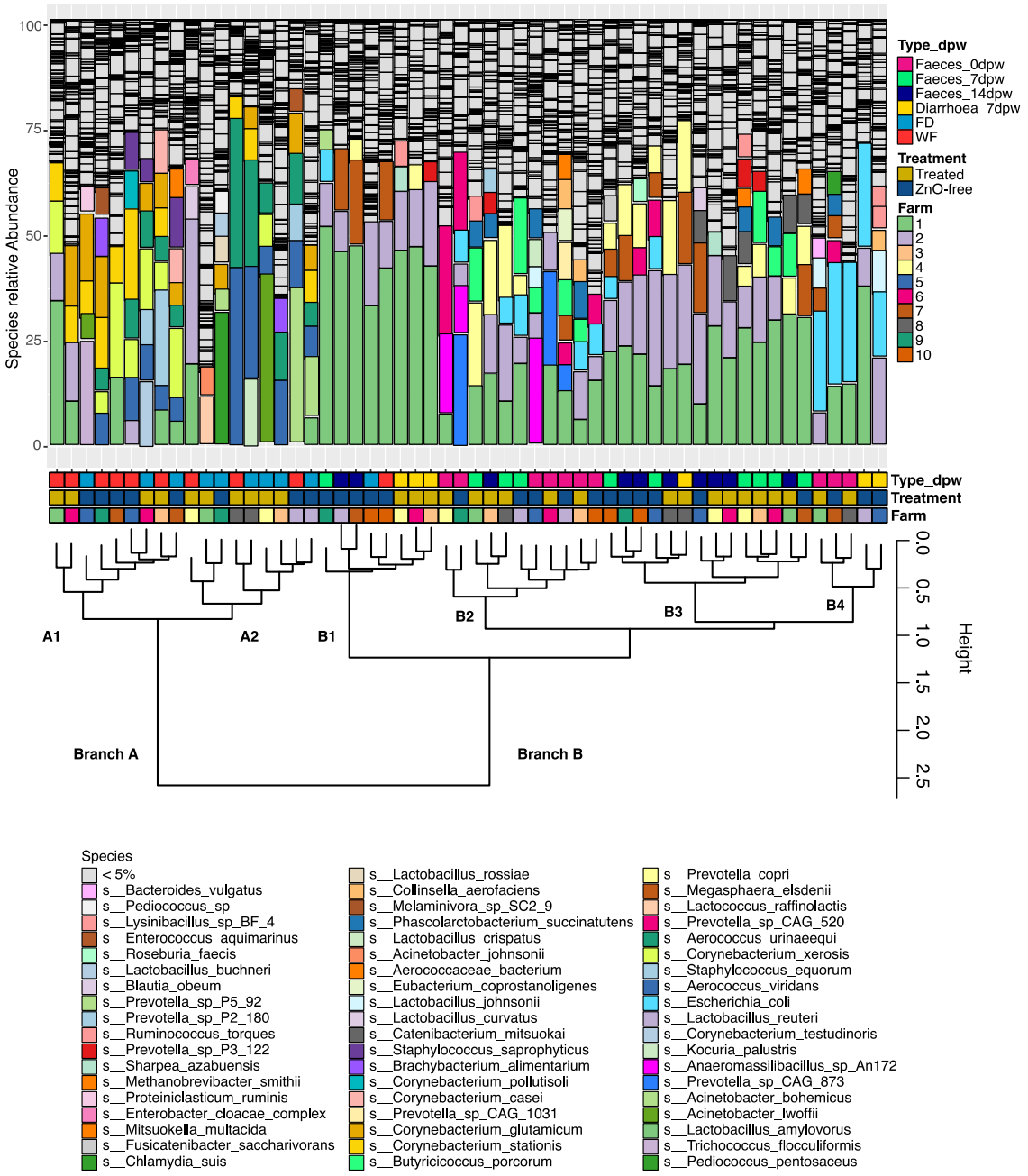
Factor	Species		Pathways	
	R^2	p.val	R^2	p.val
Farm	0.035	0.999	0.055	0.999
Type	0.672	0.001	0.583	0.001
Dpw	0.256	0.001	0.330	0.001
Treatment	0.004	0.786	0.003	0.821
Type_dpw	0.762	0.001	0.610	0.001
Type_dpw_treatment	0.789	0.001	0.674	0.001

Chapter 3. Study II. Supplementary Table S2. Results of the permutation multivariate ANOVA test performed in the ordination analysis.

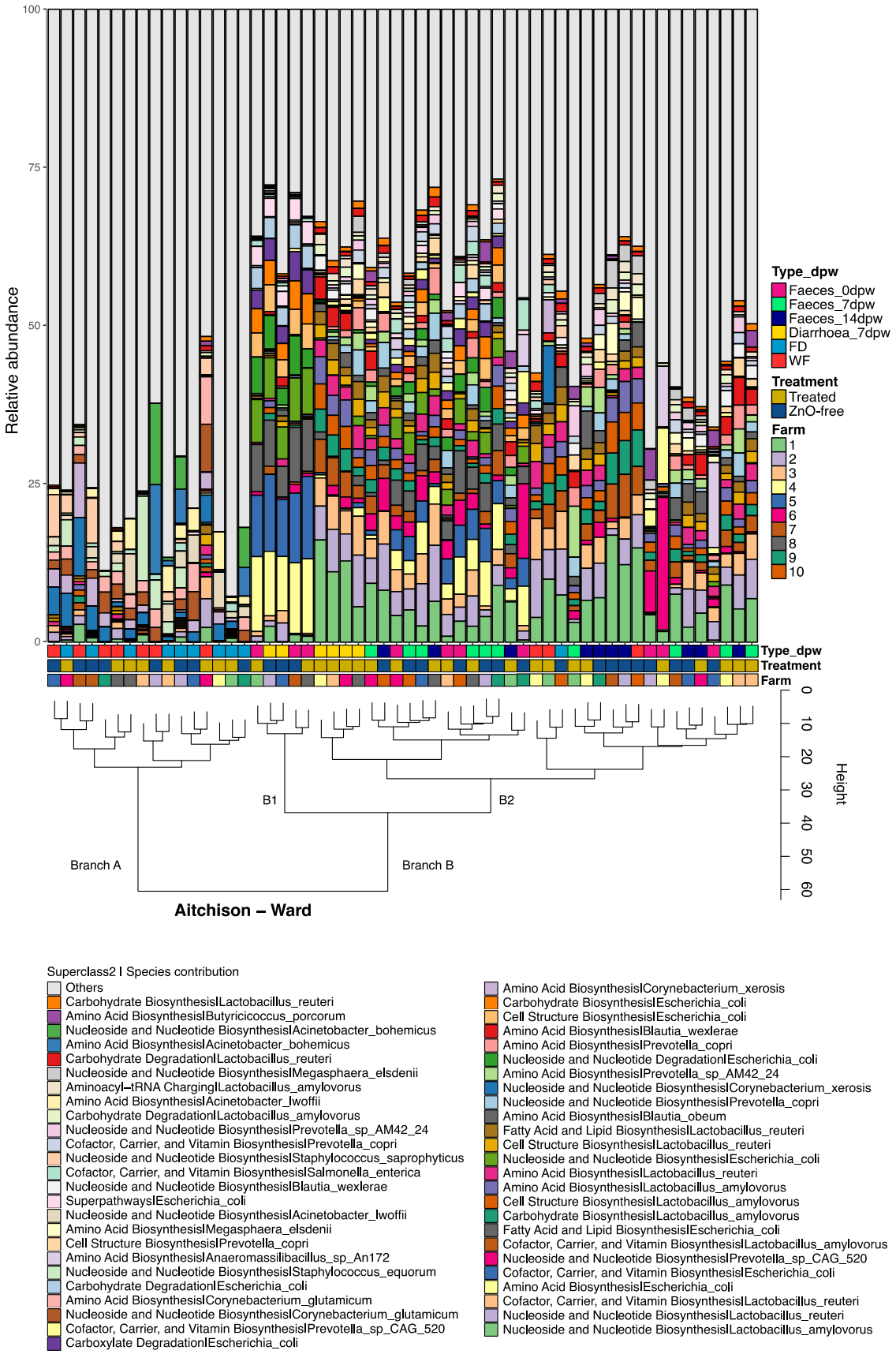
Factor/Levels	Species		Pathways	
	R^2	p.val	R^2	p.val
Treatment	0.010	0.294	0.014	0.324
Type_dpw	0.560	0.001	0.318	0.001
Faeces 0dpw vs Faeces 7dpw	0.252	0.002	0.094	0.059
Faeces 0dpw vs Faeces 14dpw	0.359	0.002	0.156	0.002
Faeces 0dpw vs Diarrhoea 7dpw	0.405	0.003	0.133	0.052
Faeces 0dpw vs WF	0.507	0.002	0.209	0.002
Faeces 0dpw vs FD	0.561	0.002	0.272	0.002
WF vs FD	0.065	0.280	0.072	0.117
Faeces 7dpw vs Diarrhoea 7dpw	0.217	0.009	0.100	0.110
Faeces 7dpw vs Faeces 14dpw	0.081	0.157	0.066	0.144
Faeces 7dpw vs WF	0.495	0.002	0.257	0.002
Faeces 7dpw vs FD	0.584	0.002	0.336	0.002
Diarrhoea 7dpw vs Faeces 14dpw	0.133	0.076	0.121	0.033
Diarrhoea 7dpw vs WF	0.377	0.002	0.238	0.002
Diarrhoea 7dpw vs FD	0.479	0.003	0.307	0.003
Faeces 14dpw vs WF	0.456	0.002	0.273	0.002
Faeces 14dpw vs FD	0.560	0.002	0.354	0.002
Type_dpw:Treatment	0.046	0.388	0.086	0.068
Diarrhoea 7dpw ZnO-free vs Diarrhoea 7dpw Treated	0.480	0.070	0.541	0.110

Chapter 3. Study II. Supplementary Table S3. Results of analysis of relative abundances between type and dpw levels.

Species	Type and dpw comparison	*P*
Lactobacillus amylovorus	Faeces_0dpw vs Faeces_7dpw	0.006
	Faeces_0dpw vs Faeces_14dpw	0.003
Lactobacillus reuteri	Faeces_0dpw vs Diarrhoea_7dpw	0.006
	Faeces_0dpw vs Faeces_14dpw	0.015
Escherichia coli	Faeces_0dpw vs Faeces_14dpw	0.006
Prevotella copri	Faeces_0dpw vs Faeces_7dpw	*P* < 0.001
	Faeces_0dpw vs Faeces_14dpw	*P* < 0.001
Prevotella sp. CAG 520	Faeces_0dpw vs Faeces_7dpw	0.043
	Faeces_0dpw vs Faeces_14dpw	0.037
	Faeces_0dpw vs Diarrhoea_7dpw	0.001
	Faeces_7dpw vs Diarrhoea_7dpw	0.033
Anaeromassilibacillus sp. An172	Faeces_0dpw vs Faeces_7dpw	*P* < 0.001
	Faeces_0dpw vs Faeces_14dpw	*P* < 0.001
	Faeces_0dpw vs Diarrhoea_7dpw	*P* < 0.001
Prevotella sp. CAG 873	Faeces_0dpw vs Faeces_7dpw	*P* < 0.001
	Faeces_0dpw vs Faeces_14dpw	*P* < 0.001
	Faeces_0dpw vs Diarrhoea_7dpw	0.001
Catenibacterium mitsuokai	Faeces_0dpw vs Faeces_7dpw	0.009
	Faeces_0dpw vs Faeces_14dpw	0.016
Ruminococcus torques	Faeces_0dpw vs Faeces_7dpw	0.010
	Faeces_0dpw vs Faeces_14dpw	0.010
	Faeces_0dpw vs Diarrhoea_7dpw	0.010
Prevotella sp. P3 122	Faeces_0dpw vs Faeces_7dpw	*P* < 0.001
	Faeces_0dpw vs Faeces_14dpw	0.004
	Faeces_7dpw vs Faeces_14dpw	0.037
	Faeces_0dpw vs Diarrhoea_7dpw	0.037
	Faeces_7dpw vs Diarrhoea_7dpw	0.037
Methanobrevibacter smithii	Faeces_0dpw vs Faeces_7dpw	0.018
	Faeces_0dpw vs Faeces_14dpw	0.006
	Faeces_0dpw vs Diarrhoea_7dpw	0.001
Lactobacillus johnsonii	Faeces_0dpw vs Faeces_7dpw	0.034
Bacteroides vulgatus	Faeces_0dpw vs Faeces_7dpw	*P* < 0.001
	Faeces_0dpw vs Faeces_14dpw	*P* < 0.001
	Faeces_0dpw vs Diarrhoea_7dpw	*P* < 0.001
Blautia obeum	Faeces_0dpw vs Faeces_7dpw	0.015
	Faeces_0dpw vs Faeces_14dpw	0.009

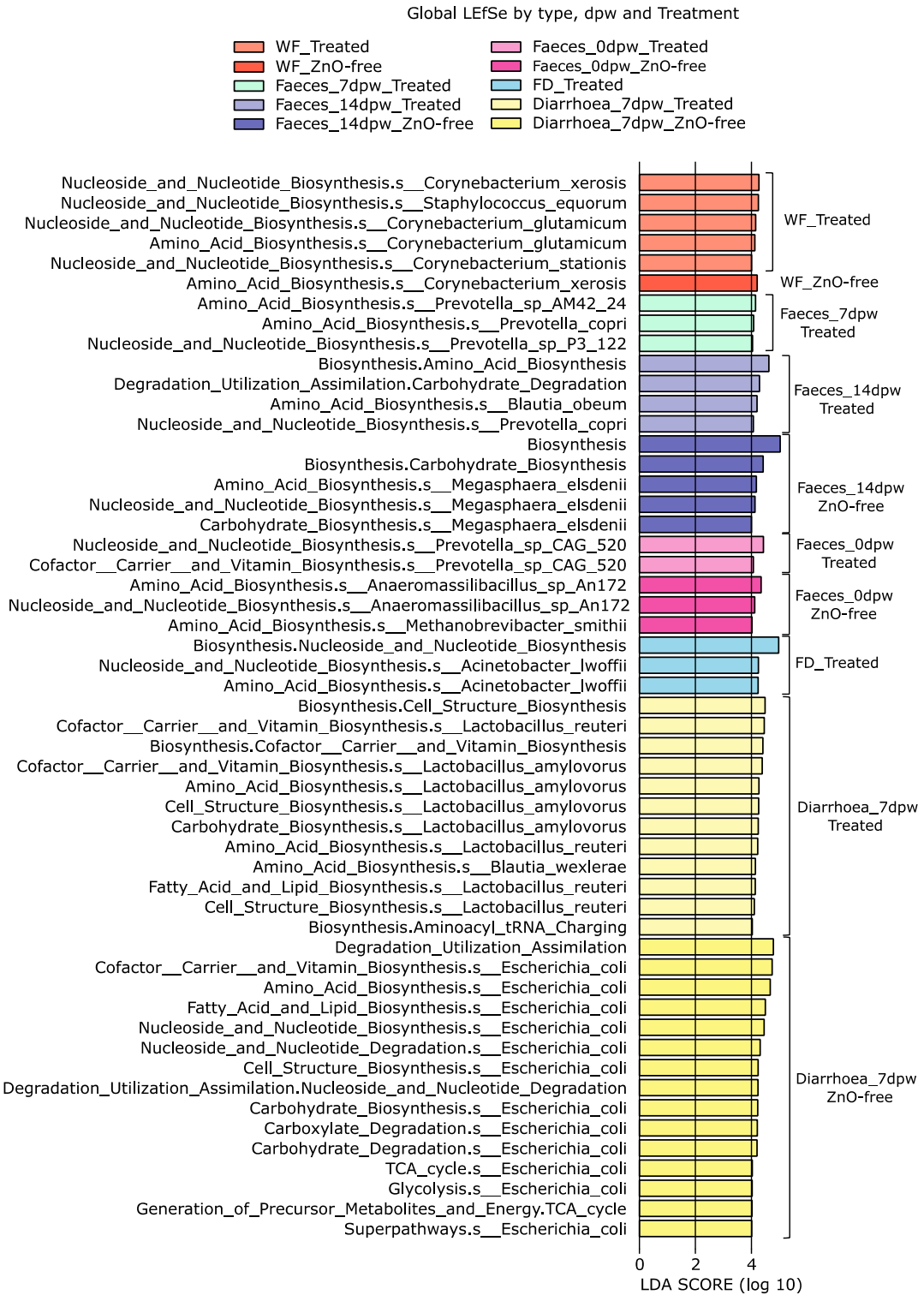


Chapter 3. Supplementary Figure S1. Stacked bar plot of the relative abundance of the main species in each sample from 10 commercial farms. Profiles of samples are ordered by Ward clustering of the squared Weighted Unifrac distances between samples. Cluster dendrogram represents the similarity between samples regarding its microbial composition. Variables information in each sample (from lower to upper level: Farm, Treatment, Type-dpw) are indicated in the coloured squares below the bars. Taxonomic identification of sequences was performed using Metaphlan3.



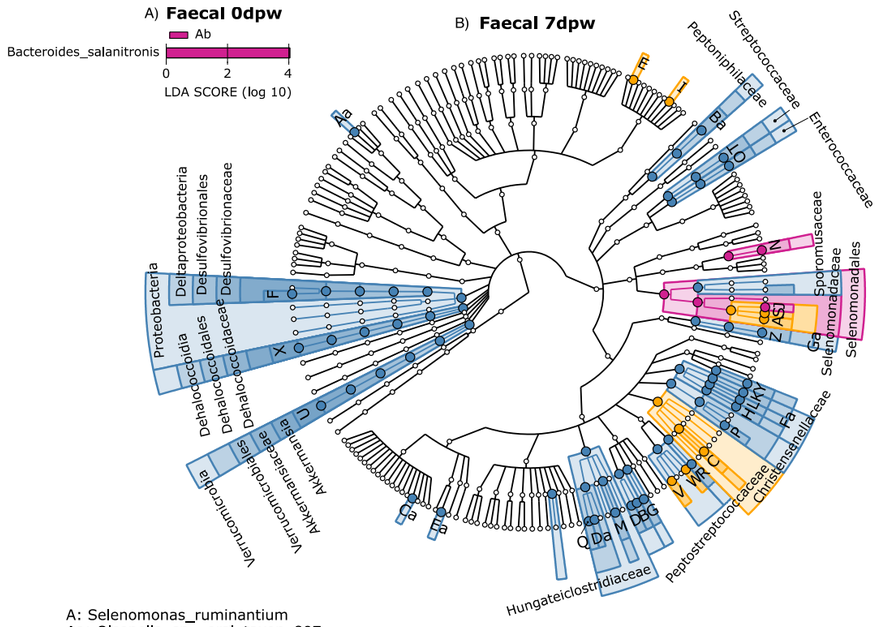
Chapter 3. Supplementary Figure S2. Stacked bar plot of relative abundance values, showing the species contribution to each metacyc superclass2 grouped pathways in environmental and faecal samples from weaned pigs. Samples are clustered by their (continues in next page)

functional abundance profile using Ward clustering and the squared Aitchison distances between samples. Cluster dendrogram represents the similarity between samples regarding its functional and species composition. Variables information in each sample (from lower to upper level: Farm, Treatment, Type and day post-weaning) are indicated in the coloured squares below the bars. Metabolic profiling was performed using HUMAnN3.



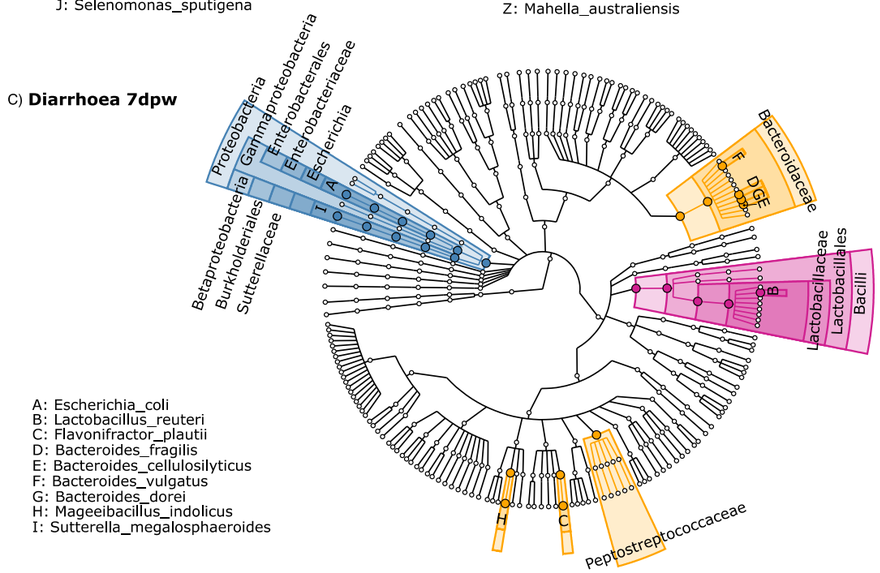
Chapter 3. Supplementary Figure S3. Differences in superclass2 grouped pathways and species abundance, returned by LEfSe analysis, most likely explaining the differences among dietary treatments in each sample type and day post-weaning.

Chapter 4: Study III: Sequencing the microbiome of post-weaning pigs in commercial farms that failed to remove in-feed antibiotics and zinc oxide

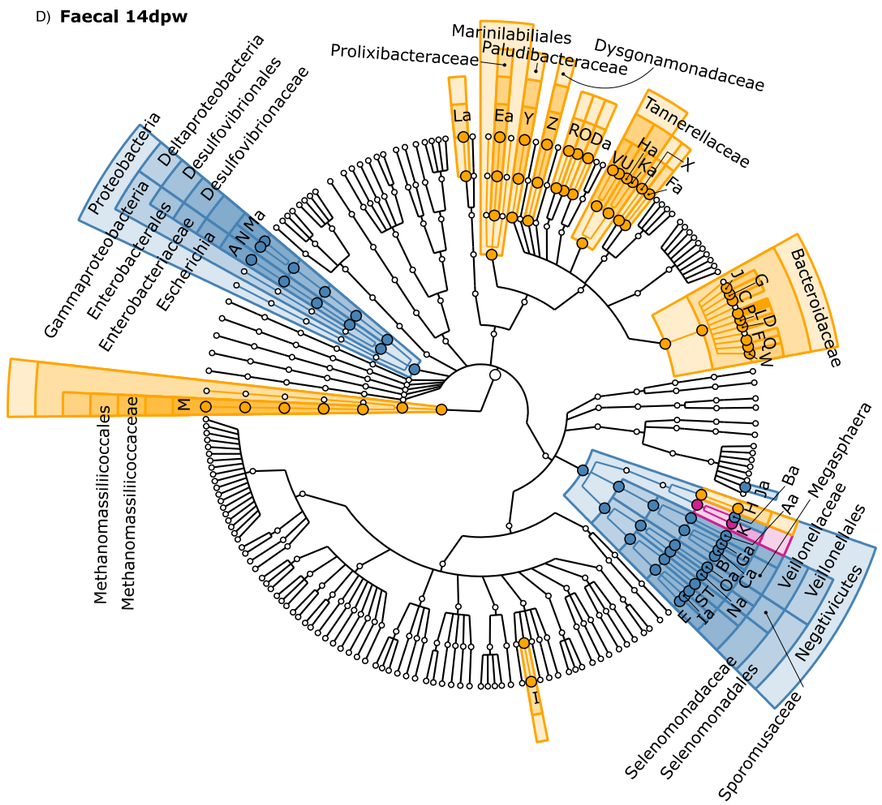


A: Selenomonas_ruminantium
Aa: Olsenella_sp__oral_taxon_807
B: Ruminococcus_bicirculans
Ba: Murdochiella_vaginalis
C: Clostridioides_difficile
Ca: Clostridium_sp__BNL1100
D: Ruminococcus_albus
Da: Hungateiclostridium_saccincola
E: Bacteroides_vulgatus
Ea: Alkaliphilus_oremlandii
F: Desulfovibrio_piger
Fa: Clostridiales_Family_XIII__Incertae_Sedis
G: Caproiciproducens_sp__NJN_50
Ga: Thermoanaerobacterales_Family_IV__Incertae_Sedis
H: Aminipila_sp__JN_39
I: Bacteroides_dorei
J: Selenomonas_sputigena

K: Mogibacterium_diversum
L: Eubacterium_sulci
M: Pseudoclostridium_thermosuccinogenes
N: Dialister_sp__Marseille_P5638
O: Enterococcus_faecium
P: Christensenella_massiliensis
Q: Hungateiclostridium_clariflavum
R: Acetobacterium_woodii
S: Selenomonas_sp__oral_taxon_920
T: Streptococcus_suis
U: Akkermansia_muciniphila
V: Eubacterium_callanderi
W: Eubacterium_limosum
X: Dehalococcoides_mccartyi
Y: Dehalobacterium_formicoaceticum
Z: Mahella_australiensis



A: Escherichia_coli
B: Lactobacillus_reuteri
C: Flavonifractor_plautii
D: Bacteroides_fragilis
E: Bacteroides_cellulosilyticus
F: Bacteroides_vulgatus
G: Bacteroides_dorei
H: Mageeibacillus_indolicus
I: Sutterella_megalosphaeroides



A: Escherichia_coli
Aa: Dialister_sp__Marseille_P5638
B: Megasphaera_elsdenii
Ba: Acidaminococcus_intestini
C: Bacteroides_salanitronis
Ca: Megasphaera_hexanoica
D: Bacteroides_fragilis
Da: Mucinivorans_hirudinis
E: Selenomonas_ruminantium
Ea: Draconibacterium_orientale
F: Bacteroides_cellulosilyticus
Fa: Fermentimonas_caenicola
G: Bacteroides_thetaiotaomicron
Ga: Megasphaera_stantonii
H: Phascolarctobacterium_faecium
Ha: Parabacteroides_sp__CT06
I: Clostridioides_difficile
Ia: Selenomonas_sp__oral_taxon_920
J: Bacteroides_vulgatus
Ja: Lactobacillus_delbrueckii
K: Acidaminococcus_fermentans

Ka: Parabacteroides_distasonis
L: Bacteroides_helcogenes
La: Ornithobacterium_rhinotracheale
M: Candidatus_Methanomethylophilus_alvus
Ma: Desulfovibrio_fairfieldensis
N: Desulfovibrio_piger
Na: Methylomusa_anaerophila
O: Butyricimonas_faecalis
Oa: Pelosinus_fermentans
P: Bacteroides_heparinolyticus
Q: Bacteroides_caecimuris
R: Odoribacter_splanchnicus
S: Selenomonas_sputigena
T: Megamonas_hypermegale
U: Tannerella_forsythia
V: Tannerella_sp__oral_taxon_HOT_286
W: Bacteroides_caccae
X: Petrimonas_mucosa
Y: Paludibacter_propionicigenes
Z: Proteiniphilum_saccharofermentans

Treatment
Ct Ab Zn

Supplementary figure 1. Differences in species abundance, returned by *LEfSe (Linear discriminant analysis Effect Size)*, most likely explaining the differences among dietary treatments in days 0, 7 and 14dpw. A) Species associated with each dietary treatment in the analysis of Faecal 0dpw species data. B) Taxa associated with each dietary treatment in the analysis of Faecal 7dpw data. Significant species are coloured according to the treatment to which they are associated to, and are annotated in the cladogram as letters, which can be identified bellow. C) Taxa associated with each dietary treatment in the analysis of Diarrhoea 7dpw data. Significant species are coloured according to the treatment to which they are associated to, and are annotated in the cladogram as letters, which can be identified bellow. D) Taxa associated with each dietary treatment in the analysis of Faecal 14dpw data. Significant species are coloured according to the treatment to which they are associated to, and are annotated in the cladogram as letters, which can be identified bellow.