Determinants of the gut microbiota development in piglets and its relationship to performance

By

Clare Helen Gaukroger

BSc (Hons) Animal Science

A thesis submitted for the degree of Doctor of Philosophy (PhD)

School of Natural and Environmental Sciences, Newcastle University

Submitted December 2020

Abstract

The role of the microbiota in host health and metabolic phenotype is of increasing interest, with perturbations to the microbiota in early life influencing long term health conditions. The aim of this thesis was to establish factors affecting the neonatal piglet microbiota development and to identify microbiota markers associated with superior piglet growth.

Longitudinal analysis revealed that, whilst piglet age was the main determinant of microbiota development over the first 8 weeks of life, differences in faecal microbiota richness and genera abundance were associated with piglet birthweight. The abundance of several identified genera was higher in piglets with superior growth rates during early life.
The sow is an important source of microbiota seeding to neonatal piglets. Sow faecal microbiota changed significantly during the periparturient period and differed between parities, with primiparous sows exhibiting a lower microbiota diversity than multiparous sows. Early life piglet microbiota community composition was more like the maternal areolar skin microbiota immediately after birth but became increasingly similar to the maternal faecal microbiota with time. In a reciprocal cross-fostering model between primiparous and multiparous sows, a litter specific neonatal piglet microbiota existed for the first three days of life, with siblings separated by cross-fostering retaining a more similar microbiota composition than non-siblings in the same litter. Non-fostered primiparous progeny had lower neonatal microbiota diversity and pre-weaning growth, whilst cross-fostered piglets developed a more diverse neonatal microbiota.
Administration during the neonatal period of an autogenous *Enterococcus faecium* strain, previously associated with superior piglet growth was unsuccessful in improving pre- or post-weaning performance, but reduced diarrhoea occurrence.

In conclusion, early life microbiota markers associated with birthweight and growth have been identified. Sow microbiota sources, sow parity and standard management practices, such as cross-fostering influence piglet microbiota development. Exploiting this knowledge could help to design management strategies aimed at improving piglet performance through microbiota manipulation.

Declaration

I planned and conducted all the experiments performed as part of my PhD studies, as well as all the microbiota sample DNA extractions, data analysis. Bioinformatics procedures were conducted by myself for chapter 2, whilst chapter 3 – 5 were outsourced to Baylor College of Medicine, Texas, USA to be run through their pipeline. The *Enterococcus faecium* genome was analysed by the bioinformatics team at FERA Science, York, UK in support of my IAFRI scholarship.

This thesis has been composed by myself and has not been submitted as part of any previous application for a degree. All sources of information have been specifically acknowledged by means of referencing.

Clare Helen Gaukroger

Acknowledgments

Firstly, I would like to thank the Institute for Agri-Food Research and Innovation, a joint venture between Newcastle University and Fera Science Ltd, without the studentship funding none of this would have been possible, I am eternally grateful.

To my Newcastle University supervisors, Professor Sandra Edwards, Professor Ilias Kyriazakis and Dr Christopher Stewart, I can never thank you enough for the support and scientific guidance you have given me over the course of my PhD. I feel extremely fortunate to have been surrounded by each of you and for your expertise in your respective areas of research. You are the busiest people I know and yet you were always there for me when I needed support. I have learnt so much from each of you, for which I feel extremely fortunate, very few people get to work with the best! To my Fera Science Ltd supervisor, Dr Ian Adams, I thank you for helping me to navigate the initially foreign lab environment and equipping me with the skills required to become proficient in a lab setting. Your guidance and knowledge related to 16S rRNA gene sequencing has been invaluable.

I would not have been able to conduct the on-farm experimental work without the help of the Cockle Park Technical and Pig Unit staff. Despite interrupting commercial practice, you have always accommodated my experimental work. In particular, a big thank you goes to Mark Brett, Emma Malcolm, Louisa Craig, Darren Blomfield and Hazel Baker who have always been there to assist with data collection and provide invaluable knowledge. You have all kept me entertained on night checks, weekends and during the holidays, but most importantly when patiently waiting for pigs to poop! I would also like to thank Debra Patterson for her organisational skills and kindness. A special thank you goes to John Walshaw for his assistance with bioinformatics analysis, I have learnt so much and I am so grateful for your kindness and patience when fixing any issues I had. I must extend my thanks to Sam McGreig and John for their assistance with *Enterococcus faecium* genome analysis, without your expertise this would not have been possible. I would also like to thank Maciek Misuria for his statistics advise and expertise over the course of my PhD. I must also express my gratitude to Daniel Smith at Baylor College of Medicine, having never used R before starting my PhD you have provided the most amazing support and guidance, not to mention having developed some brilliant R packages for microbiota analysis.

I would like to thank Dr Erin Lewis for her friendship, as somebody who understands the highs and lows of a PhD, being able to confide in you during the lows has helped so much and I will always be grateful to IAFRI for introducing us. Furthermore, thank you to my friends for understanding when I have been absent over the years.
To Shaun, thank you for your support, helping to grow my self-belief, and your expertise in computer programming. A final thank you must go to my amazing parents and family for their unwavering support throughout my life and especially during my PhD, I hope I have made you proud.

Whilst thinking about writing my acknowledgements the proverb “It takes a village to raise a child” resonated with me as it became increasingly apparent that completing my PhD studies would not have possible without an extended support network. Thank you everyone.

Publications and conference abstracts

Peer-reviewed publications

Gaukroger, C.H., C.J. Stewart., S.A. Edwards., J. Walshaw., I.P. Adams and I.Kyriazakis. 2020. Changes in Faecal Microbiota Profiles Associated with Performance and Birthweight of Piglets. *Frontiers in Microbiology.* 11:917.

Gaukroger, C.H., S.A. Edwards., J. Walshaw., A. Nelson., I. P Adams., C.J. Stewart and I. Kyriazakis. 2021. Shifting sows: longitudinal changes in the periparturient faecal microbiota of primiparous and multiparous sows. *Animal*. 15(3):100135.

Conference and abstracts

Gaukroger, C.H., S.A. Edwards., I.P. Adams., S. Rushton and I. Kyriazakis. (2018). Associations between birthweight, performance and faecal microbiota of piglets pre and postweaning. 14th International Symposium Digestive Physiology of Pigs, Brisbane, 21-24th August. Theatre Presentation.

List of abbreviations

16S rRNA	16S ribosomal ribonucleic acid
AC	Abdominal circumference
ADG	Average daily liveweight gain
ADFI	Average daily feed intake
AHDB	Agriculture and Horticulture Development Board
AIAO	All-in-all-out
BiW	Birthweight
BiW:CC	Birthweight: cranial circumference
BMI	Body mass index
CP	Crude protein
CRL	Crown rump length
D/d	Day
DE	Digestible energy
DFM	Direct fed microbials
E. faecium	*Enterococcus faecium*
FCR	Feed conversion ratio
FI	Feed intake
GALT	Gut associated lymphoid tissue
GF	Germ free
GIT	Gastrointestinal tract
glmer	General linear mixed effect model
iNKT	Invariant natural killer T-cells
IUGR	Intra-uterine growth restricted
KEGG	Kyoto encyclopaedia of genes and genomes
kg	Kilogram
LBW	Low birthweight (0.80 – 1.25kg)
Lmer	Linear mixed effect model
LW	Liveweight (body weight)
m	Metre
mg	Milligram
ml	Millilitre
NBW	Normal birthweight (1.50 – 2.00kg)
NE	Net energy

OTU	Operational taxonomic unit
PCR	Polymerase chain reaction
PEDv	Porcine epidemic diarrhoea virus
PI	Ponderal index
PWD	Post-weaning diarrhoea
PWSY	Pigs weaned per sow per year
ROS	Reactive oxygen species
SD	Standard deviation
SE	Standard error
SI	Small intestine
µ	Micro
v	Version
WW	Weaning weight
ZnO	Zinc oxide

Table of Contents

List of tables

List of figures

Chapter 1. General introduction

1.1 Increasing pig production, but at what cost?

As the global human population rises, so has the demand for pork and subsequently pig production. To meet consumer demand and increase profitability, pigs have been selected for faster and leaner growth and sows for increased productivity. Weaning weight has been identified as a predictor of lifetime performance, with heavier weaning weights associated with increased subsequent growth rates (Collins *et al.*, 2013; Collins *et al.*, 2017; Douglas *et al.*, 2013, Montoro *et al.*, 2020) and reduced days to slaughter (Mahan and Lepine, 1991; Wolter and Ellis, 2001; Cabrera *et al.*, 2010; He *et al.*, 2016; López-Vergé *et al.*, 2019; Montoro *et al.*, 2020). This lowers the cost of production, as feed accounts for approximately 70% of production costs. Thus, increasing weaning weights is an important goal for pig producers in order to boost productivity and farm profitability.

To further meet the demands for increasing pig production, sows have been selected for an increased number of piglets weaned per sow per year (PWSY) and consequently the development of the hyperprolific sow, as seen today in commercial practice. Bjerre *et al.* (2010) reported that the number of piglets per litter has increased by 0.3 pigs annually between 1992 and 2007 in Denmark, whilst in the UK the number of piglets born alive per litter has increased annually by 0.2 pigs between 2010 to 2020 (AHDB, 2020a). In light of the increased litter size, the subsequent sow performance benchmark, PWSY, has followed suit. In the UK, whilst lower than some European countries, PWSY has increased from 22.8 to 27.7 between 2010 to 2020, thus an extra 0.49 pigs annually. Koketsu *et al.* (2017) forecasted that PWSY could reach between 30-40 in the future, a figure already seen commercially by some producers, particularly in Europe. The rapid increase in litter size, arising from genetic improvements to dam lines, has come at the expense of reduced average birth weight, neonatal piglet viability and litter uniformity, with the production of more low birthweight piglets born per litter, which have been exposed to varying degrees of intra-uterine growth restriction (IUGR) (Quiniou *et al.*, 2002; Milligan *et al.*, 2002; Quesnel *et al.*, 2008; Beaulieu *et al.*, 2010; Foxcroft *et al.*, 2006; Baxter *et al.*, 2008; Campos *et al.*, 2012).

1.1.1 Low birthweight piglets – physiological characteristics associated with intra-uterine growth restriction

A recent study on a UK herd, conducted by Matheson *et al.* (unpublished) as part of the EU ProHealth project, quantified the proportion of low birthweight (LBW) piglets (< 1.25kg),

concluding LBW piglets to equate to 28.47% of 20, 959 piglets born. Meanwhile, Feldpausch *et al.* (2019) reported the proportion of piglets below 1.11kg to be 15.2%, in accordance with earlier research reporting that piglets with a birthweight below 1.1kg contribute to 15-20% of piglets born alive (Wu *et al.*, 2004). LBW piglets are associated with increased pre-weaning mortality rates (Baxter *et al.*, 2008; Calderón Díaz *et al.*, 2017a; Zeng *et al.*, 2019; Feldpausch *et al.*, 2019), with pre-weaning mortality increasing from 5% to 85% for piglets with a birthweight of 1.80kg to 0.61kg, respectively (Quiniou *et al.*, 2002). Similarly, Hawe *et al.* (2020) reported piglets below 1.00kg at birth to have a 21% pre-weaning mortality, 50% of which was due to starvation, whilst piglets with a birthweight between 1.30-1.70kg had a 6% mortality rate. Higher pre-weaning mortality rates associated with LBW piglets can be attributed to reduced vigour at birth, poor thermoregulatory ability, increased latency to first suckle of colostrum, reduced colostrum intake and more time spent at the udder, increasing the risk of crushing (Weary *et al.*, 1996; Herpin *et al.*, 2002; Baxter *et al.*, 2008; Amdi *et al.*, 2013, 2016; Klaaborg and Amdi, 2020). Piglets with low birthweight can be divided into two classes: those who are small for gestational age and symmetrical in shape, or those who are IUGR (intra-uterine growth restricted) and display morphological asymmetry as a result of not reaching foetal growth potential (Bauer *et al.*, 1998a; Rutherford *et al.*, 2013). IUGR is due to prenatal nutrient restriction imposed on the foetus by the sow, as she has a limited uterine capacity which has not increased at a rate proportionate to her increased prolificacy (Town *et al.*, 2004, 2005; Foxcroft *et al.*, 2006, 2009), resulting in differences in the proteome of the placenta and endometrium of IUGR foetuses (Chen *et al.*, 2015).

IUGR will not only reduce the birth weight (BiW) of the foetus compared with its genetic potential, but will also alter the development and physiology of the foetus. Prenatal nutrient restriction induces a "brain sparing effect" with regards to *in utero* growth and development, with IUGR foetuses prioritising the development of the brain and heart over other organs such as the liver, gastro-intestinal tract (GIT) and the development of muscle fibres (Town *et al.*, 2004, 2005; Roza *et al.*, 2008; Beaulieu *et al.*, 2010). When these piglets are born, they appear asymmetrical in shape, exhibiting a characteristic "dolphin-like" head shape with a steep rounded forehead, bulging eyes, shorter snout and wrinkles around the mouth (Chevaux *et al.*, 2010; Hales *et al.*, 2013; Amdi *et al.*, 2013). These defining features of head shape have been used to identify piglets with higher mortality rates and poorer performance amongst LBW pigs (Hansen *et al.*, 2019).

As a result of the physiological adaption to *in utero* nutrient restriction, differences in the proteome of IUGR and normal birthweight piglets exists with respect to the small intestine (SI), liver and skeletal muscle in neonatal and pre-weaned piglets. IUGR piglets are more susceptible to oxidative stress and exhibit reduced absorption and metabolism of nutrients as a result of altered development of the SI, liver and muscles (Wang *et al.*, 2008, 2010, 2013a). Whilst significant changes to IUGR piglets occur at the proteome level, physiological changes are also apparent at the whole animal level, including reduced liver weights in relation to BiW and brain weight (Bauer *et al.*, 1998b; Town *et al.*, 2005; Alvarenga *et al.*, 2013; Amdi *et al.*, 2013). IUGR piglets consume less colostrum relative to BiW, which can be attributed to their reduced relative stomach capacity rather than a lower gastric emptying rate (Amdi *et al.*, 2016; Lynegaard *et al.*, 2020). Moreover, IUGR has a negative effect on skeletal muscle development, with a reduced total myofiber number in the foetus and at birth (Foxcroft *et al.*, 2006; Wang *et al.*, 2013a). At slaughter, differences in muscle characteristics continue to be apparent in IUGR piglets, including a reduction in secondary muscle fibre numbers (Beaulieu *et al.*, 2010) and muscle fibre percentage (with an increase in the proportion of connective tissue) (Alvarenga *et al.*, 2013), larger myofibril size (Beaulieu *et al.*, 2010) and reduced loin area (He *et al.*, 2016). IUGR piglets also display altered hormonal patterns in the nursery and finisher phase, with reduced levels of plasma insulin-like growth factor 1 (IGF-1) and insulin in the nursery stage and lower density of IGF-1 receptors in the SI (Gondret *et al.*, 2005; Michiels *et al.*, 2013; He *et al* 2016). IUGR finisher pigs exhibit lower plasma concentrations of insulin and leptin (He *et al.*, 2016), which may contribute to the reduced growth rate and increased fat deposition associated with IUGR LBW piglets. LBW piglets also have poor mucosal immunity with lower tumour necrosis factor alpha (TNF-α) and interferon gamma (IFN-γ) pre- and post-weaning (Dong *et al.*, 2014; Lo Verso *et al.*, 2020) and altered proportions of leukocytes in the systemic immune system (Lessard *et al.*, 2018; Lo Verso *et al.*, 2020). However, the most widely reported change in IUGR piglet physiology is related to the intestines, with numerous studies reporting delays to SI maturation, reduced absorption capacity and thus nutrient utilization (Wang *et al.*, 2010; Ferenc *et al.*, 2017). During early life IUGR piglets have a relatively longer and thinner SI (D'Inca *et al.*, 2010, 2011; Michiels *et al.*, 2013; Dong *et al.*, 2014). SI villus morphology is also negatively affected by IUGR; during early life, duodenum, jejunum and ileum villus height is reduced (Wang *et al.*, 2010; D'Inca *et al.*, 2010; Alvarenga *et al.*, 2013). Moreover, villi appear damaged and jagged (Dong *et al.*, 2014) and villus width is reduced (D'Inca *et al.*, 2010; Wang *et al.*, 2010). This reduction in villus area in IUGR piglets during early life is estimated to be between 20-30% (D'Inca *et al.*, 2011). Change in IUGR piglet SI physiology may be explained by a decrease in

the cell proliferation: apoptosis rate in epithelial cells (D'Inca *et al.*, 2010) in early life, coupled with a delayed rate in removal of foetal type enterocytes, and is accompanied by a reduction in crypt mitosis and increased apoptosis (Ferenc *et al.*, 2017). However, alterations to the IUGR piglet SI still exist outside of the neonatal period, with smaller villus heights observed at 21 days of age (Wang *et al.*, 2010) and thinner SI reported post-weaning (Michielis *et al.*, 2013). Changes to the physiology of IUGR piglets, particularly in the gastro-intestinal tract (GIT), are important factors contributing to poor post-natal growth rate in LBW IUGR piglets.

LBW piglets are associated with alternations to carcass characteristics and meat quality at slaughter, arising from these changes in metabolism and muscle physiology. LBW pigs reportedly produce fatter carcasses with lower lean meat content, increased intra-muscular fat and larger subcutaneous adipocytes (Gondret *et al.*, 2006, Rehfeldt and Kuhn, 2006; Rehfeldt *et al.*, 2008; Zeng *et al.*, 2019). Krueger *et al.* (2014) suggested that increased carbohydrate oxidation and reduced fat oxidation in LBW pigs may induce earlier onset of fat deposition and thus poorer carcass composition at slaughter. Moreover, meat quality has also been identified as being poorer in LBW pigs, producing less tender pork (Gondret *et al.*, 2006; Nissen and Oksbjerg, 2011), proposed to be as a result of increased myofiber size in LBW pigs (Gondret *et al.*, 2005; 2006). However, other studies have reported contradictory results, with no effect of BiW on carcass composition and quality (Bérard *et al.*, 2008; Beaulieu *et al.*, 2010; Lanferdini *et al.*, 2018); this may be attributed to differences in IUGR severity of LBW pigs between studies.

1.1.2 Low birthweight piglets – a problem to pig unit profitability

Aside from the aforementioned changes to carcass composition and quality, LBW pigs are associated with poorer lifetime performance (Quiniou *et al.*, 2002; Bérard *et al.*, 2008; Beaulieu *et al.*, 2010; Douglas *et al.*, 2013, 2014a, b; Lanferdini *et al.*, 2018; Hawe *et al.*, 2020) and poorer feed efficiency (Gondret *et al.*, 2005; Bérard *et al.*, 2008), having obvious negative implications for farm profitability. However, poorer performance, resulting in increased liveweight variation within a batch of pigs presents additional management problems, as reviewed by van Barnewald and Hewitt (2016) and summarised below. LBW pigs are more susceptible to disease. Not only will this increase the cost of production if a group of pigs require a course of medication, but the efficacy of the treatment programme may be compromised by these LBW pigs, as liveweight variation will make effective group dosing problematic. LBW pigs can create a large standard deviation in liveweight within a group,

making it difficult to align a diet specification to the nutrient requirements of all individuals within a batch of pigs. This variability in liveweight within a group also makes selection for slaughter a more time consuming and laborious task for the stockperson. Commercially, light pigs may be held back pre-weaning, or in weaner accommodation, before moving on to the next production stage with a younger pig cohort to increase building efficiency by limiting start weight variability. However, mixing different ages of pigs has a negative impact on health and performance by increasing disease transmission between batches. Calderón Díaz *et al.* (2017b) reported that pigs which were delayed by over 1 week at the nursery, grower and finisher phases, namely LBW and piglets reared by primiparous sows, displayed increased lameness, pleurisy, pericarditis, meat condemnation and produced a carcass 10kg lighter than those pigs who went through the AIAO system with no delays. Calderón Díaz *et al.* (2017b) concluded that pigs who need to be held back from the next stage of production should be reared in a separate pen away from younger batches of pigs, although this could reduce efficient building utilisation. However, the alternative, which would involve sending to slaughter the whole pen of pigs of varying liveweight, would result in a considerable penalty at the slaughterhouse, reducing profit. In order to avoid the issues associated with LBW pigs and costs later in the production cycle, it is important to minimise the variation in size within a batch of pigs as early as possible (Huting *et al.*, 2017; López-Vergé *et al.*, 2018). This should be done by promoting the compensatory growth of LBW pigs, as opposed to slowing the relative growth rates of normal birthweight (NBW) pigs (Douglas *et al.*, 2013; van Barnevald and Hewitt, 2016).

LBW pigs have been defined as pigs with a BiW of 2 standard deviations (SD) below the mean by McMillen *et al.* (2001). However, some LBW pigs are able to express a degree of natural compensatory growth, reducing the body weight variability within a group of pigs post-weaning, whilst others remain permanently stunted. Therefore, BiW cannot be considered the only factor for predicting future performance of these pigs (Douglas *et al.*, 2013). Paredes *et al.* (2012) concluded that LBW pigs, of less than 2.5 SD from the mean, have potential to compensate for their BiW as they grow, whilst more recently Surek *et al.* (2019) classified piglets between 1.25kg – 2.30kg as those who can exhibit compensatory growth. However, Douglas *et al.* (2013) demonstrated that natural compensatory growth was possible in piglets below 1.25kg BiW. Moreover, Zeng *et al.* (2019) and Montoro *et al.* (2020) concluded that, providing LBW pigs recorded a pre-weaning average daily gain (ADG) above the mean for the batch, they could exhibit compensatory growth post-weaning. Recently, morphometric markers to estimate the future performance of LBW pigs have been established. These can be utilised by both farmers and researchers to aid with the identification of LBW pigs that might benefit

from intervention. Douglas *et al.* (2016) concluded that measures of body mass index (BMI) and abdominal circumference (AC) at piglet processing were the best predictors of pre-weaning performance, whilst AC and ponderal index (PI) were the best predictors for post-weaning performance to 70 days of age. Similarly, Huting *et al.* (2018) reported that BMI was the best predictor of pre-weaning performance, whilst birthweight:cranial circumference (BiW:CC) was the best predictor of post-weaning performance. Therefore, LBW pigs recording a larger AC, BMI/PI and BiW:CC at birth are more likely to exhibit superior growth rates. However, an explanatory mechanism for why some LBW pigs are able to exhibit compensatory growth whilst other remain stunted is yet to be established.

1.2 Weaning – the perfect storm

1.2.1 Challenges of weaning

Under natural conditions, piglets are not weaned by the sow until between 8.5 – 17.2 weeks of age (Newberry and Wood-Gush, 1985; Jensen and Recén, 1989; Bøe, 1991), with smaller litters being weaned later (Bøe, 1991). Weaning under commercial conditions (at approximately 28 days in systems in the UK) presents piglets with an array of stressors including handling and transportation, and loss of sow milk (including bioactive compounds and growth factors) which is replaced by a solid diet that is less palatable and digestible and contains different dietary antigens (Pluske *et al.*, 1997; Campbell *et al.*, 2013). Weaned piglets are also presented with environmental stressors attributed to a different environmental microbiota and potential pathogen load, and differences in the pen layout; social stress arises from establishing a new social hierarchy within the pen (Campbell *et al.*, 2013). As a result of these stressors, the immediate post-weaning period is characterised by a degree of anorexia immediately post-weaning, the extend of which depends on pre-weaning creep feed intake. The stressors associated with weaning also predispose piglets to developing diarrhoea. Taken together, the initial post-weaning period is associated by a growth-check. Bruininx *et al.* (2002) demonstrated that, whilst 50% of piglets with high creep feed intakes pre-weaning initiated post-weaning feed intake within 4 hours, for those piglets who ate very small amounts or no creep feed pre-weaning, post-weaning feed intake was not initiated until 6.9 hours, with 5% of piglets still not eating by 50 hours post-weaning. Indeed, Le Dividich and Herpin (1994) reported that piglets failed to consume their maintenance energy requirements until 5 days post-weaning and did not achieve pre-weaning levels of energy intake until 2 weeks post-weaning. Post-weaning anorexia has detrimental effects on GIT architecture, inducing villous atrophy and thus reducing villous height:crypt depth ratio (Pluske *et al.*, 1996a, b, 1997), hence surface area for nutrient absorption. Villous height is reduced for up to

5 days post-weaning before beginning to recover, although villous morphology shifts from finger-like projections to a wider more tongue-like structure (Pluske *et al.*, 1997; Dong and Pluske, 2007). Furthermore, post-weaning anorexia is accompanied by increased intestinal inflammation, with negative effects on villous function and structure which are mitigated upon resumption of feed intake (McCracken *et al.*, 1999). Similarly, Piè *et al.* (2004) reported that the period of post-weaning anorexia was correlated with increased proinflammatory cytokine levels (interleukin (IL)-1ß, IL-6 and TNF-α). Although the levels of these proinflammatory cytokines were reduced between days 2 – 6 post-weaning, the level of TNF-α remained high in the proximal small intestine. Consequently, post-weaning anorexia is associated with reduced barrier function and increased intestinal permeability (Spreeuwenberg *et al.*, 2001), which does appear to be mitigated by the resumption of feed intake (Boundry *et al.*, 2004). Increased intestinal permeability can lead to translocation of bacteria, toxins and dietary antigens leading to inflammation, increased propensity to enteric health problems and can result in hypersensitivity to dietary antigens (Dong and Pluske, 2007; Campbell *et al.*, 2013; Pluske *et al.*, 2018). The immediate post-weaning period is also associated with reduced brush border and pancreatic enzyme activity (Pluske *et al.*, 1997; Pluske *et al.*, 2003; Lallès *et al.*, 2004), further reducing digestion and absorption of nutrients. A further consequence of increased inflammation is amplified production of reactive oxygen species (ROS), including nitric oxide, which is transformed to nitrate in the lumen where it can confer growth advantages to *Escherichia coli* (Gresse *et al.*, 2017).

Post-weaning anorexia can be followed by gorging, particularly in genotypes with high feed intakes, which will increase gastric pH and reduce protein digestion, increasing substrate availability to proteolytic bacteria, including *E. coli*, subsequently inducing microbiota dysbiosis. A review by Zeng *et al.* (2017) noted increased Enterobacteriaceae in response inflammation, whilst Wei *et al.* (2017) reported increased *E. coli* abundance in the jejunum to be correlated with increased ROS at 7 days post-weaning. Microbiota dysbiosis (corresponding to higher *E. coli* abundance) and increased intestinal permeability arising from post-weaning anorexia, in addition to poorer mucosal active immunity during the immediate post-weaning period (Lallès *et al.*, 2007), provide the perfect storm to facilitate PWD, particularly via ß-haemolytic enterotoxigenic *E. coli* (ETEC) at 3-10 days post-weaning (Pluske *et al.*, 1997; Kim *et al.*, 2012). PWD will exacerbate the post-weaning growth check. It is imperative to minimise the post-weaning growth check, as Kats *et al.* (1992) reported that pigs which gained >227g/d during the first week post-weaning reached slaughter weight 6-10 days faster compared with those who gained 0-150g/d during the first week post-weaning.

Thus, it is essential to encourage pre-weaning creep feed intake and rapid feed intake post-weaning in order to mitigate the negative chain reaction on piglet health and performance associated with post-weaning anorexia.

1.2.2 Pre-weaning management interventions to increase weaning weight

To promote post-weaning performance, pre-weaning growth rates must be optimised, particularly in LBW piglets. Several common management practices, including cross-fostering and provision of creep feed, can be utilised during the suckling period in order to promote performance, reduce within-litter variation in liveweight (LW) and the extent of post-weaning anorexia.

Cross-fostering is utilized to deal with large litters and/or litter heterogeneity, moving piglets on to foster sows depending on piglet BiW, vigour and sow udder and teat quality, in order to create litter homogeneity and equalisation of piglet numbers to functional teats (Baxter *et al.*, 2013). Rearing LBW in homogenous litters reduces competition between litter mates and can result in lower mortality rates (Milligan *et al.*, 2001; Deen and Bilkei, 2004; Cecchinato *et al.*, 2008; Camargo *et al.*, 2013; Muns *et al.*, 2014). Creation of homogenous LBW litters has beneficial effects on piglet performance (English and Bilkei, 2004) compared with those reared in mixed BiW litters, with LBW piglets weighing an extra 500 – 600g at weaning (Douglas *et al.*, 2014a, Huting *et al.*, 2017). The improvement in performance associated with rearing LBW in homogenous litters persisted until slaughter (Douglas *et al.*, 2014a), with such LBW on average 3.7kg heavier than those from heterogenous BiW litters (Huting *et al.*, 2017). However, the creation of homogenous BiW litters has a negative effect on the performance of NBW piglets, who exhibit 970g lower weaning weights and 2.5kg lower slaughter weights than NBW rearing in heterogenous BiW litters (Huting *et al.*, 2017). In heterogenous BiW litters, NBW are at a competitive advantage and more likely to obtain an anterior or middle teat, with posterior teats associated with lower milk yield and consequently performance (Skok *et al.*, 2007; Pluske *et al.*, 2007; Huting *et al.*, 2017). Moreover, LBW pigs are less efficient at massaging and draining teats (King *et al.*, 1997), with milk production by each teat dependant on the duration and intensity of udder massage (Algers and Jensen, 1991). Thus, Huting *et al.* (2017) suggested that LBW in homogenous litters may display improved growth rates due to more even milk production across the teats with stimulation comparable across all teats, with the reverse being true for NBW piglets in homogenous litters.

Cross-fostering should be conducted between 9-12 hours and 48 hours of age to ensure adequate colostrum intake from the biological mother, and thus acquired immunity, especially if the piglets are destined to be cross-fostered on to a nurse sow, or a sow who has farrowed earlier in the batch (Baxter *et al.*, 2013). Whilst later cross-fostering does occur to remove struggling piglets in order to increase their performance, cross-fostering throughout lactation to even up growth rates within a batch of pigs should be avoided (Baxter *et al.*, 2013). This does not increase growth rates of piglets during lactation (Robert and Martineau, 2001; King *et al.*, 2020), with teat order needing to repeatedly be re-established, causing disruption to suckling bouts (Pedersen *et al.*, 2011; Skok and Škorjanc, 2014). Moreover, increased piglet fighting can be seen as a result of late fostering (Jensen, 1994; D'Eath, 2005) and disruption to sow behaviour, especially in freedom farrowing pens (D'Eath, 2005, King *et al.*, 2020), with piglets and sows able to recognise foreign litter mates by smell from the first week of life (Horrell and Hodgson *et al.*, 1992a, b).

The provision of creep feed pre-weaning provides an additional source of nutrients to support optimal piglet growth when sow milk yield is insufficient (Pluske *et al.*, 2005), especially with later weaning ages. Moreover, creep feed, as opposed to supplementary milk, is used to ease the transition to the post-weaning diet of solid feed, with the aim to reduce latency to initial post-weaning feed intake, minimising the post-weaning growth check (Collins *et al.*, 2013). This is thought to be the beneficial mechanism behind creep feed intake, as creep feed alone does not prevent damage to the GIT associated with weaning (Bruininx *et al.*, 2004). Creep feed intakes are highly variable between and within litters (Barnett *et al.*, 1989; Pajor *et al.*, 1991; Bruininx *et al.*, 2002, 2004; Sulabo *et al.*, 2010; Collins *et al.*, 2013; Huting *et al.*, 2017), but increase over time, particularly from day 19 of lactation (Pluske *et al.*, 2007; Sulabo *et al.*, 2010; Collins *et al.*, 2013; Huting *et al.*, 2017; Choudhury *et al.*, 2020). Piglets who display reduced growth rates between birth and day 19 of lactation have increased creep feed intake between days 21 – 28 of lactation (Appleby *et al.*, 1992; Huting *et al.*, 2017). Furthermore, creep feed intake is higher in piglets suckling posterior teats (Algers *et al.*, 1990; Huting *et al.*, 2017). Thus, Huting *et al.* (2017) concluded that creep feed intake is dependent on whether milk intake is adequate to support the demands of the suckling piglet for growth. Most studies report creep feed intake to have no effect on average weaning weight (Bruininx *et al.*, 2004; Sulabo *et al.*, 2010; Collins *et al.*, 2013; Huting *et al.*, 2017). Piglets classified as being "eaters" pre-weaning exhibit a reduced latency to initiate feed intake post-weaning and have a higher feed intake for the first week post-weaning (Bruininx *et al.*, 2002, 2004). Furthermore, piglets classified as "eaters" have a higher post-weaning ADG than "non-eaters"

(Pajor *et al.*, 1991; Bruininx *et al.*, 2002, 2004; Sulabo *et al.*, 2010, Collins *et al.*, 2013; Huting *et al.*, 2017, 2019). The reported duration of improvement in post-weaning ADG of "eaters" varies from up to 4 weeks (Kuller *et al.*, 2007a) and 10 weeks (Huting *et al.*, 2019). Piglets classified as creep feed "eaters" have increased small intestinal net absorption at 4 days post-weaning (Kuller *et al.*, 2007b). A reduction in post-weaning diarrhoea was observed when creep feed was provided from days 5-10 of lactation, as opposed to from day 15 (Yan *et al.*, 2011). Earlier provision may be associated with an increased number of piglets eating creep feed, arising from social learning and increased familiarisation with creep feed (Oostindjer *et al.*, 2014). However, several factors can affect creep feed intake, including piglet BiW and rearing sow parity. LBW pigs consume very little creep feed, as demonstrated by Huting *et al.* (2017, 2019). Furthermore, creep feed intake of NBW pigs in uniform BiW litters is associated with rearing sow parity; piglets reared on 1st and 3rd - 5th parity sows had numeric increases in creep feed consumption during lactation when compared to those reared by 2nd parity sows, associated with the assumed lower milk production and udder/teat quality, respectively. This was related to a significant increase in feed intake in the first week post-weaning, and increased feed intake, ADG and LW up to 10 weeks post-weaning in NBW pigs reared by 3rd-5th parity sows (Huting *et al.*, 2019).

1.3 The gastrointestinal tract microbiota – the increased research interest and the importance to host health

Many of the observed effects on piglet performance and health in the pre- and post-weaning period might be mediated by influences on the gut microbiota. The microbiota can be defined as the assembly of microorganisms belonging to different Kingdoms, including Bacteria, Archaea, Protozoa, Fungi and Algae (Berg *et al.*, 2020). In contrast, the term microbiome encompasses the assembly of microorganisms present in the microbiota, as well as the collective microbiota gene functions and "theatre of activity" which includes structural elements, metabolic and signal molecules and surrounding environmental conditions (Berg *et al.*, 2020). Technological advances in DNA sequencing methods (see 1.3.1) and reduced costs of sequencing have resulted in an exponential increase in research studies on the microbiota, and more recently microbiome, in the last decade. The microbiota has been increasingly considered as a metabolic 'organ' due to its interaction with host physiology, immune function, metabolism, endocrinology and behaviour (Guinane and Cotter, 2013).

1.3.1 Microbiota analysis methods

Historically, studies used culture-based microbiology techniques to identify microbiota species. However, culture-based techniques are limited in their capacity to only detect species capable of growth on the media utilised, and these techniques are not representative of the growth conditions present in the GIT (Ames *et al.*, 2017). Technological advancements have resulted in the development of high throughput DNA sequencing methods, which have enabled the microbiota community assemblage to be determined using culture-independent techniques (Osman *et al.*, 2018). Subsequently, two methods of high throughput DNA sequencing are now utilised - 16S ribosomal RNA (rRNA) and shotgun metagenomic sequencing. The 16S rRNA gene is considered a housekeeping gene and is present in most Prokaryotes (Bacteria and Archaea). The 16S rRNA gene contains highly preserved regions utilised for primer binding and hypervariable regions (V1 – 9) which are unique to each Prokaryote and utilised for taxonomy identification purposes (Clarridge, 2004; Ames *et al.*, 2017; Osman *et al.*, 2018; Bharti and Grimm, 2019). 16S rRNA gene sequencing enables microbiota phylogeny and taxonomy to be determined, although taxonomic resolution is typically limited to the genus level (Ames *et al.*, 2017). In comparison to the single Prokaryote gene sequencing utilised in 16S rRNA gene sequencing, shotgun metagenomics sequences the whole microbiota genome within a sample. Shotgun metagenomic sequencing generates not only increased taxonomic resolution, down to the species/strain level, but also genetic capacity of the microbiome, facilitating formal analysis of the interaction between the host and microbiota (Osman *et al.*, 2018; Bharti and Grimm, 2019). However, shotgun metagenomic sequencing is considerably more expensive than 16S rRNA gene sequencing, limiting sample size and its usage in low budget research. In comparison, 16S rRNA gene sequencing provides a cost-effective method with which to study the microbiota community profile, including the ability to detect rarer taxa, increasing the accessibility of microbiota research as part of wider scientific studies.

1.3.2 Common microbiota terms

Alpha diversity refers to the richness in diversity of a microbiota sample. Alpha diversity measures commonly reported throughout the thesis include observed OTUs (operational taxonomic units) and Shannon diversity. Briefly, observed OTUs refer to the number of OTUs (each a cluster of 16s rRNA sequences with $\geq$97% similarity) and thus genus within a microbiota sample. Shannon diversity is a measure of alpha diversity which accounts for the evenness of the OTU abundance within the sample, thus indicating whether a sample is characterised by a few predominant OTUs or many less abundant OTUs.

Beta diversity refers to the similarity in the microbiota community composition between two samples. Beta diversity measures commonly referred to in the thesis include Bray Curtis, weighted and unweighted UniFrac. Beta diversity measures range from 0 to 1, with a value of 1 meaning samples are identical based on the sample characteristics compared within each measure used. Bray Curtis measures are calculated based on the similarity in shared OTUs between samples but also the relative abundance. UniFrac measures of beta diversity are based on the differences in the phylogenetic tree information between samples (Lozupone and Knight, 2005), with weighed UniFrac distances also considering relative abundance of each OTU.

1.3.3 Host-microbiota interaction

In the absence of microbiota dysbiosis, the GIT microbiota has a symbiotic relationship with the host. As reviewed by Gérard (2016), the microbiota has fundamental roles in the maturation and modulation of the host immune system, growth and differentiation of intestinal epithelial cells, fermentation of complex polysaccharides to monosaccharides and short chain fatty acids (SCFAs), synthesis of vitamins and minerals, catabolism of toxins and protection against pathogen invasion (Flint *et al.*, 2008; Gérard, 2016; O'Callaghan *et al.*, 2016). Both persistent and transient microbiota dysbiosis can mediate negative effects on the host, with long term implications for immune and metabolic disease if microbiota dysbiosis occurs during the "critical window" in early life (Scholtens *et al.*, 2012; El-Aidy *et al.*, 2013; Zeissig and Blumberg, 2014).

Colonisation of the neonatal GIT with a diverse microbiota is important to develop the innate and adaptive immune system, with disruption to the microbiota colonisation, including antibiotic usage, altering the propensity to develop disease in later life (Gensollen *et al.*, 2016; Ximenez and Torres, 2017). The interaction between the microbiota and the intestinal mucosa induces maturation of the gut-associated lymphoid tissue (GALT) (Gensollen *et al.*, 2016). Compared with colonized mice, germ free (GF) mice possess an immature GALT, thymus and spleen, a lower number of lymphocytes and a reduction in antimicrobial peptides and IgA concentrations (Kabat *et al.*, 2014; Gensollen *et al.*, 2016). Antibiotics in early life, inducing microbiota dysbiosis and consequently modification of the mucosal immune system, are associated with the development of asthma (Risnes *et al.*, 2011) and food allergies (Cahenzli *et al.*, 2013; Azad *et al.*, 2014), thus immune states of hypersensitivity and hyperinflammation (Stiemsma and Michels, 2018). Early life GF or antibiotic treatment in mice results in an increase in invariant natural killer T-cells (iNKT) cells (Olszak *et al.*, 2012) and has been

associated with asthma and colitis development (Olszak *et al.*, 2012; Russell *et al.*, 2012; Gollwitzer *et al.*, 2014). However, this increase in iNKT cells could be mitigated by seeding a GIT microbiota of GF mice, provided that this occurred during the first 2 weeks of life (Olzsak *et al.*, 2012). This effect is supported by the reduction in the propensity to develop asthma in children exposed to farms and thus a wider variety of environmental microbes during early life (Riedler *et al.*, 2001).

In neonatal piglets, treatment with antibiotics has been shown to alter the expression of immune related processes on day 8 and 55 of life (Schokker *et al.*, 2014; 2015). To establish host-microbiota homeostasis, the commensal microbiota is thought to regulate the mucosal immune system via several mechanisms in order to generate tolerance. These include, altering the Ig collection via the B cell lineage in the lamina propria (Wesemann *et al.*, 2013; Ximenez and Torres, 2017) and adequate stimulation of sIgA (Kamada and Núñez, 2014; Ximenez and Torres, 2017). Generating tolerance to the commensal microbiota avoids the development of hypersensitivity of the immune system and subsequent inflammation. This is also mediated through the action of CD4+ and CD25+ T-lymphocytes, resulting in the secretion of the anti-inflammatory cytokine IL-10, which helps to retain tolerance to the commensal microbiota by inhibiting the activation and proliferation of commensal-reactive T-cells (Kamada and Núñez, 2014; Neu *et al.*, 2007). The intestinal microbiota interacts with the host via activation of host pattern recognition receptors, including toll-like receptors (TLR) or nucleotide-binding and oligomerization domain-like receptors via cell wall components or secondary metabolites, such as SCFAs (Bhattaria, 2018). Downregulation of TLRs by the commensal microbiota is important in early life to promote mucosal immune system tolerance (Gensollen *et al.*, 2016).

The SCFAs produced by the microbiota, particularly butyrate, are an important energy source for enterocytes, increasing epithelial cell integrity (Bhattarai, 2018) and the density of the capillary network in the villi (Stappenbeck *et al.*, 2002; Gérard, 2016). However, Yu *et al.* (2016) demonstrated that the microbiota origin is also important in promoting mucosal development and tight junction integrity. GF mice colonised with the microbiota of low birthweight infants displayed intermittent expression of tight junction proteins and reduced villus height and crypt depth compared with those mice colonized with the microbiota of normal weight infants (Yu *et al.*, 2016). Thus, reduced GIT maturation and increased permeability of GF mice when colonised with low birthweight infant microbiota.

The microbiota also has an important role in host metabolic phenotype. Early studies demonstrated obesity in humans to be associated with reduced alpha diversity of the microbiota, lower Bacteroidetes and higher Actinobacteria abundance (Ley *et al.*, 2006; Turnbaugh *et al.*, 2009; Le Chatelier *et al.*, 2013). In mice, diet induced obesity was associated with higher abundance of Firmicutes, particularly class Mollicutes, and lower Shannon diversity index (Turnbaugh *et al.*, 2008). However, the association between obesity and the microbiota is not just a response to diet change; the microbiota can also induce obesity. When GF mice colonised with a caecal microbiota transplant from an obese mouse were compared to those with a lean donor, the metabolic pathways of the obese microbiota recipient mice were enriched in carbohydrate metabolism and increased phosphotransferase, indicative of increased energy sequestration (Turnbaugh *et al.*, 2008).

Meanwhile, microbiota metabolites can have a beneficial effect on host metabolism. The SCFAs produced by microbial fermentation of complex polysaccharides are able to act as signal regulating molecules for energy harvest, appetite and fat storage regulation by the host (Conterno *et al.*, 2011; Gérard, 2016). SCFAs are able to bind to receptors on endoepithelial cells, altering endocrine function of the host by increasing gut peptide YY and glucagon-like peptide 1, whilst reducing ghrelin production (Delzenne *et al.*, 2011), resulting in increased satiety and reduced feed intake (Gérard, 2016). Butyrate and propionate can increase leptin expression on adipocytes, modulating appetite (Soliman *et al.*, 2011). Moreover, Lin *et al* (2012) reported butyrate and propionate to protect the host from diet-induced obesity via mechanisms other than binding to endoepithelial cell receptors. Therefore, SCFAs elicit protective effects against the onset of type 1 diabetes (De Goffau *et al.* 2014; Vatanen *et al.*, 2018).

In summary, avoidance of early life perturbations to the microbiota, resulting in dysbiosis or reduced diversity and SCFA production, are essential to the development of a healthy mucosal immune system and prevention of immune and metabolic disorders later in life. Furthermore, the microbiota also affects aspects of mental health and the development of social skills (Desbonnet *et al.*, 2014; Heijtz *et al.*, 2011).

1.4 Development of the piglet microbiota

1.4.1 Longitudinal microbiota development in piglets

Increasing efforts have been made to identify the succession pattern of the GIT microbiota of the pig and factors which affect this (Inoue *et al.*, 2005; Konstantinov *et al.*, 2006; Thompson *et al.*, 2008). At present, research suggests that the early GIT microbiota is highly variable between individual piglets, even between siblings, with the highest degree of variability seen

in the first week or so of life (Thompson *et al.*, 2008; Bian *et al.*, 2016; Mach *et al.*, 2015). It is thought that this might be because the initial microbiota, seeding an effectively sterile gut, is formed at random from a much larger microbiota community presented by both the sow and the pen (Curtis and Sloan, 2004). Initially, the microbiota is colonised by facultative anaerobes, predominantly Enterobacteriaceae and Clostridiaceae, although quickly superseded by Steptococcaceae. From 3 days of age until weaning, facultative anaerobes and anaerobes then colonise the GIT and become the most abundant type of bacteria, namely from the Lactobacillaceae family. Post-weaning (and towards the end of lactation), the microbiota is predominated by Ruminococcaceae, Lachnospiraceae and Prevotellaceae (Konstantinova *et al.*, 2006; Petri *et al.* 2010; Bian *et al.*, 2016). At the phylum level the microbiota is dominated by Firmicutes and Bacteroidetes across all ages of pigs (Kim *et al.*, 2011; Pajarillo *et al.*, 2015; Mach *et al.*, 2015; Bain *et al.*, 2016; Chen *et al.*, 2017; Wang *et al.*, 2019a). Although, during early life Protobacteria and Fusobacteria are among the most abundance phylum, their relative abundance declines during the suckling period and is diminished post-weaning, whilst Bacteroidetes and the Firmicutes:Bacteroidetes ratio is increased during the same period (Zhao *et al.*, 2015; Niu *et al.*, 2015; Chen *et al.*, 2017; Pollock *et al.*, 2018). The post-weaning period is also characterised by an increase in the Ternicutes; this phylum is associated with fibre degradation, and thus its abundance mirrors the change in increased crude fibre concentration and lower digestibility of grower-finisher diets (Niu *et al.*, 2015). Frese *et al.* (2015) reported changes in the microbiota at the family level in piglets up to 42 days of age, noting changes to mainly occur in response to weaning. Across all stages, the piglet microbiota was characterised by Enterobacteriaceae, Lachnospiraceae, Bacteroidaceae, Lactobacillaceae, Clostridiaceae, Ruminococcaceae, Prevotellaceae, Erysipelotrichaceae, Streptococcaceae and Enterococcaceae. However, in response to weaning, the relative abundance of Clostridaceae, Bacteroidaceae and Enterobacteriaceae declined, whilst the relative abundance of Lactobacillaceae, Ruminococcaceae, Viellonellaceae and Prevotellaceae increased (Frese *et al.*, 2015). At the genus level, the most abundant genera vary between studies, although generally the suckling piglet microbiota is characterised by *Bacteroides, Oscillibacter, Escherichia/Shigella* and *Fusobacterium* (Pajarillo *et al.*, 2015; Frese *et al.*, 2015; Mach *et al.*, 2015; Bain *et al.*, 2016; Chen *et al.*, 2017). However, the post-weaning microbiota is predominated by *Prevotella, Blautia, Ruminococcus, Subdoligranulum and Roseburia* (Kim *et al.*, 2011; Pajarillo *et al.*, 2015; Frese *et al.*, 2015; Mach *et al.*, 2015; Hu *et al.*, 2016a; Bian *et al.*, 2016; Chen *et al.* 2017; Pollock *et al.*, 2018).

The early life microbiota seems to be colonised by pathobiont species from the Proteobacteria and Fusobacteria phyla, but it would appear that the host must present conditions compatible for pathobiont species to elicit pathogenic behaviour (Chen *et al.*, 2017). Throughout the literature, two genera are repeatedly reported to characterise the change in microbiota associated with weaning. *Bacteroides* is the predominant genus associated with suckling piglets, due to its ability to produce enzymes which can metabolise milk oligosaccharides that are not digested by piglets (Pajarillo *et al.*, 2015; Frese *et al.*, 2015). In comparison, *Prevotella* is the predominant genus associated with weaned pigs, as this genus is capable of producing xylanase, mannanase and ß-glucanase (Flint and Bayer, 2008), metabolising complex polysaccharides present in plant cell walls. Moreover, Pajarillo *et al.* (2015) suggest that increased *Prevotella* abundance corresponds to increased mucin availability immediately post-weaning. The abrupt change in diet form and composition associated with weaning causes perhaps the most dramatic change in the piglet microbiota, compared with gradual changes associated with age, or changes in diet composition between production stages (De Rodas *et al.*, 2018).

Microbiota richness (number of operation taxonomic units (OTUs)) increases with age (Pajarillo *et al.*, 2015; Kim *et al.*, 2015; Zhao *et al.*, 2015, Zhao and Kim, 2015; Frese *et al.*, 2015; Chen *et al.*, 2017; De Rodas *et al.*, 2018; Wang *et al.*, 2019a; Ke *et al.*, 2019). Conversely, some studies report that microbiota evenness (relative abundance/representation of each OTU compared with the others detected in a sample) plateaus in weaned pigs (Kim *et al.*, 2015; Zhao and Kim, 2015). Although beta diversity distance measures (microbiota community composition variability between samples, with lower beta diversity being indicative of more similar community composition between samples) vary between studies, throughout the literature the microbiota community composition shows a clear progression with age, becoming increasingly more homogenous between conspecifics, particularly post-weaning (Zhao and Kim, 2015; Mach *et al.*, 2015; Bian *et al.*, 2016; Hu *et al.*, 2016a; Chen *et al.*, 2017; De Rodas *et al.*, 2018; Wang *et al.*, 2019a; Ke *et al.*, 2019).

The microbiota profile also differs with GIT site. Li *et al.* (2019) reported lower microbiota diversity and richness in the ileum compared to the colon of pre-weaned pigs. Moreover, beta diversity differs between different GIT segments (Zhao *et al.*, 2015; Holman *et al.*, 2017), and between GIT segments and faeces, with a 75% similarity in the microbiota between faeces and the large intestinal microbiota and 38% similarity between the faeces and the small intestine (Zhao *et al.*, 2015). The small intestine microbiota is comprised of mainly aerobes and

facultative anaerobes, namely Proteobacteria and Firmicutes, compared with that of the large intestine which consists of anaerobes including Firmicutes, Spirochaetes and Bacteroidetes, whilst the faeces composition is predominantly Firmicutes and Bacteroidetes (Zhao *et al.*, 2015). Frese *et al.* (2015) proposed that the piglet microbiota is constantly seeded with environmental microbes, with changes in abundance, or species presence and absence, related to substrate availability and GIT environment suitability to facilitate colonisation. Moreover, this notion was supported by Kim *et al.* (2015) who reported that, despite the microbiota changing with growth stage of pigs, a core microbiota of over 50% of all OTUs was retained from piglets up to sows. Similarly, Wang *et al.* (2019a) reported a core microbiota present in pigs between 11 and 174 days of age.

1.4.2 Factors affecting the development of the early life microbiota

The microbiota colonization of neonatal pigs begins during expulsion, with ingestion of vaginal microbes; piglets born via caesarean section develop a microbiota which produces lower concentrations of SCFAs in the small and large intestine (Wang *et al.*, 2013b). The GIT is subsequently seeded with microbes present in the maternal pen environment, sow faeces, skin and mucosal surfaces (Mach *et al.*, 2015; Chen *et al.*, 2017; Liu *et al.*, 2019a). Moreover, milk composition/neonatal nutrition can alter the microbiota profile, as demonstrated when comparing the microbiota of formula vs sow milk-fed piglets (Poroyko *et al.*, 2010; Li *et al.*, 2012; Sugiharto *et al.*, 2015; Poulsen *et al.*, 2017). Pre-weaning formula feeding resulted in increased *Escherichia*, *Shigella*, *Enterococcus* and *Ruminococcus* abundance, as well as an increased predisposition to colonisation with *Clostridium difficile* (Grześkowiak *et al.*, 2018).

The microbiota can also be affected by breed (Pajarillo *et al.*,2015; Bian *et al.*, 2016; Xiao *et al.*, 2017; Bergamaschi *et al.*, 2020a). A recent study by Xiao *et al.* (2018) demonstrated differences in the microbiota between Landrace and Jinhua pigs, divergent in metabolic phenotype, with the most predominant differences associated with microbiota in the jejunum and ileum. Jinhua pigs possessed microbiota metabolic pathways enriched in fatty acid biosynthesis in line with their inherent fatty body composition (Xiao *et al.*, 2018). Furthermore, variability in milk composition between breeds can alter the microbiota of piglets (Bian *et al.*, 2016). Differences between breed types were related predominantly due to a difference in lactose content of the milk, although differences in protein and fat content were also drivers of microbiota community composition in suckling piglets (Bian *et al.*, 2016). Thus, despite a core microbiota being present in pigs, when comparing between microbiota studies and interpreting

results it is important to consider piglet genetics, age and origin of microbiota sample in relation to the GIT.

Katouli *et al.* (1997) reported that the microbiota fingerprint of piglets closely resembled the sow for the first 2 weeks of life, before developing a microbiota fingerprint more similar to littermates. However, continuous microbiota seeding of neonates by the sow and maternal pen environment continues to be important in shaping the microbiota outside of the first 48 hours of life. As demonstrated by Schmidt *et al.* (2011), continued interaction with the sow and maternal pen was required after 48 hours of life in order to develop a stable and adult-like microbiota by 56 days of age. Pigs separated from the sow after 48 hours, in either an outdoor or indoor production system, and reared in an isolator developed a significantly different microbiota profile, even after solid feed intake was initiated at weaning (Schmidt *et al.*, 2011).

1.5 The importance of the gastrointestinal tract microbiota for piglet performance and health

1.5.1 Microbiota markers for pig performance

Due to the interaction of the microbiota with the host immune, hormonal system and metabolic phenotype, it is unsurprising that an increasing number of studies have identified characteristics in the microbiota to be associated with piglet performance and health. However, only limited research has been conducted identifying microbiota markers for early life piglet performance. Ding *et al.* (2019) reported that microbiota richness at weaning was higher in piglets with superior ADG, accompanied by a higher abundance of *Lactobacillus* in the caecum and colon, and *Moraxella* and *Selenomonas* in the ileum. Similarly, increased microbiota richness and higher Firmicutes:Bacteroidetes ratio in post-weaning pigs was associated with heavier pigs at 9 weeks of age (Han *et al.*, 2017). Mach *et al.* (2015) and Ramayo-Caldas *et al.* (2016) reported pigs to diverge into two different microbiota enterotypes, categorised by a high abundance of either *Prevotella* (+ *Moraxella* (Ramayo-Caldas *et al.*, (2016)) or *Ruminococcaceae* (+ *Treponema* (Ramayo-Caldas *et al.*, (2016)), with pigs in the *Prevotella* enterotype exhibiting higher LW, secretory IgA concentrations and alpha diversity. The microbiota in the *Prevotella* enterotype had increased Kyoto Encyclopedia of Genes and Genomes (KEGG) ortholog pathways for carbohydrate metabolism and for the production of glycoside hydrolase and polysaccharide lyase (Ramayo-Caldas *et al.*, 2016). *Prevotella*, and the co-occurring genera within the enterotype, are thought to increase performance by metabolizing carbohydrates and fibre into monosaccharides and SCFAs (Ramayo-Caldas *et al.*, 2016). More recently, Lu *et al.* (2018)

reported that the Shannon diversity index at 15 weeks of age was negatively correlated with back fat at 18 and 22 weeks of age and ADG between weaning – 14 and 14 – 22 weeks of age. However, microbiota markers associated with compensatory growth in pigs are yet to be identified.

In contrast to the growth rate literature, a larger number of studies have been conducted to determine associations between the microbiota and feed efficiency in grower/finisher pigs. Whilst differences in taxa abundance between high and low feed efficiency pigs exist, results are not consistent across studies (Vigors *et al.*, 2016, 2020a; Tan *et al.*, 2017; Yang *et al.*, 2017; McCormack *et al.*, 2017, 2019a; Quan *et al.*, 2018; Metzler-Zebeli *et al.*, 2018; Si *et al.*, 2020; Bergamaschi *et al.*, 2020b). Differences in taxa associated with feed efficiency between studies can be partially explained by GIT tract segments used, as microbiota markers for feed efficiency differ depending on sampling site, even within the same study (McCormack *et al.*, 2017, 2019a; Quan *et al.*, 2018). A further explanation for differences in the literature is the age of sampling, as demonstrated by McCormack *et al.* (2019a), whereby the genera markers for high feed efficiency pigs were different between pigs at 70 and 135 days of age. The lack of consistent results between studies using the same microbiota sample type is most likely due to the variability in experimental pig age, genotype, diet, management, farm/herd health status which will all affect the microbiota composition. However, even when measures are taken to limit variability in the aforementioned factors between farms and batches of pigs, different genera markers for feed efficiency still exist. This is most likely due to differences in the specific farm environment and maternal microbiota, with genera markers for high feed efficiency only shared between 2/3 farms or between batches of pigs (McCormack *et al.*, 2019a). Moreover, Vigors *et al.* (2020a) demonstrated that microbiota associated with differences in feed efficiency were predominantly farm specific, although they reported an increase in the abundance of BS11 and *Ruminococcus flavefaciens*, and lower abundance of *Collinsella* to be associated with high feed efficiency for both of the farms studied. Bergamaschi *et al.* (2020b) reported *Oscillibacter* to be negatively correlated with average daily feed intake (ADFI) and feed conversion ratio (FCR) at 73 days of age across three different breeds. Furthermore, the increased abundance of *Cellulosilyticum* has been identified in the caecum and faeces of pigs with high feed efficiency in multiple studies (McCormack *et al.*, 2017, 2019a; Reyer *et al.*, 2020), thus possesses the potential to be a reliable microbiota marker for higher feed efficiency. Collectively, based on the results reported in the literature, the KEGG ortholog pathways indicate that higher feed efficiency in pigs is related to enriched carbohydrate and amino acid metabolism by the microbiota (Yang *et al.*, 2017; Tan *et al.*, 2017; Quan *et al.*, 2018; McCormack *et al.*, 2017).

1.5.2 Microbiota markers for pig health

The microbiota also plays an important role in piglet health, particularly in the susceptibility to post-weaning diarrhoea. Dou *et al.* (2017) was able to distinguish between piglets who developed post-weaning diarrhoea 4 weeks before it happened, based on differences in the microbiota profile of piglets at 7 days of age. Healthy piglets were categorised by a higher microbiota richness but lower evenness, and an increase in the abundance of Prevotellaceae, Lachnospiraceae, Ruminococaceae and Lactobacillaceae compared to diarrhoeal piglets (Dou *et al.*, 2017). The outcome of porcine reproductive and respiratory syndrome and porcine circo-virus 2 infection can be improved if piglets have a higher alpha diversity in the presence of non-pathogenic *E. coli* (Niederwerder *et al.*, 2016; Niederwerder, 2017). The presence of a healthy and diverse microbiota is also required to reduce the severity of *Mycoplasma hyopneumonia* infection (Schachtshneider *et al.*, 2013). A more diverse microbiota with a higher abundance of *Ruminococcus 2* was shown to reduce the severity of lung lesions caused by *M. hyponeumonia* at 70 days of age following post-weaning infection (Nair *et al.*, 2019). Several studies have identified differences in the microbiota of healthy piglets and those suffering from PEDv (Porcine epidemic diarrhoea virus) (Liu *et al.*, 2015; Koh *et al.*, 2015; Song *et al.*, 2017; Huang *et al*, 2018; Tan *et al.*, 2019). PEDv diarrhoeal piglets commonly have a microbiota characterised by increased abundance in the phyla Proteobacterium and Fusobacterium and a reduction in Bacteroidetes (Liu *et al.*, 2015; Song *et al.*, 2017; Huang *et al.*, 2018; Tan *et al.*, 2019). At the genera level, PEDv is associated with a lower abundance of *Prevotella, Parabacteroides, Bacteroides* and *Butyricomonas* and higher abundance of *Veillonella* and *Fusobacteria* (Liu *et al.*, 2015; Koh *et al.*, 2015; Song *et al.*, 2017; Huang *et al.*, 2018; Tan *et al.*, 2019).

1.6 Interventions to modify the microbiota

1.6.1 Direct interventions – early life faecal microbial transplant

As antibiotic treatment of neonates is not routinely practiced in the UK, the neonatal microbiota can be manipulated via two primary pathways. 1) Modulation of the sow microbiota in gestation and lactation by pre- or probiotic administration (discussed in 1.6.7), or 2) Early life probiotic treatments, or the use of direct fed microbials from a healthy donor animal. Faecal microbial transplant (FMT) is gaining increasing interest, particularly as the microbiota associated with aspects of performance are heavily influenced by farm origin (McCormack *et al.*, 2019a; Vigors *et al.*, 2020a). FMT refers to the transfer of faeces from a healthy individual into a recipient, with the aim of modulating the GIT microbiota via competitive exclusion to re-establish a healthy microbiota profile, particularly in sick individuals (Nowland and Kirkwood, 2020).

FMT in early life has demonstrated beneficial effects when the FMT donor is the rearing sow (Cheng *et al.*, 2019 Wan *et al.*, 2019), as opposed to a grower-finisher pig (McCormack *et al.*, 2018). Cheng *et al.* (2019) reported that FMT between days 1-3 of life increased ADG to 21 days of age, reduced diarrhoea occurrence, increased sIgA concentration and reduced intestinal permeability, whilst the microbiota metabolism pattern shifted to increased SCFA production. These findings were also reported by Hu *et al.* (2018) when piglets were treated with FMT between days 1 - 11 of age, characterising FMT pigs to have increased *Prevotella*, *Ruminococcus* and *Oscillospira* abundances and lower *Sutterella*, *Escherichia/Shigella* and *Bacteroides* abundances. The metabolic effects of FMT are not limited to the GIT microbiota; following FMT treatment between days 1 – 6 of life, the liver of FMT piglets displayed lower fatty acid catabolism and amino acid biosynthesis. However, FMT composition will vary with age and nature of the donor pigs, with the composition of the sample relatively unknown. Consequently, the donor animal must be carefully selected as the sample could present a biosecurity risk for disease transmission, particularly if the FMT is destined for sick pigs who will have a compromised immune system (Nowland *et al.* 2020). Screening of the potential FMT samples to ensure absence of pathogenic bacteria may help to identify suitable donor samples, however this may increase the expense and limit practicality of use on-farm.

1.6.2 Direct interventions - zinc oxide use in post-weaning diets to facilitate pathogen exclusion

To prevent diarrhoea post-weaning by reducing microbial and pathogenic load on the GIT, (Dibner and Richards, 2005), antimicrobial growth promoters were historically used in post-weaning diets. However, due to increasing concern over antimicrobial resistance, the use of anti-microbial growth promoters was banned in the EU from the 1st of January 2006 (Regulation 1831/2003/EC). Since the EU ban on the use of antimicrobial growth promoters, pharmacological doses of zinc oxide (ZnO; < 3100ppm) have been increasingly relied upon in weaned pig diets to mitigate post-weaning diarrhoea occurrence and severity (Poulsen, 1995; Ou *et al.*, 2007; Molist *et al.*, 2011; Hu *et al.*, 2014; Lei and Kim, 2018). High levels of ZnO have growth promoting effects when used for short periods (< 2 weeks) post-weaning, increasing ADG and ADFI (Jensen-Waern *et al.*, 1998; Hill *et al.*, 2001; Shelton *et al.*, 2011; Hu *et al.*, 2014; Wang *et al.*, 2019b). Whilst the antidiarrheal and growth promoting mode of action of high level ZnO is not fully understood, several mechanisms have been proposed, including reduced bacterial adhesion of *E. coli* and preventing the pathogen induced inflammatory response (Roselli *et al.*, 2003). Ou *et al.* (2007) proposed that the antidiarrheal effect is due to ZnO reducing the gene expression of stem cell factor in mucosal mast cells

and consequently histamine release from the mucosa and submucosa. High ZnO levels increase intestinal epithelial cell integrity (Hu *et al.*, 2013; Wang *et al.*, 2019b) and reduce intestinal inflammation (Hu *et al.*, 2013; 2014). Furthermore, high levels of ZnO help to improve SI morphology post-weaning, increasing villous height and villous height:crypt depth ratio (Hu *et al.*, 2013, 2014; Lei and Kim, 2018), consequently increasing the absorption of dietary nutrients. However, high levels of ZnO affect gut microbiota composition, reducing not only *E. coli* in the distal SI (Wang *et al.*, 2019b) but also lactic acid bacteria and *Lactobacilli* abundance, whilst increasing the abundance of *Enterococci* (Højberg *et al.*, 2005). A more recent study by Yu *et al.* (2017), utilising 16S rRNA sequencing, reported high levels of ZnO alter microbiota alpha and beta diversity and taxa abundance. High levels of ZnO feeding, and subsequently high ZnO excretion in pig slurry, have raised environmental concerns (Romeo *et al.*, 2014). However, of most concern is the increase in bacterial antimicrobial resistance genes associated with high ZnO feeding (Cavaco *et al.*, 2011; Bednorz *et al.*, 2013a; Vahjen *et al.*, 2015; Ciesinski *et al.*, 2018; Pieper *et al.*, 2020). Consequently, high level ZnO feeding for pharmacological use will be banned in the EU from June 2022 (Directive 2001/182/EC and Regulation number 726/2004/EC). Thus, it is imperative to find an alternative to replace high level ZnO without compromising post-weaning performance. Whilst no "silver bullet" has been discovered, combined changes to management, nutrition and the use of feed additives discussed below present options to rear piglets ZnO free, depending on farm health status.

1.6.3 Direct interventions - formulating weaner diets to improve gut health

With the impending ban on ZnO and pressure to reduce antibiotic usage in pig production, there has been a move to formulating post-weaning diets to not only be highly digestible and palatable, to improve performance and encourage feed intake, but formulated to enhance gut health and promote a healthy microbiota community composition.

Post-weaning diets can often be formulated to contain a high crude protein (CP) content (>20%) to support growth. However, the GIT is still immature and protease production limited (Lindemann *et al.*, 1986; Pluske *et al.*, 2003), with the absorption of amino acids limited as a result of villous atrophy and reduced villous height:crypt depth ratio. Moreover, the stomach pH of weaned pigs is increased, and gorging of feed post-weaning following a period of anorexia also increases gastric pH, reducing pepsin activation and promoting pathogen proliferation (Kim *et al.*, 2012). Reduced protein digestion and absorption results in increased protein fermentation in the posterior GIT and proliferation of proteolytic bacteria

(including *E. coli*) and subsequently increased production of branched chain fatty acids, ammonia, volatile phenols, indoles and amines (Williams *et al.*, 2001; Kim *et al.*, 2012; Rist *et al.*, 2013). The increased production of these metabolites can be harmful to the intestinal epithelium and increase permeability (Kim *et al.*, 2012). Consequently, high protein diets are considered a risk for PWD. Research has shown that reducing the CP % of weaner diets can reduce PWD, especially under ETEC challenge (Wellock *et al.*, 2007; Heo *et al.*, 2008, 2009, 2010), lowering plasma urea and faecal ammonia levels and improving intestinal morphology and integrity (Opapeju *et al.*, 2009). Heo *et al.* (2008) suggested feeding diets with 18% CP for the first 5 -7 days post-weaning to reduce the propensity to PWD, although lower CP diets must be balanced with crystalline amino acids in order to account for any amino acid deficiencies presented by feeding a lower CP diet, to avoid compromising piglet performance.

PWD can also be mitigated with the addition of non-starch polysaccharides (NSP), specifically insoluble NSP such as wheat bran. Insoluble NSP helps to retain the balance of saccharolytic:proteolytic bacteria in the microbiota. Inclusion of 4% wheat bran, under *E. coli* K88 challenge conditions, reduced PWD and increased the volatile fatty acid concentration of faeces, especially when wheat bran was coarsely ground (Bach Knudsen, 1997; Molist *et al.*, 2010, 2012). Chen *et al.* (2013) formulated weaner diets with 10% wheat bran and reported a reduction in PWD, lower abundance of *E. coli* and increased abundance of *Lactobacillus* and *Bifidobacterium* in the ileum and colon, respectively, as well as improved intestinal morphology and tight junction integrity. Viscosity of NSP must also be considered, with increased viscosity associated with increased susceptibility to ETEC (Choct, 1997; Kim *et al.*, 2012). Xylanase reportedly reduces the viscosity of jejunal digesta and increases the production of total SCFA, improving intestinal morphology and integrity and apparent ileal digestibility of NSP (Tiwari *et al.*, 2018; Duarte *et al.*, 2019). Moreover, supplementation of protease along with xylanase to post-weaned diets improved piglet performance (Duarte *et al.*, 2019). Kim *et al.* (2012) proposed that, to limit PWD, diets immediately postweaning should be formulated to limit the use of viscous or soluble NSP and include at least 0.2 - 0.8% insoluble NSP.

1.6.4 Direct interventions – Acidifiers

Acidifiers can be used in post-weaning diets to lower gastric pH, increasing pepsin activation and dietary protein digestion, prohibiting the proliferation of pathogens and reducing the amount of protein available for fermentation lower down the GIT. However, organic acids can also exert direct antimicrobial properties by diffusing across bacterial cells walls and

dissociating to release H+ and RCOO- ions, increasing cytoplasmic pH and inducing bacterial cell death (Heo *et al.*, 2013). Butyrate is an important organic acid, particularly in weaned pigs, as it is the preferred energy source for colonocytes and can increase the proliferation of the intestinal epithelium (Huang *et al.*, 2015). Provision of sodium-butyrate to weaned pigs reduced post-weaning diarrhoea and intestinal permeability and beneficially altered the microbiota composition by increasing the relative abundance of Lachnospiraceae and Ruminococcaceae, both important bacterial families for fermentation of complex polysaccharides (Huang *et al.*, 2015). Liu *et al.* (2018) suggested using a combination of free organic acids, such as formic acid to reduce gastric pH, in combination with an organic acid salt (such as sodium-butyrate) to increase release of organic acids down the length of the GIT in order to promote gut health.

1.6.5 Direct interventions – substrate induced changes via prebiotics

Prebiotics can be defined as "Live microorganisms that, when administered in adeaute amounts, confer a health benefit on the host" (Gibson *et al*., 2017). A beneficial microbiota composition, activity and consequently piglet performance can be induced by the inclusion of non-digestible fermentable ingredients including inulin, fructo-oligosaccharides, galacto-oligosaccharides, mannan-oligosaccharides, lactulose and lamarin, which are classified as prebiotics (Liu *et al.*, 2018; Guevarra *et al.*, 2019; Vigors *et al.*, 2020b). Prebiotics help to increase the saccharolytic:proteolytic microbiota abundance, increasing the production of short chain fatty acids (SCFAs) and reducing luminal GIT pH (Liu *et al.*, 2018). This was demonstrated by Awati *et al.* (2006), whereby the inclusion of inulin reduced microbial protein fermentation. Vigors *et al.* (2020b) reported that a 300ppm lamarin-rich microalgae extract beneficially changed the microbiota profile of weaned pigs, reducing the abundance of the potentially pathogenic family, *Enterobacteriaceae,* and increasing the abundance of *Prevotella.* Lactulose is a synthetic prebiotic, but elicits beneficial effects on post-weaning performance, intestinal morphology, increases the abundance of *Lactobacillus* and colonic butyric acid concentration (Guerra-Ordaz *et al.*, 2014), as well as increasing IgM and IgA levels in response to *Salmonella* typhimurium infection (Naqid *et al.*, 2015).

1.6.6 Direct interventions – addition of beneficial microbes via probiotics

Probiotics have been defined by the World health Organisation as "live microorganisms which, when administered in adequate amounts, confer a health benefit on the host" (FAO/WHO, 2001). Probiotics can have beneficial effects, particularly in reducing PWD, by directly modulating microbiota composition. Probiotics have several modes of action

including modulation of the microbiota by competitive exclusion or microbial inhibition, modulating host immune response, reducing diarrhoea and increasing nutrient digestion (Liao and Nyachoti, 2017). Increasing research has suggested that the use of probiotic lactic acids bacterial strains can have beneficial effects on weaned pig performance and health, when used immediately post-weaning. Klingspor *et al.* (2013) reported *Enterococcus faecium* NCIMB 10415 to increase nutrient absorption and improve intestinal barrier function in weaned pigs, when included in post-weaning diets. Zhang *et al.* (2010) demonstrated *Lactobacillus rhamnosus* GG to have an immunomodulatory effect on weaned piglets, reducing PWD, increasing secretory IgA concentration and reducing serum IL-6 concentration in response to *E. coli* K88 challenge. Similarly, *L. acidophilus* has been shown to mitigate lipopolysaccharide induced inflammation post-weaning (Qiao *et al.*, 2015). Moreover, a combination of *L. reuteri* and *L. fermentum* at a 0.1% inclusion rate to weaner diets increased *Lactobacillus* and reduced *E. coli* faecal concentrations and diarrhoea score, whilst promoting ADG, attributed to increased apparent total tract digestibility of nitrogen and gross energy (Zhao and Kim, 2015). *Saccharomyces cerevisiae* also has probiotic effects in weaned pigs, reducing post-weaning diarrohea occurrence, duration and *E. coli* shedding (Trckova *et al.*, 2014). This anti-diarrhoeal mode of action is thought to be mediated by *S. cerevisiae* cell wall components, notably α-$_D$-mannans, which can bind to mannose receptors on *E. coli* and *Salmonella* reducing their adhesion to the intestinal mucosa (Daudelin *et al.*, 2011; Liu *et al.*, 2018).

1.6.7 Indirect interventions – manipulation of the sow microbiota

Due to the importance of the sow and home pen in the development of the neonatal microbiota, altering the sow microbiota profile offers a potential mode by which to modulate the neonatal microbiota in a less labour-intensive manner than altering the microbiota directly at the piglet level. The provision of yeast derivatives to sows in gestation at a rate of 2g/kg was able to reduce the abundance of potentially pathogenic bacteria, including *Helicobacter, Desulfovibrio* and *Escherichia/Shigella*, with progeny of yeast derivative-fed sows having higher microbiota diversity at one-week of age (Hasan *et al.*, 2018). Moreover, by modulation of the sow microbiota with probiotics in gestation, it is possible to indirectly alter the microbiota of neonatal piglets. *Lactobacillus acidophilus* and *Bifidobacterium lactis* provided to gestating sows for 7 days pre-farrowing resulted in these two genera colonising 75% and 80% of the litter by 14 days of age, respectively (Buddington *et al.*, 2010); these genera were not detected in sow faeces prior to probiotic treatment. Similarly, Menegat *et al.* (2019) demonstrated that *Bacillus subtilis C-3102* supplementation to sows in gestation and lactation increased *B. subtilis*

and *Bacillus* counts in piglets on day 18 of lactation. However, administering sows with a faecal microbiota transplant (FMT) from pigs with a high feed efficiency had a negative effect on body weight, absorptive capacity and gut health of progeny (McCormack *et al.*, 2018; 2019b).

1.6.8 Early life microbiota interventions

As described in the preceding sections for weaned piglets, the microbiota development of neonatal piglets can also be modulated directly through the use of antibiotics, probiotics and FMT. Schokker *et al.* (2014, 2015) demonstrated that antibiotic use in 4-day old neonates changed the microbiota composition at 8 and 176 days of age. Early life probiotic treatment can modulate the microbiota of piglets during the period of developmental plasticity. Hou *et al.* (2015) reported that orally dosing neonates with *L. reuteri 15007* over 4 days (in succession or every 4th day) increased the abundance of *Bifidobacterium* and reduced the abundance of *E. coli* in the ileum and proximal distal colon. Similar results have been obtained when repeatedly orally dosing neonatal piglets with single or multi species/strains of lactic acid bacteria, resulting in lower abundances of pathogenic bacteria and improvements to ADG, intestinal integrity and morphology and antioxidant capacity, whilst reducing incidences of diarrhoea, although the most striking effect appears to be on the development of the immune system (Liu *et al.*, 2014; Hou *et al.*, 2015; Liu *et al.*, 2017; Wang *et al.*, 2019a, b, Xin *et al.*, 2020; Yang *et al.*, 2020; Wang *et al.*, 2020). Aside from bacteria, *Saccharomyces cerevisiae* treatment throughout the pre-weaning period altered the caecal microbiota community composition of piglets. Although the microbiota diversity was reduced, the abundance of SCFA-producing bacteria and piglet ADG increased, attributed to increasing abundance of *Prevotella* (Kiros *et al.*, 2019).

1.7 Summary of literature review and identification of knowledge gaps

From this literature review, it is clear that weaning weight is positively correlated with lifetime performance. Efforts to increase efficiency of pig production must focus on maximising pre-weaning performance and building robust piglets to cope with the stressors of weaning, especially in the face of the impending EU ban on the pharmacological use of ZnO. However, the increasing proportion of LBW pigs (particularly LBW IUGR) per litter present a challenge, as they are associated with poorer growth and farm system inefficiencies. Despite this, a proportion of LBW pigs are able to exhibit catch up growth; this ability has been correlated with morphometric measures at birth, although a mechanistic explanation for this association is yet to be ascertained. LBW pigs present a less mature GIT at birth, which persists post-weaning, and have altered immune function; this may alter the GIT microbiota development in

LBW pigs. In turn, the GIT microbiota plays an integral role in host metabolism and immune function. Perturbations to the microbiota in early life can have long term effects on the microbiota composition and subsequently piglet performance and health.

There is currently a gap in the literature establishing associations between the early life microbiota and the pre- and post-weaning performance of pigs. Research has yet to establish whether there are associations between the microbiota and LBW piglets which can exhibit catch up growth. Moreover, there seems to be a lack of understanding of how current early life management strategies aimed at promoting pre-weaning growth affect the microbiota development of piglets, and whether these effects are birthweight dependant, given the differences in GIT physiology and immune function of LBW pigs. Addressing this knowledge gap will not only help to further characterise LBW pigs who can exhibit catch up growth, but will help to identify how the microbiota of LBW pigs can be manipulated to increase performance. To do this, however, requires a deeper understanding of factors which affect gut microbiota development of LBW pigs during early life, in order to identify how it can be manipulated to benefit performance. This forms the topic of the PhD.

1.8 Thesis aims

The overall aim of the thesis was to investigate how the piglet microbiota develops in early life and how this relates to characteristics of the sow, birthweight and growth trajectory of piglets.

The specific objectives of this thesis were:

- To determine, through longitudinal analysis, how the piglet microbiota develops over time and to identify microbiota markers associated with low birthweight piglets who were able to exhibit superior growth rates compared to those who remain stunted (Chapter 2).

- To conduct a detailed analysis of the sow microbiota changes during the periparturient period, including differences associated with sow parity, as maternal microbiota sources seed the neonatal gastrointestinal tract (Chapter 3).

- To determine how cross-fostering, and thus exposure to increased sources of maternal and pen microbes, affects microbiota development and performance of neonates, and whether microbiota changes are affected by piglet birthweight and sow parity.

Furthermore, to identify the relative importance of maternal microbial sources on the microbiota development of neonatal piglets (Chapter 4).

- To investigate whether a direct-fed autogenous *Enterococcus faecium* microbial treatment during early life alters the microbiota development and subsequently performance of piglets, and to determine whether this effect is birthweight dependant (Chapter 5).

Chapter 2. Changes in faecal microbiota profiles associated with performance and birthweight of piglets

2.1 Introduction

The gut microbiota is now recognised for its fundamental role in moderating host health and phenotype. Increasing evidence suggests that perturbations to the neonatal microbiota development can result in a higher propensity to develop certain health disorders, including metabolic disorders and problems linked to the immune system (Schokker *et al.*, 2014, 2015; Gensollen *et al.*, 2016; Stiemsma and Michels, 2018). Furthermore, the neonatal microbiota can affect preterm infant growth (Grier *et al.*, 2017) and gastrointestinal tract (GIT) physiology of piglets (Lallès *et al.*, 2014). Thus, the neonatal period can be identified as one of the critical stages in which changes to the microbiota can have long term consequences on host health or phenotype.

As the demand for efficient pigmeat production increases, one response from the pig industry has been to increase litter size. However, this comes at the expense of average individual birthweight and litter uniformity, with a larger proportion of low birthweight (LBW) pigs born per litter (Quiniou *et al.*, 2002; Martineau and Badouard, 2009). LBW pigs are exposed to varying degrees of intrauterine growth restriction (IUGR) (Foxcroft *et al.*, 2006; Baxter *et al.*, 2008; Rutherford *et al.*, 2013), as uterine capacity to deliver nutrients to the foetuses has not increased at a rate proportionate to sow prolificacy. IUGR foetuses prioritise brain and heart development over other organs, such as the liver, GIT and the development of muscle fibres (Rehfeldt and Kuhn, 2006; Roza *et al.*, 2008; Amdi *et al.*, 2013). Poor development of the GIT of IUGR pigs persists pre- and post-weaning (D'Inca *et al.*, 2011; Dong *et al.*, 2014), with reduced rate of GIT maturation thought to negatively affect performance (Wang *et al.*, 2005). LBW pigs represent a considerable economic problem to pig producers as a result of increased morbidity and mortality (Hales *et al.*, 2013; Ferrari *et al.*, 2014; Feldpausch *et al.*, 2019), higher propensity to develop enteric health problems, poorer feed efficiency as well as carcase yield and quality with increased adiposity (Rehfeldt *et al.*, 2008; D'Inca *et al.*, 2011; Nissen and Oksbjerg, 2011; Zhang *et al.*, 2018a). However, a proportion of LBW pigs exhibit compensatory growth within the same environment as those who remain stunted (Paredes *et al.*, 2012; Douglas *et al.*, 2013; Huting *et al.*, 2018), although an explanatory mechanism for this phenomenon is yet to be proposed.

Due to the difference in performance and health of LBW and normal birthweight (NBW) pigs and the importance of the microbiota in modulating host health, recent research has begun to explore the differences in the microbiota of LBW and NBW pigs. Early studies have shown increased bacterial adhesion in the ileum and colon of IUGR LBW pigs during early life (D'Inca *et al.*, 2010, 2011). More recent studies have demonstrated the microbiota community composition of the jejunum, ileum, colon (Li *et al.*, 2019; Zhang *et al.*, 2019a) and faeces (Li *et al.*, 2018) is different between LBW and NBW pigs up to 5 weeks of age. Whilst studies have identified microbiota characteristics related to growth of pre-weaned and weaned pigs (Mach *et al.*, 2015; Ding *et al.*, 2019), as well as feed efficiency of grower-finisher pigs (McCormack *et al.*, 2017; Han *et al.*, 2018; Quan *et al.*, 2018), no published studies have compared the faecal microbiota of LBW piglets able to exhibit compensatory growth to those who remain stunted, in comparison to NBW pigs. Identifying early-life microbiota markers for LBW pigs able to exhibit compensatory growth could have important implications for the pig industry with regards to informing management interventions.

2.1.1 Study aims and hypotheses

This study aimed to characterise the microbiota development longitudinally and capture microbiota changes associated with early life and weaning. Specific aims included identifying how the early-life microbiota development was affected by birthweight and therefore identify microbiota markers associated with piglet birthweight. Furthermore, the study aimed to identify characteristics of the microbiota associated with growth rate of LBW and NBW pigs during the pre- and post-weaning period. The study firstly hypothesised that the longitudinal microbiota profile would be significantly different between LBW and NBW piglets due to differences in GIT physiology and consequently the environment for microbiota colonisation. Secondly, the study hypothesised that LBW piglets capable of exhibiting compensatory growth could be identified by significant differences in the microbiota profile from those LBW piglets who remain stunted.

2.2 Materials and methods

2.2.1 Experimental design

A total of 26 experimental piglets (dam line Large White x Landrace and sire line Hylean Synthetic, Hermitage Seaborough, Ltd., United Kingdom) from 10 experimental sows from 2 consecutive batches were used in this study. The experiment consisted of a 2 × 2 factorial design with repeated measures taken at 10 different time points: the experimental factors were piglet birthweight (low birthweight (LBW; 0.80 - 1.25kg, n = 13) or normal birthweight

(NBW; 1.50 - 2.00kg, n = 13)) and piglet performance between birth and 56 days of age, denoted as ADG class ("poor" or "good"). All piglets received a post-hoc ADG class depending on their daily live weight gain (DLWG) for the entire experimental period compared to their birthweight (BiW) class average. Piglets within each BiW class were defined as having a "poor" ADG class if their DLWG was below the BiW class average and a "good" ADG class if their DLWG was above the BiW class average, calculated between birth – 56 days of age. Experimental piglets formed 13 sibling pairs of LBW and NBW piglets, with three sows contributing two sibling pairs to the experimental cohort due to the antibiotic free status of their piglets.

2.2.2 Animal housing and management

Gestating sows were managed in a 3-week indoor batch farrowing system and housed in straw yards in groups of 5 sows of similar size and parity. During gestation, sows were fed a home-milled mash gestation diet based on barley and soyabean meal (13.14 MJ digestible energy (DE) /kg, 13.82% crude protein (CP) and 0.62% standard ileal digestible (SID) lysine). They received approximately 2-2.50 kg/head at 0730 h daily throughout gestation.

Multiparous sows were moved from solid floored straw yards to a conventional part-slatted farrowing pen with a farrowing crate at approximately 109 days of gestation, with primiparous sows entering at 111 days. The later entry of primiparous sows into the farrowing house was standard practice on the commercial unit, reducing the amount of time primiparous sows spent in farrowing crates in an effort to reduce stress. Prior to entry, the farrowing pen was washed and disinfected (concentration = 0.03% PhenoPharm, East Riding Farm Services, UK) and allowed to dry for a minimum of 7 days. Farrowing crate dimensions were: entire pen 1.80m width x 2.42m length, creep area 1.11m length x 0.80m width and sow crate 0.6m width x 1.77m length to the feed trough. All sows were wormed with Bimectin (5ml primiparous and 8ml multiparous intramuscularly (IM), Bimeda, Llangefni, UK) upon entry to the farrowing house and received a FarrowSure Gold vaccine against porcine parvovirus, erysipelas and leptospirosis on the day before weaning (2ml IM, Zoetis, Surrey, UK), which occurred at ~28 days post-partum. Following housing in the farrowing crates, sows received approximately 0.70 kg of the gestation diet fed twice daily at 0745 h and 1500 h until farrowing. Sows which had not farrowed by 116 days of gestation were induced with intramuscular injection of 2ml of a prostaglandin analogue (Planate, Intervet UK, Walton, UK). On the day after farrowing sows were transferred to a home-milled mash lactation diet (13.98 MJ DE/kg, 18.50% CP and 0.95% SID lysine) initially as a 2.0 kg/head/day allowance, which was increased to appetite by 0.5

kg/head/day until a 10 kg/head/day limit was reached. Water was available *ad libitum* through a nipple drinker.

The farrowing house was maintained at 21°C, whilst an enclosed heated creep area (infrared heat lamp) was available to piglets; this contained wood shavings as bedding for the first week of life. Water was available to piglets *ad libitum* through piglet nipple drinkers positioned at the rear of the sow. Piglets were teeth clipped within the first 12 hours of life, then received an iron injection (1ml IM Gleptosil 200mg iron/ml, CEVA Animal Health Ltd, Amersham, UK) and were tail docked at ~4 days of age. Piglets received the first of their 2-stage vaccination program against *Mycoplasma hyopneumoniae* (1ml M+PAC, Intervet, UK) at 7 days of age. Creep feed (16.50% DE MJ/kg, 22.50% CP and 1.7% lysine; FlatDeck 1, A-One Feeds Supplements Ltd, Thirsk, UK) was made available to experimental piglets from 10 days of age at a rate of 25g/pen/day, increasing up to 150g/pen/day during the final week of lactation. The creep feed was scattered in the creep area which had a solid floor.

At weaning (28 days of age ±1 day), experimental piglets were housed in fully slatted, temperature controlled flat deck accommodation. Room temperature was initially set to 26°C and reduced by 0.2°C/day to a minimum of 22°C, which was sustained for the remaining trial period. Experimental pigs were housed in pens of 20 piglets according to birthweight class, thus segregating sibling pairs of LBW and NBW piglets into pens of either LBW or NBW pigs only. Each pen was furnished with two nipple drinkers and two multi-space feed troughs. Feed and water were available *ad libitum*. Piglets received a three-stage weaner starter diet regime (Stage 1: 16.50 MJ DE/kg, 22.50% CP and 1.7% SID lysine (Flat Deck 1). Stage 2: 16.00 MJ DE/kg, 21.00% CP and 1.55% SID lysine (Flat Deck 150). Stage 3: 15.8 MJ DE/kg, 21.00% CP and 1.45% SID lysine (FlatDeck Turbowean); A-One Feeds Supplements Ltd, Thirsk, UK). Each of the post-weaning diets was fed for 1 week to permit a period of microbiota adaption before faecal sampling occurred. This was done as a compromise between replicating commercial practice of feeding a multi-stage weaner starter diet and avoiding changes in diet composition from confounding the microbiota differences related to BiW and ADG classes which could arise if experimental pigs were fed to the standard commercial practice of feeding starter diets on a kg/hd allowance result, attributed to the higher feed intake associated with NBW pigs. After the weaner starter diets were consumed, experimental pigs were fed a home-milled weaner meal *ad libitum* until day 56 of age (14.82 MJ DE/kg, 20.55% CP and 1.28% lysine). Neither post-weaning diets, nor creep feed contained antibiotics or pharmacological levels of zinc oxide. At the point of weaning, pigs received their 2nd stage vaccine against *Mycoplasma hyopneumoniae* in addition to a porcine circovirus type 2 vaccine, as intramuscular injections (1ml M-PAC, MSD Animal Health,

Milton Keynes, UK; 1ml Ingelvac CircoFLEX; Boehringer Ingelheim, Duluth, USA). None of the experimental piglets received any antibiotics for the duration of the study.

2.2.3 Experimental procedures

Following the methodology reported by Douglas *et al.* (2014a), all piglets from two consecutive farrowing batches were weighed, sexed and classified at first handling (within the first 6–12 h of life; day 0) as being of LBW (0.80 – 1.25 kg) or NBW (1.50 – 2.00 kg). Only pigs within the LBW and NBW criteria were individually ear tagged for identification purposes (Dentag, Toptag, United Kingdom), piglets who fell outside of the LBW and NBW categories were excluded from the study. Cross-fostering of piglets occurred within the first 24 h of life. In experimental litters cross-fostering was not conducted to create litters of homogenous piglets, only to standardise litter size according to functional teat capacity. Where the number of piglets born alive outnumbered functional teat capacity, piglets < 800g and between 1.25 - 1.50 kg were moved to non-experimental sows. Thus, experimental litters consisted only of a sows own progeny, but litter compositions were heterogenous in birthweight; no fostering of piglets into the experimental litters was permitted to reduce the introduction of foreign microbes to the pen environment.

Pigs were weighed and one faecal sample was collected from each experimental pig pre-weaning on days 4, 8, 14, 21 and 27 (weaning −1 day) of age. Sex was balanced between LBW and NBW experimental piglets. Post-weaning, pigs were again weighed and faecal sampled on days 32, 35, 42, 49 and 56 of age. All faecal samples were collected directly from the rectum during natural defecation when experimental pigs were individually isolated away from the pen and weighed. Samples were collected in 50 ml sterile plastic tubes (Sarstedt AG & Co. KG, Germany) and stored at −80°C within 2 hours of sampling until they were utilised for DNA extraction (< 3 months). Piglet average daily gain (ADG) was calculated between successive weighing days and for the entire experimental period (birth – 56 days of age).

2.2.4 16S rRNA gene sequencing

Microbial genomic DNA was extracted from < 250mg of faecal sample using the DNeasy PowerSoil HTP 96 kit (Qiagen, UK) with centrifugation, following manufactures instructions. The V4 region of the 16S rRNA gene was amplified by the Nex_16S_515 F (TCGTCGGCAGCGTCAGATGTGTATAAGAGACAGGTGYC AGCMGCCGCGGTAA) and Nex_16S_806 R (GTCTCGT GGGCTCGGAGATGTGTATAAGAGACAGGGACTACNVGG GTWTCTAAT) primers (MWG, Germany). DNA was amplified by PCR (Bio-Rad C1000 Thermo Cycler, Bio-Rad

Laboratories, Ltd., United Kingdom). The success of amplification was visualised using gel electrophoresis (1% agarose in TBE buffer). The amplified products were purified using Agencourt AMPure XP beads (Beckman Coulter, United Kingdom) and magnetic separation. Amplified products were then indexed using a second round of PCR (Nextera® XTIndex Kit v2, Illumina, United States), then purified for a second time. The concentration of amplified products was then determined using Picogreen measured on a Fluroskan Ascent Plate Reader (Thermo Fisher Scientific, United Kingdom) and pooled in equal quantities to generate a 20 nM pool. The length of the amplified products in the pool was determined using a tapestation (2200 Tapestation, Agilent Technologies, United States) and the concentration of the pool quantified by a Qubit Fluorometer (Invitrogen, Thermo Fisher Scientific, United States). The library was then diluted to create a 4 nM pool used to generate the final amplicon library. The amplicon libraries were sequenced on the Illumina MiSeq (Illumina, San Diego, CA, United States) for paired-end fragment sizes of 300 bp.

Analysis of raw sequencing reads was performed in QIIME2 (v 2018.8; Bolyen *et al.*, 2019). Paired end sequencing reads were imported into QIIME 2 in the cassava 1.8 paired-end demultiplexed Fastq format, based on unique PCR index barcodes used for each sample. PCR primers were removed using the 'cutadapt' plugin and the *trim-paired* method. Paired end reads were joined using the plugin 'vsearch' and command *join-pairs*, with a minimum of 35 mismatches in overlapping bases, minimum overlap of 210 base pairs and maximum merge length of paired end reads of 260 base pairs. A quality filter was applied to the merged reads using the 'quality-filter' plugin. Based on the default setting, merged sequences were only retained when they had a PHRED score over a threshold value of 4. Sequences were truncated where more than 3 base calls in succession had a PHRED score below the threshold value. Resulting sequences were then only retained if, after truncation, the sequences were at least 75% the length of their original sequence. Quality of sequences were then visually assessed using the plugin 'demux'. Merged sequences were denoised as an additional quality control step using the 'deblur' plugin. Deblur utilizes a positive alignment-based filtering by specifying a reference database. Although the reference database was not used for taxonomic assignment at this stage, for the purpose of this study the SILVA 16s rRNA reference database (release version 132) was selected (Quast *et al.*, 2013). The deblur steps output unique denoised sequences termed Amplicon Sequence Variants (ASVs); ASVs equate to OTUs and were referred to as this for all subsequent analysis stages. Trim length was set to 253, this resulted in an outputted OTU table and file of taxa associated with OTUs (taxonomy file). Chimeric sequences were detected using the 'vsearch' plugin and the *uchime-denovo*

method. Once identified, chimeras were removed from the OTU table and taxonomy file using the "feature-table" plugin and the *filter-features* and *feature-seqs* commands, respectively. The OTU table was then assigned taxonomy using the SILVA database and the 16S rRNA V4 region to determine taxonomy, as performed by the "feature-classifier" plugin and *classify-consensus-vsearch* method. This method was selected over *classify-consensus-blast* as it searched the whole SILVA database for taxonomic assignments before selecting the most appropriate taxonomy, as opposed to the first positive match which may not always be the most accurate taxonomic assignment. A total of 3,051,414 sequencing reads were obtained from an initial 242 piglet faecal samples run on the Illumina MiSeq (Illumina, United States). Sequencing corresponding to archaea, mitochondria and chloroplasts were removed and all sequences were rarefied to 1000 reads per sample, identifying 1700 OTUs corresponding to 19 phyla, 25 classes, 41 orders, 67 families and 158 genera. After rarefaction 180 samples were retained, with sample number for each piglet age group as follows: day 4 (n = 25), 8 (n = 15), 14 (n = 16), 21 (n = 20), 27 (n = 13), 32 (n = 18), 35 (n = 12), 42 (n = 19), 49 (n = 17) and 56 (n = 25). A phylogenetic tree was created by aligning sequences using the "alignment" plugin and the *mafft* method, any unobserved or gapped regions of columns were then masked from the alignment using the *mask* method. A tree file was then generated using the "phylogeny" plugin and the *FastTree* method to create the phylogenetic tree.

2.2.3 Statistical Analysis

All statistical analyses were conducted in R version 3.4.4. The main fixed effects selected for consideration from the raw experimental data in all statistical models were piglet age, BiW class and ADG class and their interactions. Piglet ID was nested within sow ID and specified as the random effect in all models unless stated otherwise, as these variables formed the repeated measures in the dataset. All models were tested for validity, using two diagnostic plots. The first diagnostic plot consisted of a Q-Q plot of the standardised residuals, whilst the second was a scatterplot of the standardised residuals plotted against fitted values. The 'emmeans' (v 1.3.4), 'car' (v 3.0 – 8) and 'multcomp' (v 1.4 – 13) packages were used for all post-hoc comparisons of significant fixed and interactive effects for all performance, alpha diversity and genera relative abundance models, and to generate compact letter displays, whereby least square means with different lowercase superscript letters denote significant differences ($P < 0.05$).

2.2.3.1 Piglet Performance

Longitudinal liveweights (LWs) (kg) were not normally distributed and displayed positive skew (*skewness* function 'moments' package v 0.14), thus a log10 transformation was applied to the LW data. A general linear model (glmer) ('lme4' package v 1.1 – 23) was performed, testing interactions between piglet age, and BiW class. Interactive terms were removed from the model in the absence of significance. Model diagnostic plots revealed model residuals to be normally distributed. Piglet ADG (kg) between successive time points was not normally distributed but did not display skewness, thus a glmer model was performed, testing the interaction between time period and BiW class.

2.2.3.2 Alpha diversity

The number of observed OTUs and Shannon diversity index were the two measures of alpha diversity used in this experiment. Alpha diversity values were calculated using the 'vegan' package (v 2.5). To determine the effects of longitudinal changes in alpha diversity with respect to the fixed effects, a glmer model was run for observed OTUs using a Poisson regression model in the 'lme4' package (v 1.1-21), as observed OTUs are count data. Shannon diversity index values were normally distributed and so a linear mixed effect model was performed using the 'lme4' package (v 1.1-21). Model diagnostic plots were inspected and considered to be normally distributed.

2.2.3.3 Beta diversity

Bray-Curtis distances (Anderson, 2001; Anderson and Walsh, 2013) were used as the beta diversity measure to assess the similarity of the microbiota community composition between piglet faecal samples. Bray-Curtis distances were calculated using the 'rbiom' package (v 1.0.2.9002). A PERMANOVA was performed using the Adonis function in the 'vegan' package, with 999 Monte Carlo permutations, to determine whether there were differences between microbiota community compositions based on the piglet age, BiW and ADG class.

2.2.3.4 Genera abundance

To determine how genera abundance was affected by the fixed effects of interest, relative abundances were longitudinally modelled for the top 20 most abundant genera, as these taxa were present in most pigs and time points. Taxa modelling was performed at the genera level, where taxonomic assignment permitted, by generalized linear mixed models using Template Model Builder via the 'glmmTMB' package (v 0.2.3) for 17 of the 20 taxa. Taxa abundance data were proportional, so within the model the family function was specified as *beta_family*

(link = "logit"). Model validity was assessed using the 'DHARMa' package (v 0.2.4). Models with nested random effects failed to converge for three of the 20 taxa (*Bacteroides, Escherichia–Shigella* and *Prevotella 1*) and were consequently fitted with piglet ID as a fixed variable within the model using the 'betareg' package (v 3.1-2). The validity of beta regression models fitted using *betareg* was determined through inspection of a half-normal plot of standardised residuals and a scatter graph of standardised residuals plotted against fitted values. The *Bacteroides* and *Prevotella 1* models did not display major deviations from the assumed normality or homoscedasticity assumptions of the beta regression model. However, the *Escherichia-Shigella* model violated these assumptions. Based on the Cook's Distance plot, 12 samples were removed from the dataset as these samples corresponded to a Cooks Distance of over 0.07 and to samples identified as having 0 abundance or being outliers in the dataset, subsequently diagnostic plots displayed normality.

2.3 Results

The experimental cohort displayed no symptoms of ill-health and received no antibiotics for the duration of the study. For LBW piglets the DLWG BiW class average between birth and 56 days of age was 0.28 kg/d (SE = 0.010), whilst for NBW piglets this was 0.37 kg/d (SE = 0.016). At the phylum level (**figure 2.1**), piglet microbiota was dominated by Firmicutes and Bacteroidetes across all time points, and to a lesser extent Proteobacteria pre-weaning. Proteobacteria average abundance reduced dramatically throughout lactation, from an average of 13.4% at day 8 to 4.97% at day 27. Post-weaning average Proteobacteria abundance was less than 1% at each sampling time point. Firmicutes increased in abundance steadily between day 4 (average 49.46%) and day 56 (average 81.29%) to become the most dominant phylum. Bacteroidetes displayed less variability in abundance as piglet age increased; taxa abundance fluctuated between 4 and 56 days of age (average 35.49 and 13.82%, respectively).

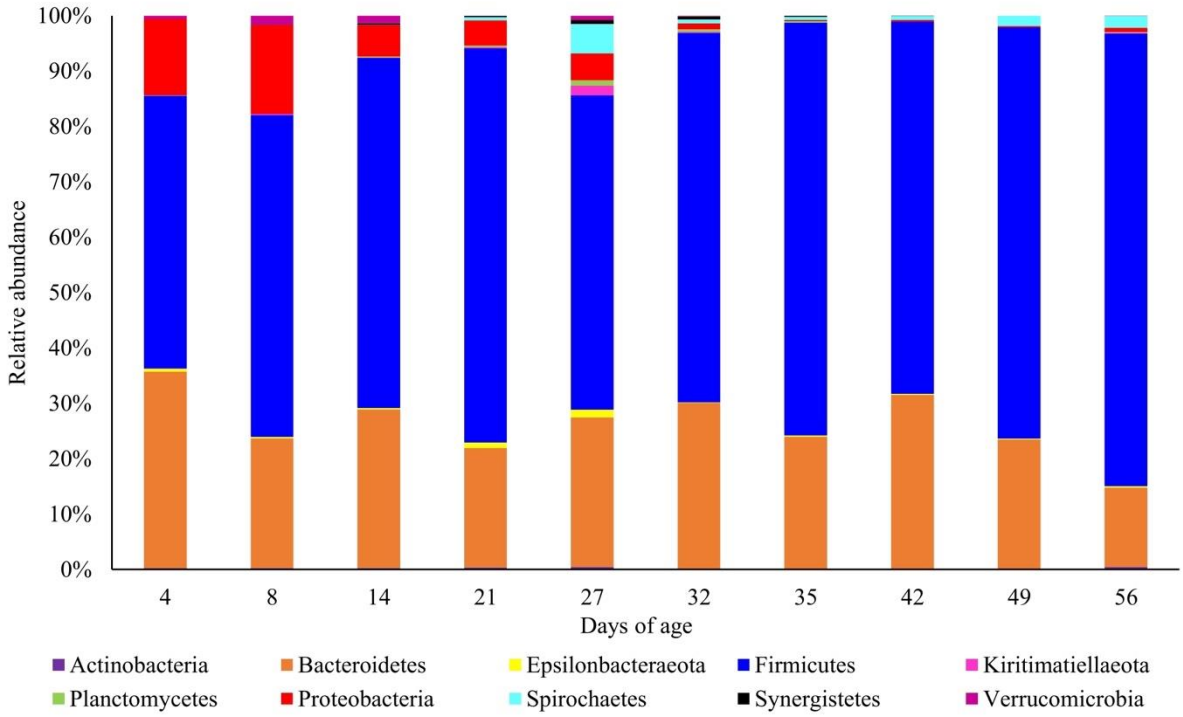


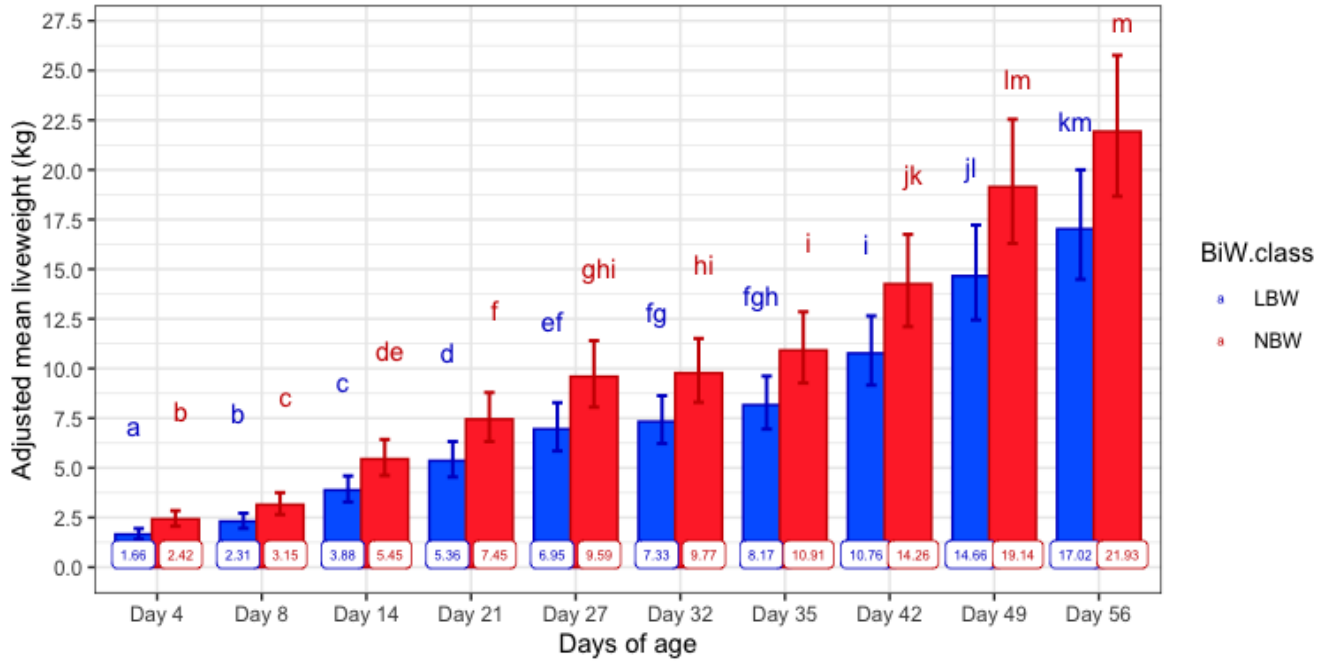
Figure 2.1 The relative abundance of the top 10 phyla according to piglet age. Weaning occurred at 28 days of age.

2.3.1 Piglet performance

There was a significant effect of piglet age ($P < 0.001$) on LW, with LW increasing significantly over time, although there was no significant difference between piglet LWs on days 27 and 32. There was also a significant effect of BiW class on piglet LW ($P < 0.001$), with LBW pigs consistently having a lower LW across all time points (**figure 2.2a**). There was no significant interaction between piglet age and BiW class on LW ($P > 0.05$).

There was a significant effect of time point on piglet ADG ($P < 0.001$). ADG was significantly lower between days 28 – 32 of age compared to all other time periods, except for birth – day 8. ADG was significantly higher between days 43 - 49 compared to all other time periods, whilst ADG between days 36 – 42 was significantly higher than all the time periods proceeding 32 days of age. There was a significant effect of BiW class on ADG, with LBW pigs having significantly lower ADG when averaged across all time periods ($P < 0.01$). However, there was no significant interaction between BiW class and time period on piglet ADG ($P > 0.05$) (**figure 2.2.b**).

a)



b)

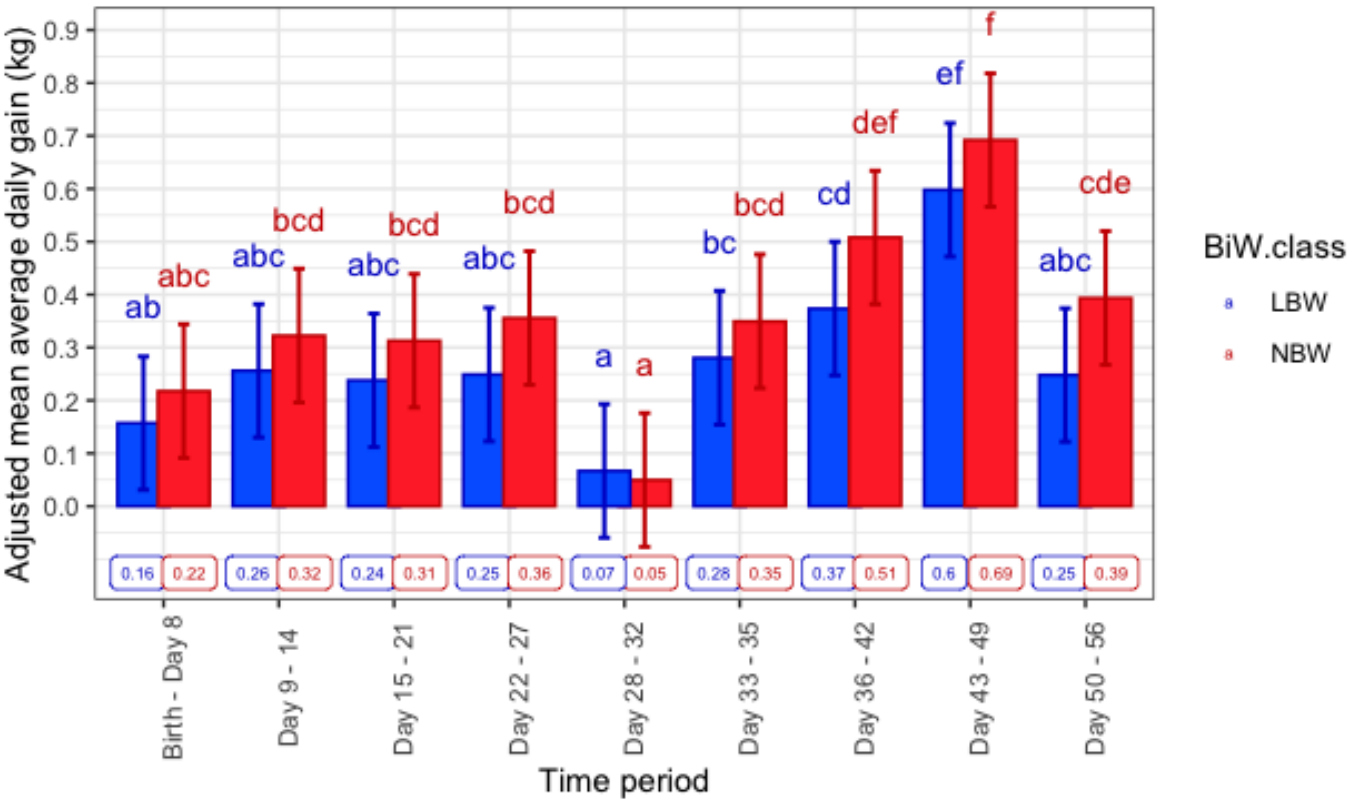


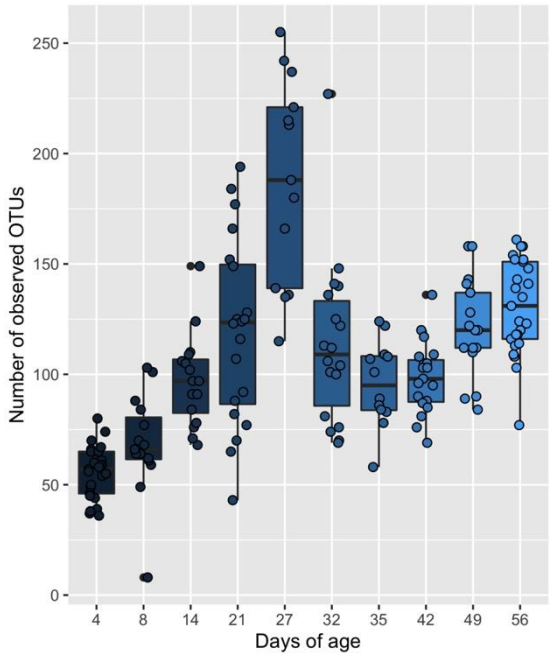
Figure 2.2. The effect on liveweight (a) and average daily gain (b) across the experimental period according to birthweight (BiW) class (LBW = low birthweight (0.80 – 1.25kg), NBW = normal birthweight (1.50 – 2.00kg). Values with different superscripts (a-m) are significantly different (P <0.05). Weaning occurred at 28 days of age and piglets were moved

from pellets to a home-milled mash on day 49. Results are presented as adjusted means and 95% confidence intervals.

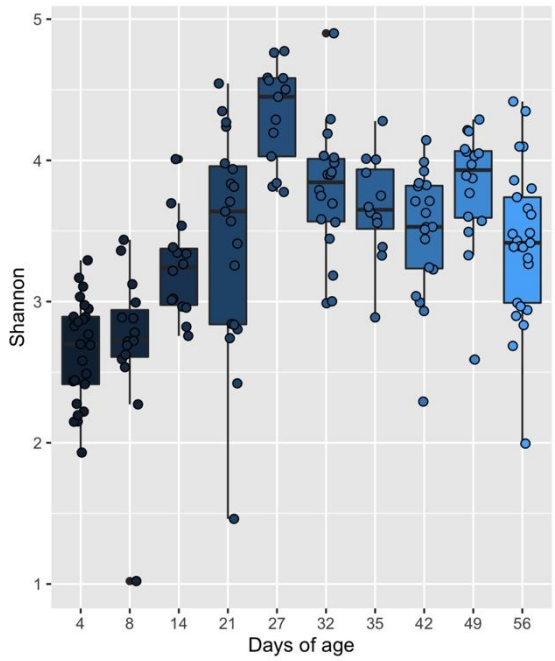
2.3.2 Alpha diversity

Piglet age significantly affected both the number of observed OTUs and Shannon diversity index ($P < 0.05$) (**figures 2.3a, b**). For both indices, alpha diversity increased exponentially until weaning where it sharply declined, followed by a steady increase and plateau in diversity. There was no significant fixed effect of BiW or ADG class on number of observed OTUs and Shannon diversity index ($P > 0.05$). There was a significant interaction between piglet age and BiW class for the number of observed OTUs. At 21 days of age NBW piglets had a significantly higher number of observed OTUs, whilst on days 27, 32 and 56 of age LBW pigs had a significantly higher number of OTUs (**figure 2.3c**; in all cases $P < 0.05$). There were three-way interactive effects between piglet age, BiW class and ADG class on the number of observed OTUs. On day 27 both "poor" and "good" LBW pigs had a higher number of observed OTUs compared to only NBW pigs with a "good" ADG class ($P < 0.05$). No other significant interactions between fixed effects were reported.

a)



b)



c)

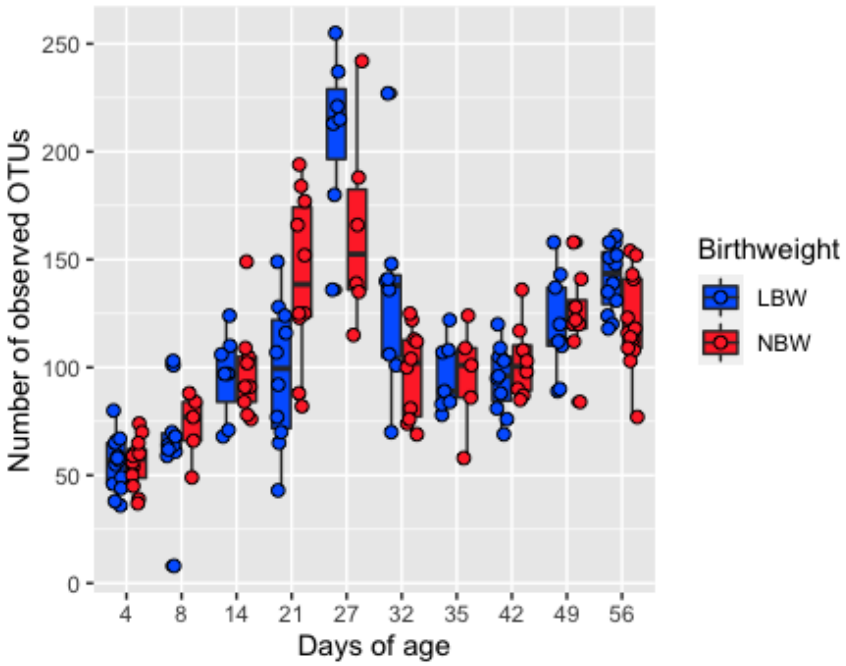


Figure 2.3. The change in number of observed OTUs (a) and Shannon diversity index (b) for all piglets according to age. The number of observed OTUs for each birthweight class (LBW = low birthweight (0.80 – 1.25kg), NBW = normal birthweight (1.50 – 2.00kg)) (c).

2.3.3 Beta diversity

Piglet age was a significant key driver in the development of the microbiota community composition ($P < 0.001$) based on the PERMANOVA results using Bray–Curtis distances. The microbiota developed from a highly varied community composition between days 4 and 8 of age to a more uniform, stable and distinct microbiome from 35 days of age (**figure 2.4**). There was no significant effect of either BiW or ADG class on the microbiota community composition ($P > 0.05$). Furthermore, there was no significant interaction between any of the fixed effects on the longitudinal microbiota community composition ($P > 0.05$).

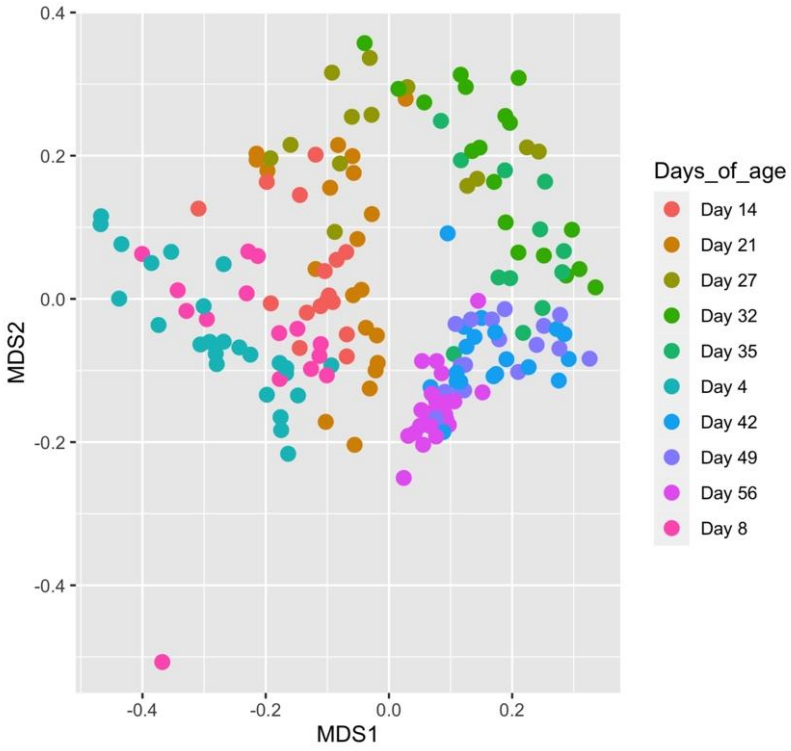


Figure 2.4. Multi-dimensional scaling plot of the longitudinal Bray-Curtis distances according to piglet age.

2.3.4 Genera abundance

All the top 20 most abundant genera were affected by age ($P < 0.01$) (**table 2.1**).

The mean relative abundance of several genera changed at critical ages in piglet management and development, such as the neonatal phase, weaning and the introduction of creep feed (**figure 2.5**). At the genus level, *Lactobacillu*s was the most dominant genus throughout the study. However, abundance sharply declined from a mean of 26.71% at 21 days of age to 4.47% at 27 days of age. Post-weaning, *Lactobacillus* abundance steadily increased to 24.68% at 56 days of age. *Bacteroides* was the second most abundant genus; abundance was highest in piglets at 4 days of age (28.82%), but steadily declined longitudinally to a mean of 0.02% in piglets at 56 days of age. *Escherichia/Shigella* was significantly abundant in neonatal piglets (10.20% at 4 days of age) but reduced as lactation progressed (2.25% at 27 days of age), post-weaning abundance declined further (0.4% average). Several initially lower abundance genera increased in mean abundance between 8 and 14 days of age, coinciding with the introduction of creep feed. These genera included: *Christensenellaceae R-7 group* (0.77% increasing to 4.49%), *Ruminococcaceae UCG-005* (0.55% increasing to 2.38%), *Rikenellaceae RC9 gut group* (0.75% increasing to 1.68%), *Prevotella 2* (0.81% increasing to

6.33%) and *Ruminococcaceae NK4A213 group* (0.75% increasing to 1.39%). Longitudinal patterns of other genera revealed an increase in mean abundance in response to weaning (27–32 days of age); these included: *Prevotella 9* (1.01% increasing to 7.65%), *Ruminococcaceae UCG-014* (1.72% increasing to 2.97%), *Subdoligranulum* (2.05% increasing to 3.19%), and *Faecalibacterium* (0.22% increasing to 2.65%).

Table 2.1. Changes in the relative abundance of the top 20 genera over time. All *P* values were FDR adjusted for multiple comparisons (Benjamini and Hochberg, 1995), *P* values below 0.05 were considered statistically significant. Significant differences in genera abundance between days of age are identified by differences in the assigned Tukey HSD compact letter displays.

Genera	Days of age										FDR adjusted *P* values
	4	8	14	21	27	32	35	42	49	56	
Unclassified Prevotellaceae	0.0285^{a}	0.0342^{ab}	0.0109^{ab}	0.0116^{ab}	0.0262^{ab}	0.0207^{b}	0.0148^{ab}	0.0179^{b}	0.0202^{b}	0.0094^{ab}	1.05E-04
Prevotella 2	0.0115^{a}	0.0081^{ab}	0.0633^{c}	0.0396^{c}	0.0213^{c}	0.0157^{bc}	0.0129^{bc}	0.0113^{bc}	0.0076^{bc}	0.0014^{ab}	3.05E-11
Prevotella 9	0.0012^{a}	0.0002^{a}	0.0018^{a}	0.0047^{ab}	0.0101^{a}	0.0765^{bc}	0.0784^{cde}	0.1657^{e}	0.0838de	0.0344^{cd}	1.99E-42
Rikenellaceae RC9 gut group	0.0025^{a}	0.0075^{ab}	0.0168^{bc}	0.0131^{bc}	0.0310^{cd}	0.0119^{abc}	0.0117^{bc}	0.0341^{de}	0.0548^{e}	0.0432^{de}	1.94E-38
Lactobacillus	0.2529^{a}	0.3592^{cd}	0.2468^{cd}	0.2671^{cd}	0.0447^{a}	0.0924^{ab}	0.1495^{abc}	0.2468^{cd}	0.2226^{bcd}	0.37824^{d}	1.64E-11
Christensenellaceae R-7 group	0.0006^{a}	0.0077^{ab}	0.0449^{de}	0.0459^{cd}	0.0747^{e}	0.0388^{bcd}	0.0213^{ab}	0.0114^{abc}	0.0139^{bcd}	0.0310^{d}	5.09E-22
Clostridium sensu stricto 1	0.0382^{c}	0.0123^{abc}	0.0204^{c}	0.0222^{bc}	0.0241^{c}	0.0064^{ab}	0.0274^{abc}	0.0079^{a}	0.0328^{abc}	0.0337c	1.67E-06
Unclassified Lachnospiraceae	0.0267^{ab}	0.0197^{a}	0.0288^{abc}	0.05995^{cde}	0.0605^{de}	0.0750^{de}	0.1249^{f}	0.0709^{de}	0.0855^{ef}	0.0453^{bcd}	9.71E-25
Eubacterium [coprostanoligenes] group	0.0412^{a}	0.0413^{ab}	0.0341^{a}	0.0419^{ab}	0.0601^{ab}	0.1035^{b}	0.0560^{ab}	0.0297^{a}	0.0221a	0.0242^{a}	2.16E-06
Faecalibacterium	0.0001^{a}	0.0001^{a}	0.0008^{a}	0.0028^{ab}	0.0022^{ab}	0.0265^{bc}	0.0354^{d}	0.0256^{cd}	0.0338^{d}	0.0077^{bc}	2.08E-30
Unclassified Ruminococcaceae	0.0019^{a}	0.0060^{ab}	0.0138^{bcd}	0.0105^{bc}	0.0170^{cde}	0.0151^{cde}	0.0263^{cde}	0.0185^{cde}	0.0262^{e}	0.0209^{de}	3.18E-23
Ruminococcaceae NK4A214 group	0.0010^{a}	0.0075^{ab}	0.0139^{bc}	0.0143^{c}	0.0189^{c}	0.0247^{bc}	0.0238^{bc}	0.0116^{bc}	0.0127^{bc}	0.0170^{bc}	5.17E-15
Ruminococcaceae UCG-002	0.0025^{a}	0.0424^{bc}	0.1226^{e}	0.0942^{de}	0.0645^{cde}	0.0308^{b}	0.0251^{bcd}	0.0171^{b}	0.0200^{bc}	0.0296^{bc}	3.47E-30
Ruminococcaceae UCG-005	0.0003^{a}	0.0055^{ab}	0.0238^{cd}	0.0279^{cd}	0.0267^{d}	0.0141^{c}	0.0168^{bc}	0.0089^{c}	0.0088^{c}	0.0109^{cd}	1.15E-19
Ruminococcaceae UCG-014	0.0002^{a}	0.0021^{ab}	0.0058^{ab}	0.0162^{bc}	0.0172^{cd}	0.0297^{c}	0.0273^{cd}	0.0345^{de}	0.0620^{e}	0.0368^{de}	1.99E-42
Ruminococcus 2	0.0036^{a}	0.0083^{ab}	0.0109^{abc}	0.0119^{a}	0.0055^{ab}	0.0085^{ab}	0.0078^{ab}	0.0220^{cd}	0.0125^{bcd}	0.03404^{d}	5.22E-19
Subdoligranulum	0.0000^{a}	0.0014^{ab}	0.0033^{ab}	0.0143^{bc}	0.0205^{cd}	0.0319^{de}	0.0269^{def}	0.0474^{ef}	0.0552^{f}	0.0300^{def}	2.86E-50
Bacteroides	0.2882^{e}	0.1456^{d}	0.1276^{d}	0.0847^{cd}	0.0672^{bcd}	0.0451^{bc}	0.0136^{ab}	0.0019^{a}	0.0002^{a}	0.0002^{a}	9.79E-14
Escherichia-Shigella	0.1020^{e}	0.0871^{de}	0.0417^{cd}	0.0305^{bc}	0.0225^{ab}	0.0014^{a}	0.0003^{a}	0.0008^{a}	0.0001^{a}	0.0033^{a}	1.02E-10
Prevotella 1	0.0002^{a}	0.0001^{a}	0.0006^{ab}	0.0019^{ab}	0.0197^{ab}	0.0488^{d}	0.0604^{d}	0.0306^{cd}	0.0178^{bc}	0.0078^{ab}	3.33E-16

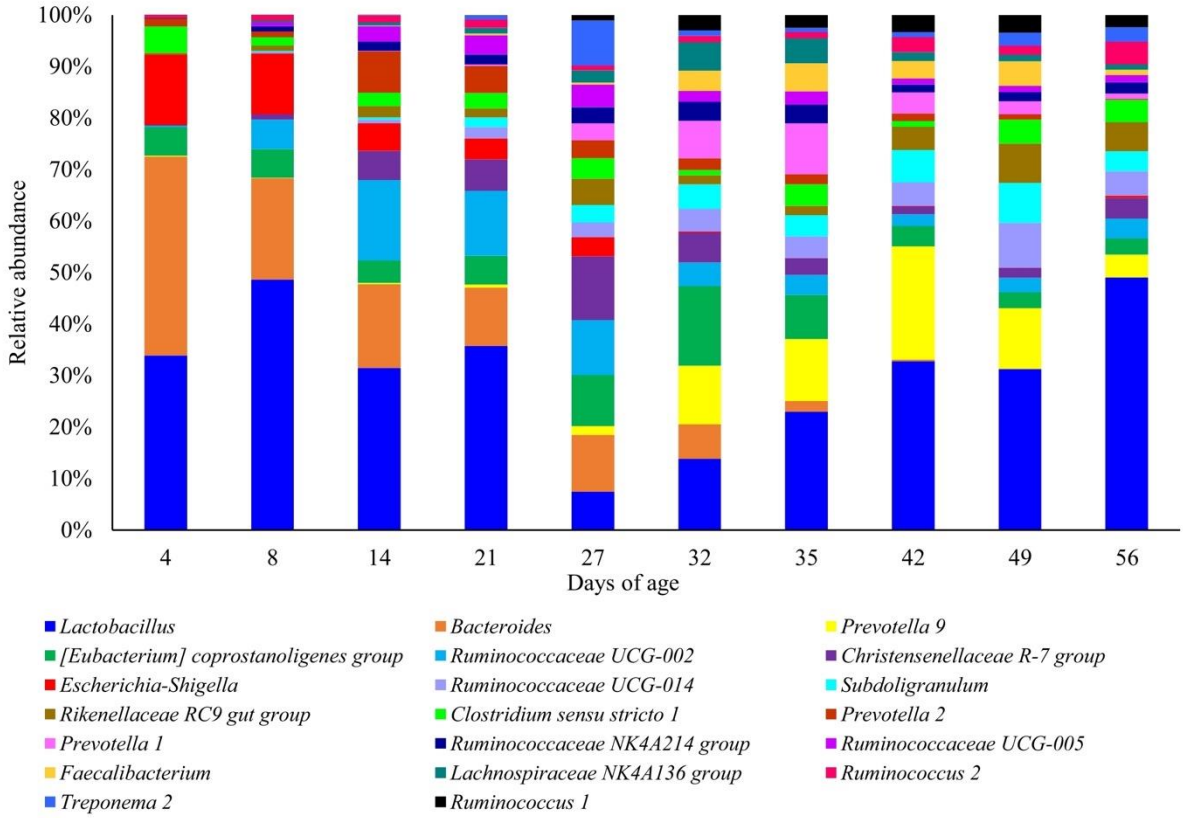
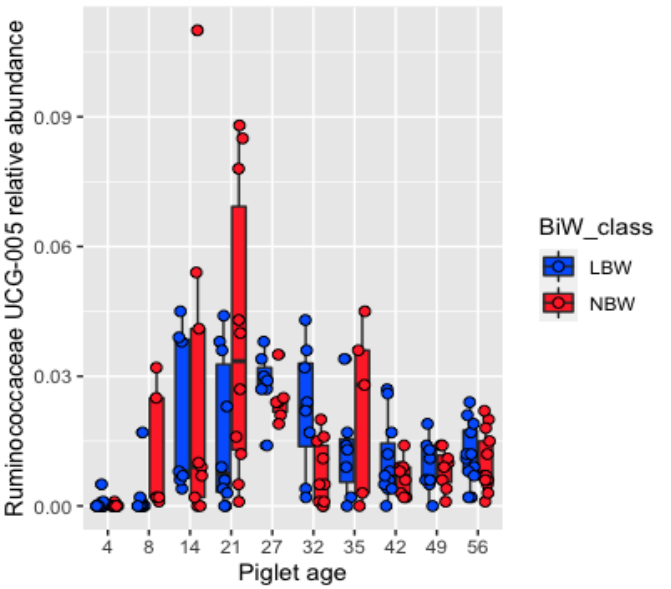


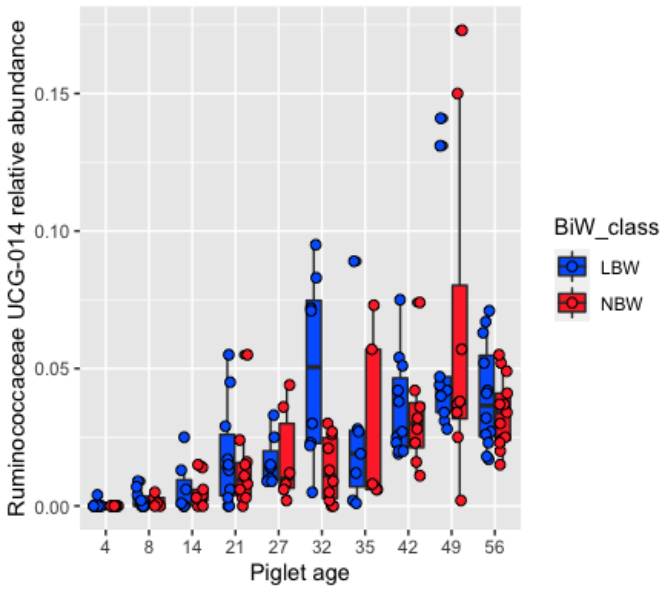
Figure 2.5. The relative abundance of the top 20 genera according to piglet age.

There were no significant fixed effects of BiW or ADG class on genera relative abundance ($P > 0.05$). However, there was a significant interaction between BiW class and piglet age on the relative abundance of *Ruminococcaceae UCG-005* ($P < 0.01$) and *Ruminococcaceae UCG-014* ($P < 0.01$). Following pairwise adjustments, LBW pigs had a lower relative abundance of *Ruminococcaceae UCG-005* at 21 days of age (LBW 1.16% $\pm$ 0.005 SE vs. NBW 3.95% $\pm$ 0.011 SE) and higher relative abundance of *Ruminococcaceae UCG-014* at 32 days of age (LBW 5.01% $\pm$ 0.012 SE vs. NBW 1.33% $\pm$ 0.004 SE) (**figure 2.6a, b**). There was also a Interaction between piglet age and ADG class. Piglets assigned to the "good" ADG class had a significantly higher relative abundance of *Lactobacillus* at 4 days of age ("good" 35.93% $\pm$ 0.065 SE vs. "poor" 16.92% $\pm$ 0.041 SE, $P < 0.05$), unclassified Prevotellaceae at 8 days of age ("good" 4.68% $\pm$ 0.018 SE vs. "poor" 2.57% $\pm$ 0.024 SE, $P < 0.05$) and *Ruminococceae UCG-005* at 14 days of age ("good" 4.25% $\pm$ 0.011 SE vs. "poor" 0.50% $\pm$ 0.001 SE, $P < 0.01$) (**figures 2.6c, d, e**). Furthermore, there was a significant interaction between piglet age, BiW and ADG class, with a higher relative abundance of *Ruminococcaceae UCG-005* segregating NBW "good" (5.03% $\pm$ 0.032 SE) from NBW "poor" (1.37% $\pm$ 0.008 SE) ADG class piglets at 14 days of age ($P < 0.001$).

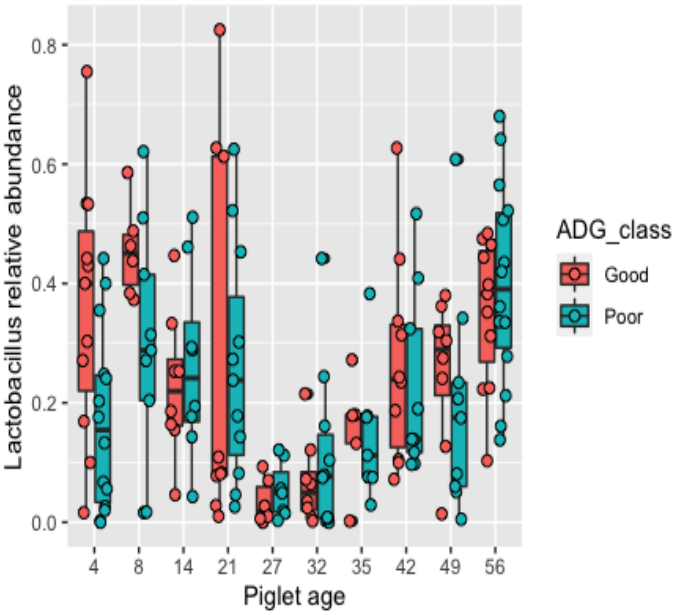
a)


Ruminococcaceae UCG-005 relative abundance
0.09
0.06
0.03
0.00
4 8 14 21 27 32 35 42 49 56
Piglet age
BiW_class
LBW
NBW

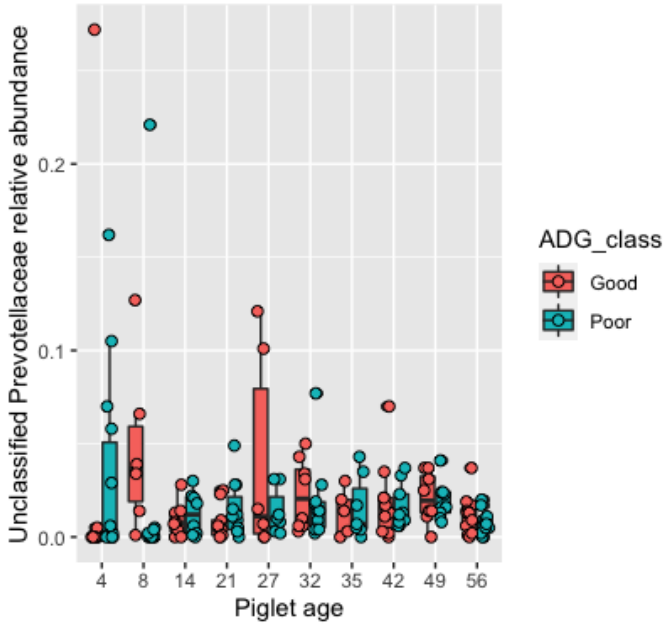
b)


Ruminococcaceae UCG-014 relative abundance
0.15
0.10
0.05
0.00
4 8 14 21 27 32 35 42 49 56
Piglet age
BiW_class
LBW
NBW

c)


Lactobacillus relative abundance
0.8
0.6
0.4
0.2
0.0
4 8 14 21 27 32 35 42 49 56
Piglet age
ADG_class
Good
Poor

d)


Unclassified Prevotellaceae relative abundance
0.2
0.1
0.0
4 8 14 21 27 32 35 42 49 56
Piglet age
ADG_class
Good
Poor

e)

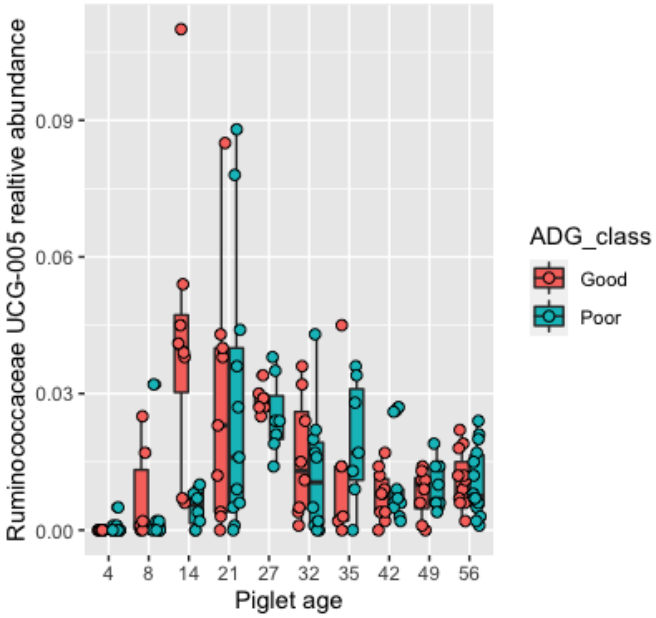


Figure 2.6. The relative abundance *Ruminococcaceae UCG-005* (a) and *Ruminococcaceae UCG-014* (b) in accordance with piglet birthweight class (LBW = low birthweight (0.80 – 1.25kg), NBW = normal birthweight (1.50 – 2.00kg)) and piglet age. The relative abundance of *Lactobacillus* (c), unclassified Prevotellaceae (d) and *Ruminococcaceae UCG-005* (e) in accordance with ADG (average daily gain) class and piglet age.

2.4 Discussion

The study hypothesised that microbiota markers could be identified which would be able to segregate LBW and NBW pigs, as well as pigs who exhibit superior growth rates from those who remain stunted. We found time-point specific, significant associations between birthweight and the number of observed OTUs between days 21, 32 and 56 days of age. *Ruminococcaceae UCG-005* and *Ruminococcaceae UCG-014* were identified as significant taxonomic markers for BiW class at 21 and 32 days of age, respectively. Several genera were identified as age specific taxonomic markers for performance pre-weaning, whilst *Ruminococcaceae UCG-005* abundance was found to significantly differentiate NBW piglets with a superior growth rate from those piglets classified as having a "poor" ADG class. Moreover, measures of microbial diversity, community composition and genera abundance were significantly affected by age. The use of qPCR to confirm the observed significant differences in the relative abundance of the taxonomic markers associated with piglet age, BiW class and ADG class was outside the scope of this study but would have provided a figure for absolute abundance of these markers opposed to relative (proportionate) abundance.

2.4.1 The effect of birthweight on piglet performance

In the present study LBW pigs had a consistently lower liveweight compared to NBW pigs and a lower ADG across the whole experimental period, apart from the immediate post-wean period, supporting published findings by others (Douglas *et al.*, 2014c; Huting *et al.*, 2017; Li *et al.*, 2018). Whilst both NBW and LBW pigs displayed a reduction in liveweight gain associated with the post-weaning growth check (Pluske *et al.*, 2003; Lallès *et al.*, 2007), NBW pigs recovered faster from the growth check, with the liveweight difference between LBW and NBW pigs progressively increasing post-weaning. This pattern in liveweight, reflecting the poorer growth rates of LBW pigs, may be associated with an immature GIT (Pluske *et al.*, 2003; Wang *et al.*, 2010; Michiels *et al.*, 2013), reduced creep feed intake during the lactation period (Huting *et al.*, 2017) and consequently post-weaning anorexia, known to induce negative effects on GIT morphology and performance (Bruininx *et al.*, 2002; Pluske *et al.*, 2003; Bauer *et al.*, 2011).

2.4.2 The longitudinal development of the early life faecal microbiota

The effects of piglet age and weaning on the microbiota have been widely reported in the literature and are considered as the main drivers for changes in microbiota diversity, community composition and taxa abundance (Mach *et al.*, 2015; Bian *et al.*, 2016; Chen *et al.*, 2017; Han *et al.*, 2018). Microbiota diversity increased pre-weaning, reaching a peak at 27 days of age (last day of lactation). However, this was followed by a decline in diversity immediately post-weaning, arising from weaning induced microbiota dysbiosis, before a recovery and plateau in diversity post-weaning, as reported by Frese *et al.* (2015) and Wang *et al.* (2019a). Weaning predominantly induces community composition divergence as a result of removal of maternal milk, which is highly digestible, high in fat and contains prebiotic milk glycans; these substrates are replaced by a solid, primarily plant-based, carbohydrate rich diet (Frese *et al.*, 2015). The uniformity of the post-weaning dietary substrates induced microbiota convergence and stability, hence the observed plateau in alpha diversity indices. The changes in beta diversity over time demonstrated neonatal community composition to display high inter-individual variability, supporting the consensus that the early life microbiota is highly stochastic and influenced by the pen and sow environment (Merrifield *et al.*, 2016; Li *et al.*, 2018). Overlap between the pre- and post-weaning community composition occurs at 27 days of age; this is likely to result from some piglets consuming creep feed during lactation. Whilst creep feed consumption increases between days 19 and 27 of lactation, intake is highly variable (Bruininx *et al.*, 2002, 2004; Collins *et al.*, 2013; Huting *et al.*, 2017) and may also explain why measures of alpha diversity were highest at 27 days of

age, with individual piglets consuming differing proportions of milk and solid feed. Irrespective of piglet birthweight and performance, the pre-weaning period was characterised by a microbiota dominated by the phyla Firmicutes, Bacteroidetes and to a lesser extent Proteobacteria. Proportions of Proteobacteria diminished as lactation progressed, while post-weaning the microbiota was dominated by Firmicutes and Bacteroidetes, as previously shown (Mach *et al.*, 2015; Chen *et al.*, 2017; Pollock *et al.*, 2018). Weaning was associated with a transient reduction in certain beneficial bacteria, such as *Lactobacillus*. The microbiota shift corresponding to weaning is widely reported (Frese *et al.*, 2015; Chen *et al.*, 2017; Gresse *et al.*, 2017; Valeriano *et al.*, 2017), characterised at the family level by a reduction in Bacteroidaceae and Enterobacteriaceae, whilst Lactobacillaceae, Ruminococcaceae and Prevotellaceae increase post-weaning, arising from changes to substrate availability within the GIT (Kim *et al.*, 2011; Pajarillo *et al.*, 2015; Frese *et al.*, 2015). Day 56 represented a period where experimental pigs were given a home-milled meal instead of commercial weaner pellets. The home-milled meal contained uncooked cereals altering the diet digestibility and thus fermentable substrates, giving rise to further microbiota changes.

2.4.3 The effect of birthweight on the early life faecal microbiota

One of the specific studies aims was to establish whether there was an effect of birthweight on the microbiota profile, the age at which this occurred and the persistency of these effects longitudinally. Abnormalities in GIT physiology of IUGR LBW pigs, thought to contribute to poor mucosal immunity at birth and ADG (Wang *et al.*, 2010; Dong *et al.*, 2014; Hu *et al.*, 2016b; Huang *et al.*, 2019a) could influence microbiota development. Indeed, microbiota establishment in LBW human infants, particularly those born prematurely, is different to that of NBW infants (Fança-Berthon *et al.*, 2010; Unger *et al.*, 2015) with similar effects reported in mice (Wang *et al.*, 2016a). Differences in the microbiota community composition have been identified in LBW pigs at different intestinal sites and in the faeces (Li *et al.*, 2018, 2019; Huang *et al.*, 2019a; Zhang *et al.*, 2019a). In the present study, differences in microbiota diversity were noted pre- and post-weaning, although inconsistent longitudinally. NBW pigs have a higher abundance of observed OTUs on day 21, but a lower abundance on days 27, 32, and 56 compared with LBW pigs, this may reflect differences in solid feed intake, particularly pre-weaning. The onset of solid feed intake characterises a key time point in microbiota development (Frese *et al.*, 2015; Mach *et al.*, 2015; Bian *et al.*, 2016; Choudhury *et al.*, 2020) and an increase in exocrine pancreatic activity and secretions, irrespective of weaning age (Pierzynowski *et al.*, 1990, 1993, 1995). However, LBW pigs with IUGR have a reduced number of pancreatic cells, which are smaller in size and immature

in neonates (Xu *et al.*, 1994). In human infants, IUGR reduces lipase activity, trypsin activity in the duodenal juice and chymotrypsin concentration in the faeces (Boehm *et al.*, 1991; Kolacek *et al.*, 1990). NBW pigs begin to eat creep feed sooner in lactation and consume higher volumes than LBW pigs (Huting *et al.*, 2017), thus should have an accelerated exocrine pancreatic development compared with LBW pigs. Although creep feed intake was not directly measured in this study, a presumed higher feed intake amongst NBW pigs, possible alterations to digestive secretions and consequently composition of fermentable substrates, is the mostly likely explanation for a higher number of OTUs present in NBW pigs on day 21 of the experiment. To validate this assumption, future studies should monitor daily creep feed intake of both LBW and NBW pigs. Poor intestinal maturation persists post-weaning, with LBW pigs slower to adapt to solid feed, indicated by the reduced thickness of the tela mucosa and tunica muscularis (Michiels *et al.*, 2013) and reduced proximal aminopeptidase A and maltase, although Huygelen *et al.* (2015) did not report a difference in brush border enzyme activity between LBW and NWB pigs. LBW pigs have also been shown to exhibit a different caecum fermentation pattern with a lower pH and increased concentrations of acetate and propionate compared to NBW pigs (Michiels *et al.*, 2013). Delayed creep feed intake, slower GIT maturation and altered fermentation patterns may cumulatively explain the significant increase in microbiota diversity on days 27, 32 and 56 arising from changes to the GIT environment and substrate availability.

No differences were observed between LBW and NBW pigs longitudinally regarding community composition (beta diversity), supporting findings reported for NBW and LBW infants, mice and pigs (Costello *et al.*, 2013; Wang *et al.*, 2016a; Li *et al.*, 2019). The present study found age specific differences in taxa abundance between LBW and NBW pigs. *Ruminococcaceae UCG-005* was higher in abundance pre-weaning for NBW pigs. Zhang *et al.* (2019a) also reported abundance to be higher in the jejunum of NBW pigs at day 21 of age, although Li *et al.* (2018) reported the abundance of *Ruminococcaceae UCG-005* to be higher in LBW pigs on days 7 and 21 of age. Both the current study and Li *et al.* (2018) report *Ruminococcaceae UCG-005* and *-014* to be within the top 50 taxa of both LBW and NBW pigs and faecal abundance to increase with age. These bacteria ferment dietary fibre and produce short chain fatty acids (SCFAs) (Song *et al.*, 2018; Liu *et al.*, 2018), and are considered stable microbiota component of the caecum and colon, irrespective of BiW class. As with these results on faecal microbiota, inconsistent findings were reported by studies comparing the ileal microbiota of LBW and NBW pigs (Li *et al.*, 2019; Zhang *et al.*, 2019a). Differences in the results may arise from factors related to BiW class criteria (<1kg in the

literature for LBW piglets), genetics, weaning age and management procedures (including age at creep feed introduction), amplicon library preparation and data analysis. In the present study, the LBW pigs were heavier at birth (0.80 – 1.25kg) compared with other studies which describe IUGR to modulate LBW pig physiology, induce GIT immaturity and compromise mucosal immunity (birthweight < 1kg) ((D'Inca *et al.*, 2010, 2011; Michiels *et al.*, 2013; Dong *et al.*, 2014). This may explain the reported minor effects of BiW class on the microbiota in the present study, as the GIT of experimental piglets may have been less detrimentally affected by IUGR than those in other studies.

2.4.4 The association between the faecal microbiota profile and piglet performance

To date, only limited research has been conducted to explore how characteristics of the early life microbiota are associated with piglet performance (Mach *et al.*, 2015; Han *et al.*, 2018; Ding *et al.*, 2019), thus an additional aim of the study. In the present study, there was an interaction between BiW class and ADG class over specific time points for observed OTUs, corresponding to late lactation and the immediate post-weaning period, although the results were not consistent over time. However, similar microbiota diversity fluctuations over time between pre-ruminant calves of high and low growth rates have also been reported (Oikonomou *et al.*, 2013). In addition, a study comparing the faecal microbiota of LBW and NBW mice able to exhibit compensatory growth reported that LBW mice exhibit microbiota dysbiosis during early life (Wang *et al.*, 2016a). Mach *et al.* (2015) identified enterotype-like clusters associated with performance of piglets, with increased pre-weaning performance associated with piglets classified within the Ruminococcaceae enterotype. In this study we identified *Ruminococcaceae UCG-005* abundance to be positively associated with performance pre-weaning; moreover, a significant interaction with BiW classes was observed, with a 3.66% higher abundance in NBW "good" pigs than NBW "poor" pigs at 14 days of age. The Ruminococcaceae family is specialised to degrade and ferment dietary fibre containing complex polysaccharides (Biddle *et al.*, 2013). A by-product of this fermentation is the production of SCFAs, with *Ruminococcaceae UCG-005* abundance positively correlated with SCFA concentration in the faeces of piglets (Liu *et al.*, 2018; Zhu *et al.*, 2020). SCFAs can be utilised by the piglet as an energy source to support growth. A higher abundance of this taxa could therefore be a marker of dietary fibre and thus solid feed intake. Presence of this genera pre-weaning will help to prime the gut to utilise plant-based carbohydrates and may help the GIT to adapt to dietary substrate changes experienced during weaning. A moderate increase in the dietary fibre content of creep feed has shown to alter the microbiota composition in pre-weaned piglets (Zhang *et al.*, 2016), although not specifically

Ruminococcaceae UCG-005 abundance. Further research exploring the specific effect of different fibre sources on *Ruminococcaceae UCG-005* abundance is required.

Unclassified Prevotellaceae was significantly higher in pigs with a "good" ADG class on day 8 only. The Prevotellaceae family is able to degrade complex carbohydrates in plant-based feeds, with abundance correlated to fibre content of the feed (Le Sciellour *et al.*, 2018). Whilst creep feed was not introduced until day 10, it was not possible to prevent piglets from eating spilled sow feed. Higher abundance of unclassified Prevotellaceae associated with "good" pigs could therefore also be an indication of early consumption of solid feed, however it could also arise from these piglets consuming larger quantities of milk. Unclassified Prevotellaceae abundance has been shown to be correlated with lactose concentration of milk (Bian *et al.*, 2016), so a higher total intake of lactose may induce the same effect. *Lactobacillus* was also significantly higher in abundance in "good" ADG class piglets at 4 days of age and only numerically higher at 8 days of age; Ding *et al.* (2019) similarly reported *Lactobacillus* abundance to be positively correlated with performance pre-weaning. Lactobacilli have a beneficial effect on weight gain of very low birth weight human infants when provided as a probiotic during early life (Vendt *et al.*, 2006; Härtel *et al.*, 2017) as well as in piglets pre- and post-weaning (Liu *et al.*, 2015; Wang *et al.*, 2019c). This effect may be mediated by increasing production of lactic acid, thus altering GIT pH, inducing a beneficial shift in microbiota taxa abundance or establishment, and reducing the degree of inflammatory response (Kim *et al.*, 2007; Liu *et al.*, 2014; Hou *et al.*, 2015). Therefore, one option to increase *Lactobacillus* abundance in early life is to administer strains of Lactobacilli orally to neonatal piglets (Yang *et al.*, 2020). The following strains have demonstrated beneficial effect on piglet performance when administered orally during early life: autogenous *L. reuteri* D8, *L.rhamnosus* GG ATCC53103, *L. fermentum* I5007 and *L. fermentum* KT260178 (Liu *et al.*, 2014; Wang *et al.*, 2019c; Wang *et al.*, 2020; Yang *et al.*, 2020). Aligning probiotic administration with routine neonatal practices, such as teeth clipping, will help to limit handling stress whilst implementing this potential intervention strategy. Although, the aforementioned studies dosed piglets across multiple time points, thus the efficacy of one dose of a selected *Lactobacillus* strain (more commercially viable solution) would need to be tested.

In conclusion, the study is the first to compare piglet birthweight and its interaction with performance in relation to longitudinal characteristics of the faecal microbiota in early life. The main findings support the premise that timing of solid feed intake modulates the

significant age-related microbiota shifts. Time-point specific microbiota markers associated with ADG and BiW classes could be identified. Future studies, with greater replication, should not only continue to define the microbiota of viable LBW pigs that exhibit compensatory growth but focus research to understand the factors which affect microbiota establishment during early life, such as the sow and pen microbiota and the effect of standard management practices, such as cross-fostering which will expose piglets to more environmental microbiota sources. However, early life appears to be the critical period in which modulations to the microbiota can induce significant beneficial effects on long term performance of both LBW and NBW pigs.

Chapter 3. Longitudinal changes in the periparturient faecal microbiota of primiparous and multiparous sows

3.1 Introduction

The neonatal piglet gastrointestinal tract (GIT) is seeded with microbes present in the uterine tract during expulsion, on the sow skin/udder and faeces, and from the pen environment. The development of the microbiota during early life is now recognised as having a significant impact on host metabolism (Mulligan and Freidman, 2017) and health (Dou *et al.*, 2017; Zhuang *et al.*, 2019). Studies aimed at assessing the impact of the sow faecal microbiota on the development of the piglet microbiota have focused on a single sow microbiota sampling time point (the day before/of farrowing) for which all comparisons are made (Bian *et al.*, 2016; Kubasova *et al.*, 2017). The underlying rationale of these studies is that microbiota seeding of the piglet GIT is assumed to occur within the immediate post-natal period. However, the period of microbiota developmental plasticity which can result in long term changes to mammalian health has not been well defined, with age matched microbiota studies between piglets and sows lacking in the literature. It is possible that the relationship between the sow and piglet microbiota extends beyond the first day of parturition. Aviles-Rosa *et al.* (2019) highlighted the importance of neonatal coprophagy for piglet health, and thus the sow faecal microbiota and metabolites, during the first week of life. Denying piglets access to sow faeces for the first 7 days postpartum resulted in lower white blood cell counts, post-weaning average daily gain and feed intake.

As gestation progresses, increased stress is placed on the sow, with changes in physiology, endocrinology, metabolism and immunity all occurring at once to support foetal delivery and the onset of lactation (Baldwin and Stabenfeldt, 1975; Père and Etienne, 2007; Farmer, 2018; Cheng *et al.*, 2018; Huang *et al.*, 2019b). Recent research has demonstrated that these changes are correlated with alterations in the microbiota from trimester one to three of gestation (Liu *et al.*, 2019b) and during the periparturient period (Cheng *et al.*, 2018; Huang *et al.*, 2019b; Shao *et al.*, 2020). Furthermore, during the periparturient period sows will transition from a gestation diet low in energy and high in fibre, to an energy-dense lactation diet. Such changes in nutrient availability and digestibility are reported to alter the microbiota profile (Sappok *et al.*, 2015). However, there is currently a lack of research investigating whether the transition from gestation to lactation affects the faecal microbiota of sows in a parity-dependant manner. Metabolic stressors occurring during late gestation and lactation are likely to be heightened in primiparous sows, which must also partition nutrients to support maternal growth and mammary gland development (Pluske *et al.*, 1998). Psychological stress is known to induce

microbiota dysbiosis in humans (Cryan and Dinan, 2012). Additional psychological stress during the perinatal period arises from housing of sows in farrowing crates, restricting movement and subsequently the ability for sows to express natural nest building behaviours associated with parturition (Jarvis *et al.*, 2002). In countries where gestation crates are no longer permitted (such as under EU Council Directive 2008/120), this psychological stress will be more profound in primiparous sows, who will not have previously been exposed to close confinement or the innate motivation to express farrowing behaviours (Jarvis *et al.*, 2001). These factors may induce a different microbiota profile in primiparous and multiparous sows during the perinatal period. Understanding parity-dependant changes in the microbiota could help to inform and tailor management strategies to promote better gut health and lactation performance.

3.1.1 Aims and hypotheses

The overall aim of the study was to understand the dynamic changes in the sow microbiota from 6 days prepartum to 8 days postpartum and determine whether these changes are affected by parity. The study firstly hypothesised that the sow microbiota profile will undergo dynamic changes in response to the transition from gestation to lactation. The study secondly hypothesised that microbiota dysbiosis associated with this transition be greater in primiparous compared with multiparous sows.

3.2 Materials and methods

3.2.1 Experimental design

A total of 29 Large White x Landrace sows (multiparous = pure Hermitage Seaborough Ltd, UK and primiparous = Hermitage Seaborough Ltd x Rattlerow Farms Ltd, UK), from eight consecutive farrowing batches were used in this study. The experiment was designed as a 2 x 4 unbalanced factorial design. The first experimental factor was sow parity (Parity), with experimental sows classified as being either primiparous (n = 13) or multiparous (second parity and above, n = 16, average parity = 2.63 (SD = 0.719)). The second experimental factor was time, with microbiota samples collected at 4 time points during the periparturient period.

3.2.2 Animal housing and management

For each sow, where possible a faecal sample was collected at four time points. Where a faecal sample could not be obtained a rectal swab was instead used to obtain a faecal microbiota sample. Research by Choudhury *et al*. (2019) determined the results of microbiota analysis of swab and faecal samples to be comparable. Rectal swabs and faecal samples are

herein collectively referred to as faecal samples throughout the study. The four sampling time points were day 109 gestation (day -6), as this was the last day in straw-yard group housing for sows; 1 day before farrowing, as the neonatal microbiome is likely to be seeded with traces of this faecal material passed by sows; 3 days postpartum, when passing of faeces has resumed; and 8 days postpartum, to collect faecal samples at the end of the periparturient period. These days are referred to as days in relation to farrowing (D0) for the duration of the chapter (D-6, D-1, D3 and D8, respectively).

Gestating sows were managed in a three-week indoor batch farrowing system and housed in solid floored (concrete) barns in groups of five sows of similar size and parity. Each pen consisted of a kennelled lying area (depth 2.50m x width 2.20m) containing straw bedding, an outside dunging area (depth 2.15m x width 2.20m), in front of which there were five individual feeding crates (length 1.84m x width 0.44m per crate). A nipple drinker in the dunging area provided *ad libitum* water. During gestation, sows were fed a home-milled mash gestation diet based on barley and soyabean meal (13.14 MJ DE/kg, 13.82% CP and 0.62% SID lysine; **Appendix 3a**). They received approximately 2 - 2.50 kg/head per day at 0730 h daily throughout gestation.

Multiparous sows were moved from gestation group housing in solid floor barns with straw to a conventional part-slatted farrowing pen with a farrowing crate at approximately 109 days of gestation, with primiparous sows entering at 111 days. The later entry of primiparous sows into the farrowing house was standard practice on the commercial unit, reducing the amount of time primiparous sows spent in farrowing crates in an effort to reduce stress. Prior to entry, the farrowing pen was washed and disinfected (concentration = 0.03% PhenoPharm, East Riding Farm Services, UK) and allowed to dry for a minimum of 7 days. Farrowing crate dimensions were as follows: entire pen 1.80m width x 2.42m length, creep area 1.11m length x 0.80m width and sow crate 0.6m width x 1.77m length to the feed trough. All sows were wormed with Bimectin (5ml primiparous and 8ml multiparous intramuscularly (IM), Bimeda, Llangefni, UK) upon entry to the farrowing house and received a FarrowSure Gold vaccine the day before weaning (2ml IM, Zoetis, Surrey, UK), which occurred at ~28 days postpartum. Following housing in the farrowing crates, sows received approximately 0.70 kg/head of the gestation diet feed twice daily at 0745 h and 1500 h until farrowing. The day after farrowing sows were transferred to a home-milled mash lactation diet (13.98 MJ DE/kg, 18.50% CP and 0.95% SID lysine; **Appendix 3a**) initially as a 2.0 kg/head per day allowance, which was increased to appetite by 0.5 kg/head per day until a 10 kg/head per day limit was reached. A change in diet was necessary in order to meet the increased nutrient requirements of the sow during lactation, and to study the change in the periparturient sow microbiota

under conditions representative of commercial practice. Individual sow feed intakes, P2 measurements and liveweights were not recorded. Water was available *ad libitum* through a nipple drinker. Cross-fostering of piglets, in order to create uniform litters of piglets based on birthweight, occurred within the first 24 hours postpartum. Litter size was set according to the number of functional teats. The number of piglets weaned per experimental sow was recorded at weaning. Piglets were managed according to Gaukroger *et al.* (2020). Veterinary records for both the sow and her litter were recorded throughout lactation. Any antibiotic treatment administered to the sows was recorded as penicillin treatment "yes" or "no" (yes n = 5 (two primiparous sows and three multiparous sows)). Sows were only treated with a three-day course of penicillin (10ml IM, Pen & Strep, Norbrook, Newry, UK) if they presented thick creamy vaginal discharge, or symptoms of mastitis, metritis, agalactia. A description of sow farrowing performance and antibiotic usage can be seen in **Appendix 3b**. No confounding differences in sow performance were observed that may cause a difference in the microbiota associated with sow parity, thus not included in the microbiota analysis.

3.2.2 16S rRNA gene sequencing

Bacterial DNA was extracted from 250mg of faeces using the DNeasy PowerSoil HTP 96 kit (Qiagen, UK) following manufactures instructions and the centrifugation-based protocol for DNA binding and column-washing steps. The V4 region of the 16S rRNA gene was amplified by polymerase chain reaction (PCR). Library generation, quality control steps and sequencing procedure were conducted in accordance with the Kozich *et al.* (2013) standard operating procedure. Briefly, amplification was performed using high fidelity Accuprime Pfx SuperMix (Invitrogen, USA) with the following conditions: 95°C 2 minutes, then 30 cycles of 95°C for 20 seconds, 55°C for 15 seconds and 72°C for 5 minutes followed by a final step of 72°C for 10 minutes. Amplicons were cleaned and normalised using the SequelPrep normalisation kit (Invitrogen, USA). Samples were pooled and quantified using the QuBit hsDNA kit (Invitrogen, USA) and fragment size was confirmed using the Agilent BioAnalyzer 2100 high sensitivity DNA kit (Agilent Technologies Inc, USA). The final library was loaded at 5pM with 10% PhiX and sequenced using an Illumina V2 500 cycle kit on the Illumina MiSeq (Illumina, USA). Sequencing was performed on the Illumina MiSeq using the 2 x 250 bp paired-end read protocol at NU-OMICS DNA sequencing facility. Following the bioinformatics methods reported by Stewart *et al.* (2018), the sequencing read pairs were demultiplexed and reads were merged using 'USEARCH' (v7.0.1090). Merging allowed zero mismatches and a minimum overlap of 50 bases. Furthermore, merged reads were trimmed at the first base with a $q \leq 5$. A quality filter was applied to the resulting merged reads and those

containing above 0.5% expected errors were discarded. Sequences were stepwise clustered into operational taxonomic units (OTUs) at a similarity cut-off value of 97% using the 'UPARSE' algorithm. Chimeras were removed using 'USEARCH' (v7.0.1090) and 'UCHIME' (v4.2). To determine taxonomies, OTUs were mapped to a version of the SILVA Database (Quast *et al.,* 2013) containing only the 16S V4 region using 'USEARCH' (v7.0.1090). Relative abundances were recovered by mapping the merged reads to the UPARSE OTUs. A custom script constructed an OTU table from the output files generated in the previous two steps for downstream analyses of taxonomic relative abundance, alpha diversity, and beta diversity. A total of 2,377,687 sequencing reads were obtained from an initial 104 sow samples run on the Illumina MiSeq. Sequences were rarefied to 3,500 reads per sample. After rarefaction 103 samples were retained, consisting of 22 phyla, 36 classes, 71 orders, 110 families and 303 genera.

3.2.3 Statistical analysis

All statistical analyses were conducted in R version 3.6.2. Fixed effects considered in all models were Day (in relation to farrowing), Parity (primiparous or multiparous) and whether experimental sows received Antibiotic treatment during lactation. Sow ID was specified as the random effect in all models as it formed the repeated measure in all analyses. One of the study aims was to determine how the microbiota profile changed over the periparturient period, thus sow microbiota variability between individuals needed to be accounted for in statistical models. Early analysis of alpha and beta diversity values revealed no significant difference in the microbiota of antibiotic treated vs non-antibiotic treated sows. Additionally, no bacterial genera were significantly different between antibiotic treated vs non-antibiotic sows. Based on the results of this analysis, antibiotic treated sows (n = 5) were retained in the dataset to increase sample size and antibiotic treatment was not considered as a fixed effect in subsequent statistical models. The number of sequencing reads for DNA extraction kit negatives and sequencing negative controls were inspected along with the microbiota community composition of DNA extraction kit negatives, sequencing negative controls and sequencing positive controls. Controls were deemed to not be representative of the sow microbiota and removed from further analysis (**Appendix 3c**).

As previously described by in Chapter 2 (Gaukroger *et al.*, 2020), post-hoc pairwise comparisons of significant fixed effects and interactions between significant fixed effects were determined using the 'emmeans' package (v 1.3.4), resulting P values were Tukey adjusted for multiplicity as part of the 'emmeans' workflow. Adjusted P values below 0.05 were considered statistically significant. All models were tested for validity, using two

diagnostic plots. The first diagnostic plot consisted of a Q-Q plot of the standardised residuals, whilst the second was a scatterplot of the standardised residuals plotted against fitted values. All plots were generated by the 'ggplot2' package (v 3.1.1).

3.2.3.1 Alpha diversity

Observed operational taxonomic units (OTUs; richness) and Shannon diversity index (evenness) were calculated using the 'vegan' package (v 2.5). Generalised linear mixed effect models (glmer; 'lme4' package v 1.1.21) were used to determine any significant longitudinal changes in taxonomic richness and diversity associated with the fixed effects (Gaukroger *et al.*, 2020). For the observed OTUs longitudinal glmer model the family function was specified as Poisson. As raw Shannon diversity index values were not normally distributed, they were subjected to a box cox normalisation using the *boxcox* function of the "MASS" package (v 7.3-51.5) to calculate the best transformation (lambda = 6), which was then applied to the Shannon values. A linear mixed effect model (LME) was then performed with normalised Shannon diversity index values with respect to the fixed effects using the 'nlme' package (v3.1-145).

3.2.3.2 Beta diversity

Beta diversity distances (weighted and unweighted UniFrac) were generated using the 'rbiom' package (v 1.0.2.9002). The *Adonis* function of the 'vegan' package (v 1.0.2.9002) was used to assess, via a PERMANOVA with 999 Monte Carlo permutations, whether any of the fixed effects caused a significant longitudinal difference in either weighted or unweighted UniFrac distances. Weighted UniFrac distances take in to account the relative abundances of taxa, whilst unweighted UniFrac distances are binary, giving equal weighting to rare and abundant taxa.

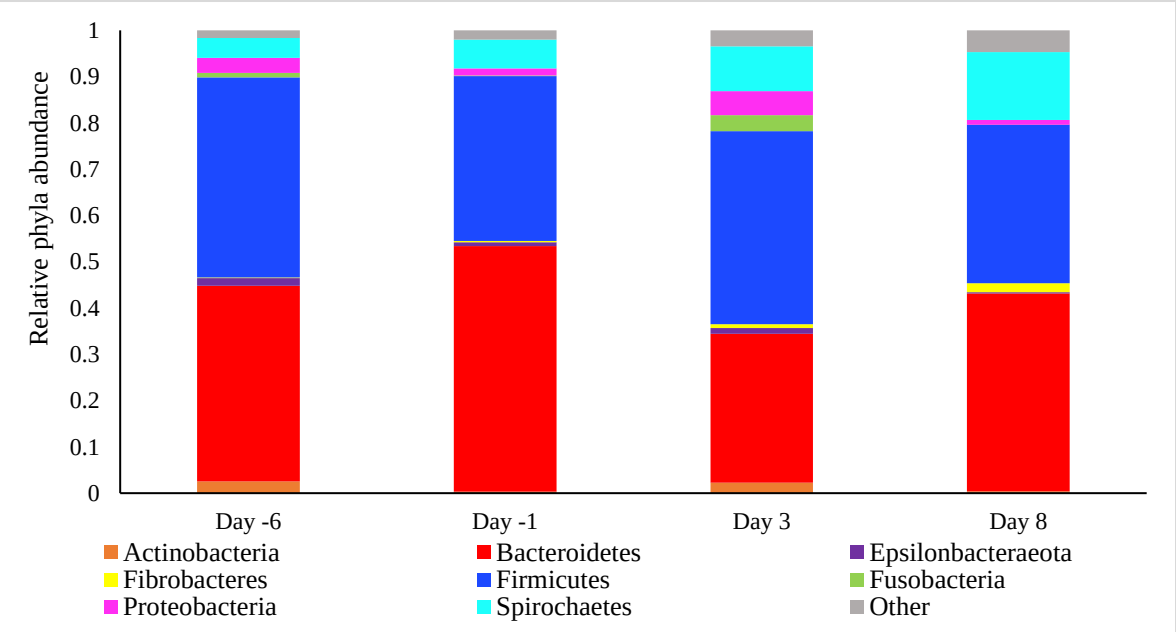
3.2.3.3 Genera abundance

To determine the longitudinal changes in individual genera abundance associated with the periparturient period (Day), the effect of Parity and any interactions between Day and Parity, LME models were performed using the 'lme4' package (v 1.1-21). Prior to running LME models, genera abundances were filtered to only retain genera with an average relative abundance $\geq$ 0.001 (0.1%) and $\geq$ 10% prevalence; retained genera (n = 77) were then arcsine square root transformed. P values were false discovery rate (FDR) adjusted for multiplicity (Benjamini and Hochberg, 1995) and resulting P values below 0.05 were considered significant.

3.3 Results

Taxonomic analysis at the phylum level showed the faecal microbiota was dominated by Bacteroidetes and Firmicutes across all timepoints, and to a lesser extent Spirochetes, Proteobacteria and Fusobacteria (on D3 only; **figure 3.1a**). At the genus level, the faecal microbiota was dominated by *Treponema 2*, *Prevotellaceae NK3B31 group*, *Prevotella 1*, *Prevotella 9*, *Phascolarctobacterium*, *Lactobacillus*, *Rikenellaceae RC9 gut group*, *Ruminococcaceae UCG-005*, *Alloprevotella* and *Bacteroides* (**figure 3.1b**)

a)



b)

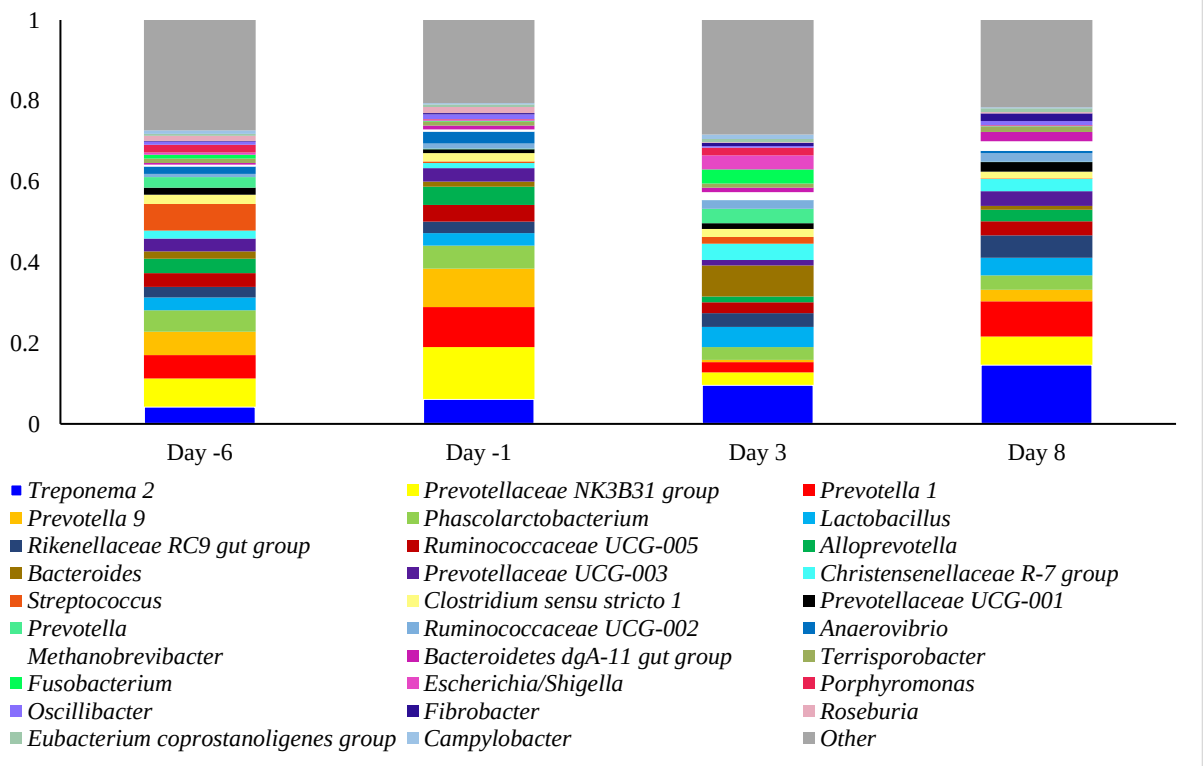
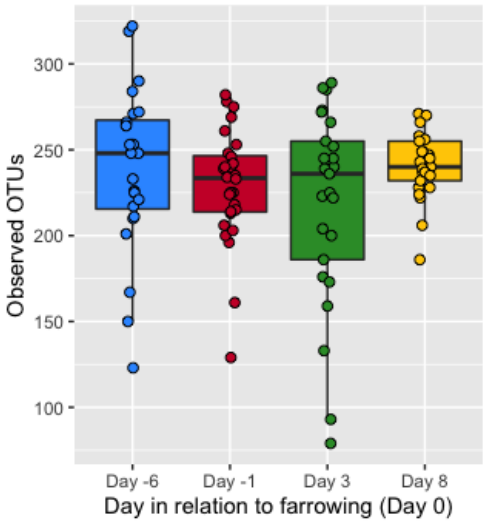


Figure 3.1. The relative abundance of the top 9 phyla (a) and 30 genera (b) present in sow faeces according to sampling day relative to farrowing (Day 0).

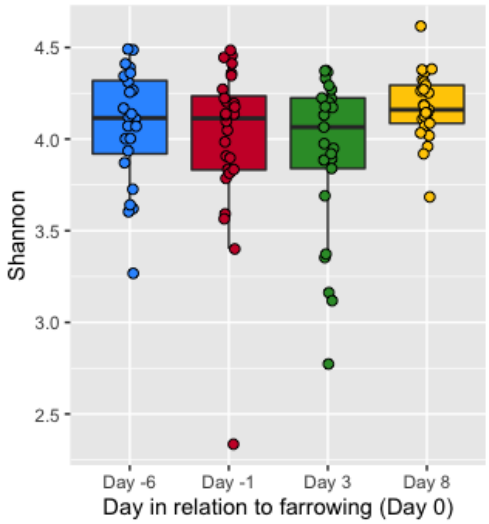
3.3.1. Alpha diversity

There was a significant effect of Day on the number of observed OTUs, with samples on D3 having a significantly lower number of observed OTUs (218 ± 11.5 SE) compared to all other sampling time points ($P < 0.05$; **figure 3.2a**). Furthermore, samples on D -1 (229 ± 6.3 SE) had significantly lower numbers of observed OTUs compared to samples taken at D -6 (239 ± 9.8 SE) and D8 (241 ± 4.0 SE). Day did not have a significant effect on Shannon diversity index values (**figure 3.2b**). There was a significant effect of Parity on alpha diversity measures. Multiparous sows had a higher number of observed OTUs (245 ± 5.3 SE vs 215 ± 6.0 SE, $P < 0.001$) (**figure 3.2c**) and Shannon diversity index (4.14 ± 0.051 SE vs 3.92 ± 0.046 SE, $P < 0.01$) (**figure 3.2d**) compared to primiparous sows. Furthermore, there was a significant interaction between Day and Parity; multiparous sows had a significantly higher number of observed OTUs on D -6 ($P < 0.001$; **figure 3.2e**), with the same trend observed on D3 ($P = 0.058$). No significant interactions between Day and Parity were observed for Shannon diversity index ($P > 0.05$).

a)



b)



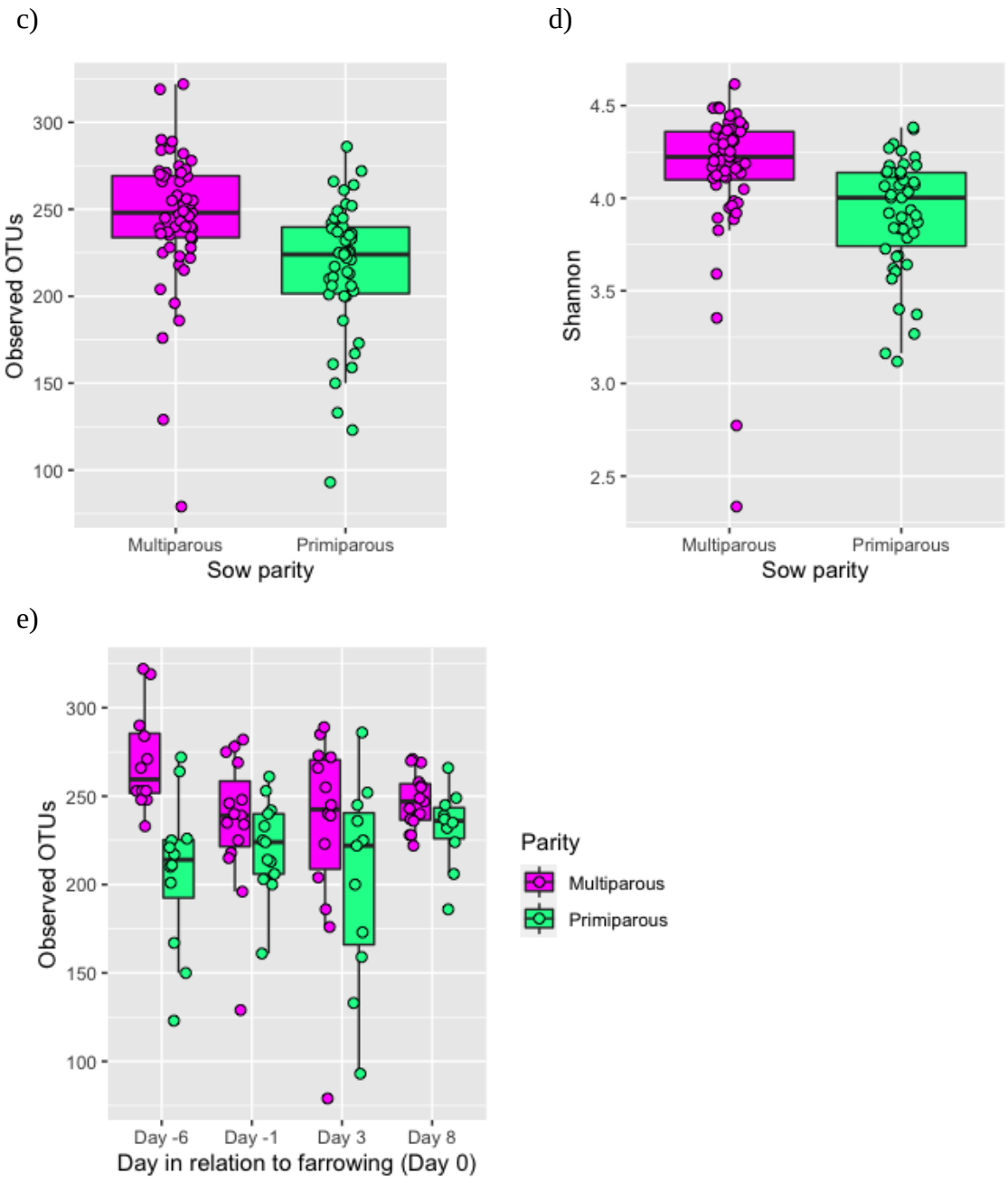


Figure 3.2. The number of observed OTUs according to sampling day (a) in relation to farrowing (day 0), sow parity (c) the interaction between sample day and sow parity (e). Shannon diversity index values according to sample day (b) in relation to farrowing (day 0) and sow parity (d).

3.3.2 Beta diversity

The was a significant effect of Day on the microbiota community composition for both weighted and unweighted UniFrac distances ($P < 0.01$; **figure 3.3a, b**), but there was no significant effect of Parity ($P > 0.05$) for either UniFrac distance.

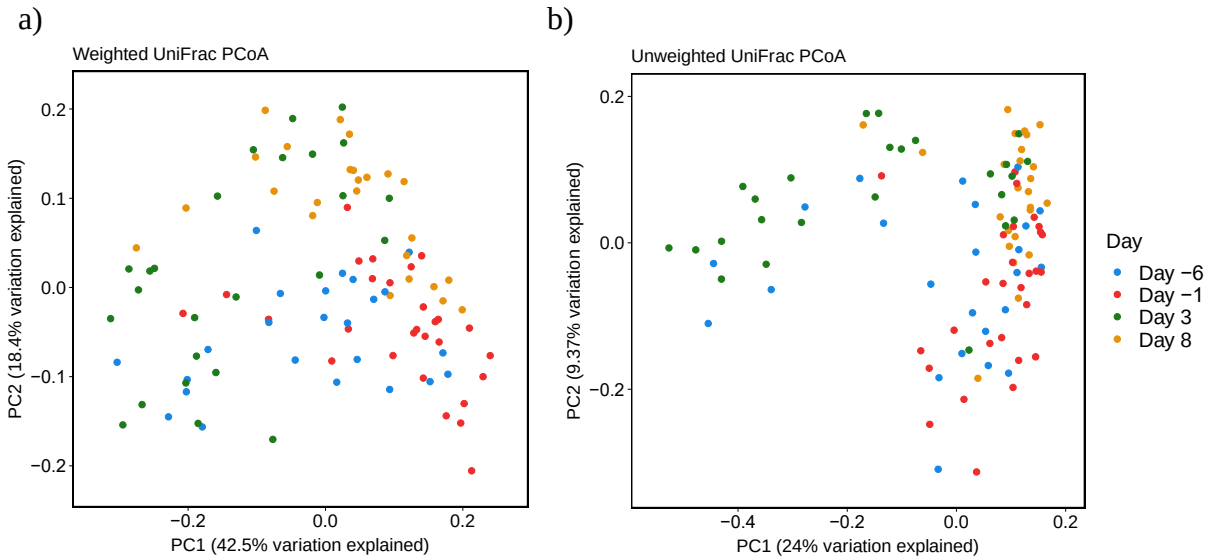
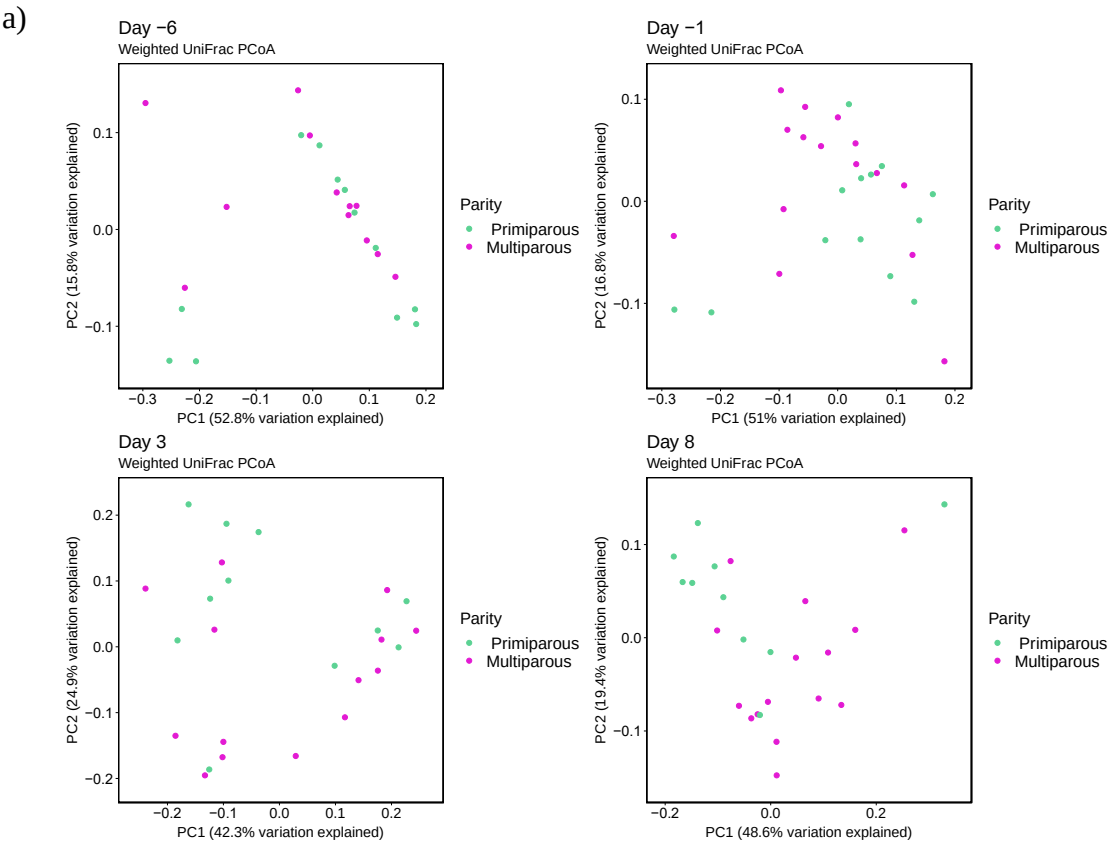


Figure 3.3. Principle coordinates analysis of weighted (a) and unweighted (b) UniFrac distances illustrating the significant changes in faecal microbiota community composition of sow faeces according to sample day in relation to farrowing (Day 0). Samples with similar microbiota community composition are positioned more closely to each other.

However, there was a significant interaction between Day and Parity for weighted UniFrac distances on D8 (*P < 0.05;* **figure 3.4a**), and on D -6, -1 and 8 for unweighted UniFrac distances (**figure 3.4b)**.



b)

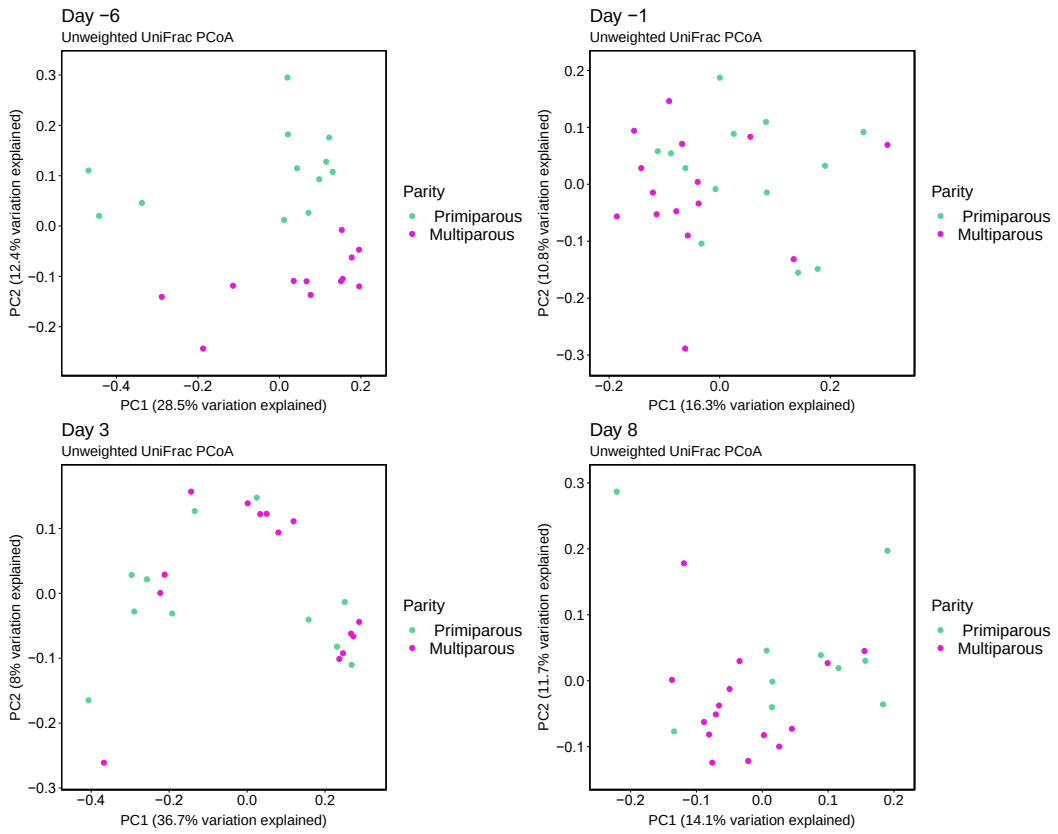


Figure 3.4. Principle coordinates analysis of weighted (a) and unweighted (b) UniFrac distances illustrating the significant changes in faecal microbiota community composition of sow faeces according sow parity on each sampling day. Sample days are labelled according to sample day in relation to farrowing (Day 0). Samples with similar microbiota community composition are positioned more closely to each other.

3.3.3 Genera abundance

There was a significant effect of Day on genera relative abundance (**table 3.1**). Based on the results of LME models, several significant longitudinal patterns in genera abundance were observed during the periparturient period. Patterns were determined by inspection of compact letter displays generated by Tukey's post-hoc pairwise comparisons of adjusted mean values for each genus, with different letters denoting significant differences in mean relative abundance between sampling timepoints. The first pattern was a significant change in genera abundance between gestation and lactation days. This pattern was characterised by a significantly lower abundance of *Roseburia*, *Prevotella 1* and *Prevotella 2* in lactation, whilst the abundance of *Christensenellaceae R-7 group*, *Ruminococcaceae UCG-002* and *Ruminococcaceae UCG-010* were significantly higher. The second pattern was associated with significant changes in genera abundance occurring on D3. This second pattern included a significant increase in *Bacteroides*, *Escherichia/Shigella* and *Fusobacterium* abundances

compared to all other time points. Conversely, several genera abundances were significantly decreased on D3 compared to all other sample days, namely *Alloprevotella*, *Prevotellaceae UCG-003* and *Ruminococcus 1* (**figure 3.5a-f**). The results of the LME models also reported two genera to be significantly different in abundance across all time points between multiparous and primiparous sows, following FDR adjustment. Multiparous sows had a significantly ($P < 0.01$) higher abundance of *Bacteroidetes dgA-11 gut group* (1.67% $\pm$ 0.200 SE vs 0.58% $\pm$ 0.142 SE) and *Prevotellaceae UCG-004* (0.29% $\pm$ 0.040 SE vs 0.08% $\pm$ 0.020 SE). No significant interactions between Day and Parity were observed for relative genera abundances after FDR adjustment for multiplicity ($P > 0.05$).

Table 3.1 The relative abundances of 61 genera present in sow faeces that were significantly different across sampling days in relation to farrowing (D0). Genera were filtered to ≥ 0.001 abundance and 10% prevalance[1].

Genera	Sampling day in relation to farrowing[2]				
	Day -6 (n = 24)	Day -1 (n = 28)	Day 3 (n = 25)	Day 8 (n = 25)	Adjusted P value[3]
Acetitomaculum	0.269±0.0741[a]	0.109±0.0199[a]	0.862±0.2950[b]	0.075±0.0333[a]	0.0016
Actinobacillus	0.579±0.0245[b]	0.033±0.0243[a]	0.303±0.1264[b]	0.034±0.0257[a]	0.0009
Actinomyces	0.030±0.0175[a]	0.002±0.0014[a]	0.475±0.1612[b]	0.034±0.0343[a]	3.64E-06
Alloprevotella	3.544±0.3575[bc]	4.528±0.4340[c]	1.458±0.2970[a]	2.854±0.3890[b]	2.74E-07
Anaerococcus	1.012±0.4704[b]	0.028±0.0217[a]	0.765±0.3377[b]	0.043±0.0422[a]	0.0002
Anaeroplasma	0.130±0.0345[ab]	0.151±0.0218[b]	0.104±0.0520[a]	0.221±0.0528[b]	0.0027
Anaerovibrio	1.844±0.3348[c]	2.963±0.2907[d]	0.086±0.0266[a]	0.513±0.1291[b]	8.47E-15
Arcanobacterium	1.289±0.5199[b]	0.071±0.0492[a]	0.648±0.2021[b]	0.035±0.0262[a]	0.0002
Bacteroides	1.817±0.5365[a]	1.292±0.1947[a]	7.697±1.855[b]	0.990±0.0963[a]	1.45E-07
Bacteroidetes dgA-11 gut group	0.426±0.1187[a]	0.951±0.2289[a]	1.071±0.2823[a]	2.263±0.3039[b]	4.02E-06
Blautia	0.318±0.0742[bc]	0.316±0.0714[c]	0.112±0.0157[a]	0.139±0.0307[ab]	0.0033
Campylobacter	0.890±0.3312[ab]	0.355±0.1645[a]	1.131±0.3045[b]	0.251±0.0523[a]	0.0050
Christensenellaceae R-7 group	1.965±0.4420[ab]	1.301±0.1660[a]	4.022±1.4966[c]	3.011±0.5090[bc]	1.73E-05
Desulfovibrio	0.248±0.0526[a]	0.257±0.0419[a]	0.737±0.1556[b]	0.245±0.0288[a]	5.46E-05
Escherichia Shigella	0.571±0.2192[a]	0.217±0.0706[a]	3.472±1.1109[b]	0.139±0.0351[a]	1.18E-05
Eubacterium coprostanoligenes group	0.521±0.0681[a]	0.641±0.0706[ab]	0.786±0.1399[ab]	0.989±0.1011[b]	0.0105
Eubacterium nodatum group	0.217±0.0545[b]	0.087±0.0112[a]	0.160±0.0390[ab]	0.101±0.0150[ab]	0.0417
Eubacterium ruminantium group	0.210±0.0429[b]	0.168±0.0265[b]	0.098±0.0286[a]	0.222±0.0431[b]	0.0130
Ezakiella	0.033±0.0144[a]	0.053±0.0531[a]	0.345±0.1908[b]	0.008±0.0080[a]	0.0033
Family XIII AD3011 group	0.394±0.0667[ab]	0.284±0.0387[a]	0.503±0.0773[b]	0.438±0.0554[ab]	0.04166
Fibrobacter	0.167±0.0394[a]	0.320±0.0764[a]	0.789±0.2278[a]	1.911±0.3677[b]	2.74E-07
Fusobacterium	0.933±0.5366[a]	0.131±0.1264[a]	3.499±0.7591[b]	0.016±0.0078[a]	3.92E-08
Helicobacter	0.767±0.3543[b]	0.417±0.3720[ab]	0.117±0.0388[ab]	0.025±0.0056[a]	0.0436
Hungatella	0.244±0.0465[b]	0.123±0.0196[b]	0.062±0.0170[a]	0.043±0.0083[a]	6.58E-06
Lachnoclostridium	0.183±0.0420[b]	0.145±0.0286[ab]	0.093±0.0466[a]	0.086±0.0164[ab]	0.0130
Lachnospiraceae NK4A136 group	0.580±0.1530[ab]	0.461±0.0770[ab]	0.515±0.1510[a]	0.955±0.1990[b]	0.0417

Genera	Day -6 (n = 24)	Day -1 (n = 28)	Day 3 (n = 25)	Day 8 (n = 25)	Adjusted P value
Lachnospiraceae UCG-008	0.201±0.0684^{ab}	0.229±0.0727^{b}	0.067±0.0164^{a}	0.094±0.0204^{b}	0.0117
Marvinbryantia	0.150±0.0376^{ab}	0.146±0.0375^{b}	0.059±0.0167^{a}	0.057±0.0155^{ab}	0.0117
Methanobrevibacter	0.419±0.2029^{a}	0.593±0.2039^{a}	1.976±0.4421^{b}	2.393±0.5151^{b}	7.52E-08
Mitsuokella	0.195±0.0613^{a}	0.805±0.2471^{b}	0.203±0.0819^{a}	0.270±0.0605^{a}	0.0003
Mobiluncus	0.363±0.1543^{bc}	0.019±0.0146^{a}	0.471±0.1577^{c}	0.046±0.0373^{ab}	4.84E-05
Murdochiella	0.230±0.1028^{b}	0.014±0.0133^{a}	0.224±0.0972^{b}	0.015±0.0106^{a}	0.0003
Oscillibacter	0.821±0.1185^{b}	1.157±0.0958^{c}	0.456±0.0806^{a}	1.030±0.0824^{bc}	3.11E-07
Parabacteroides	0.413±0.0603^{ab}	0.496±0.0494^{b}	0.315±0.0771^{a}	0.459±0.0479^{b}	0.0107
Parvimonas	0.136±0.0658^{a}	0.054±0.0530^{a}	1.168±0.0314^{b}	0.007±0.0058^{a}	2.74E-07
Peptococcus	0.200±0.0521^{bc}	0.054±0.0140^{a}	0.333±0.1005^{c}	0.089±0.0251^{ab}	0.0002
Peptoniphilus	0.563±0.2096^{b}	0.038±0.0296^{a}	0.753±0.2174^{b}	0.033±0.0278^{a}	9.61E-06
Peptostreptococcus	0.325±0.1657^{b}	0.032±0.0285^{a}	1.750±0.4600^{b}	0.009±0.0069^{a}	5.07E-08
Phascolarctobacterium	5.317±0.4210^{b}	5.678±0.4473^{b}	3.201±0.3721^{a}	3.543±0.3009^{a}	4.73E-06
Porphyromonas	1.905±0.7351^{bc}	0.204±0.1842^{a}	1.856±0.4994^{c}	0.112±0.0830^{ab}	1.88E-05
Prevotella	2.604±1.1292^{bc}	0.182±0.1743^{a}	3.566±1.3647^{c}	0.057±0.0412^{ab}	0.0001
Prevotella 1	5.777±0.7646^{b}	9.978±0.8918^{c}	2.530±0.5950^{a}	8.651±1.0370^{ab}	2.05E-09
Prevotella 2	0.285±0.0548^{b}	0.262±0.0411^{b}	0.136±0.0507^{a}	0.208±0.0622^{ab}	0.0055
Prevotella 9	5.764±1.5508^{b}	9.491±2.1593^{c}	0.509±0.1611^{a}	8.674±0.7861^{b}	4.17E-08
Prevotellaceae NK3B31 group	6.982±0.8862^{b}	12.804±0.9530^{c}	3.097±0.8306^{a}	7.038±0.7646^{b}	1.71E-12
Prevotellaceae UCG-003	3.208±0.4403^{b}	3.328±0.3230^{b}	1.358±0.4173^{a}	3.597±0.4871^{b}	9.64E-06
Prevotellaceae UCG-004	0.113±0.0364^{a}	0.143±0.0299^{a}	0.194±0.0626^{a}	0.326±0.0634^{b}	0.0010
Rikenellaceae RC9 gut group	2.573±0.3285^{a}	2.853±0.6724^{a}	3.416±0.6724^{a}	5.594±0.4643^{b}	0.0002
Roseburia	1.212±0.1835^{b}	1.466±0.1959^{b}	0.160±0.0402^{a}	0.293±0.0702^{a}	1.41E-14
Ruminococcaceae NK4A214 group	0.333±0.0528^{a}	0.522±0.0748^{a}	0.475±0.0789^{a}	0.931±0.0951^{b}	4.34E-05
Ruminococcaceae UCG-002	0.808±0.1427^{a}	1.213±0.1335^{b}	2.074±0.2425^{c}	2.181±0.1856^{c}	3.54E-08
Ruminococcaceae UCG-005	3.407±0.3935^{ab}	4.105±0.4239^{b}	2.656±0.4239^{a}	3.455±0.2760^{ab}	0.0170
Ruminococcaceae UCG-010	0.224±0.0337^{a}	0.421±0.04400^{b}	0.694±0.1509^{bc}	0.774±0.07133^{c}	9.64E-06
Ruminococcus 1	0.321±0.0753^{b}	0.183±0.0176^{b}	0.064±0.0176^{a}	0.170±0.0275^{b}	0.0002
Ruminococcus gauvreauii group	0.135±0.0313^{b}	0.127±0.0276^{b}	0.041±0.0107^{a}	0.110±0.0404^{ab}	0.01714
Clostridiales bacterium S5-A14a	0.117±0.0524^{a}	0.016±0.0163^{a}	0.386±0.0923^{b}	0.001±0.0014^{a}	9.10E-08
Streptococcus	6.629±0.9716^{c}	0.394±0.7025^{ab}	1.654±0.2016^{b}	0.191±0.1586^{a}	8.47E-15
Subdoligranulum	0.471±0.0966^{b}	0.445±0.0222^{b}	0.137±0.0223^{a}	0.216±0.0435^{a}	3.98E-05

Genera	Day -6 (n = 24)	Day -1 (n = 28)	Day 3 (n = 25)	Day 8 (n = 25)	Adjusted P value
Succiniclasticum	0.310±0.1238[a]	0.017±0.0163[a]	1.005±0.2960[b]	0.032±0.0250[a]	7.65E-07
Succinivibrio	0.204±0.0561[a]	0.458±0.0883[b]	0.289±0.1053[a]	0.262±0.0556[ab]	0.0078
Treponema 2	4.289±0.7970[a]	6.179±0.8404[ab]	9.682±0.8404[b]	14.631±1.3348[c]	4.04E-06

[1]Data are presented as means ± SEM.
[2]D-6, day 109 of gestation (n = 24); D-1, farrowing -1 day (n = 28); D3, day 3 of lactation (n = 25); D8, day 8 of lactation (n = 25).
[a-d]Significant effect of sampling day, whereby relative abundance ± SEM values with different lowercase letters are significantly different for the specified genera.
[3]P values are FDR (false discovery rate) adjusted for multiplicity using the Benjamini and Hochberg (1995) method.

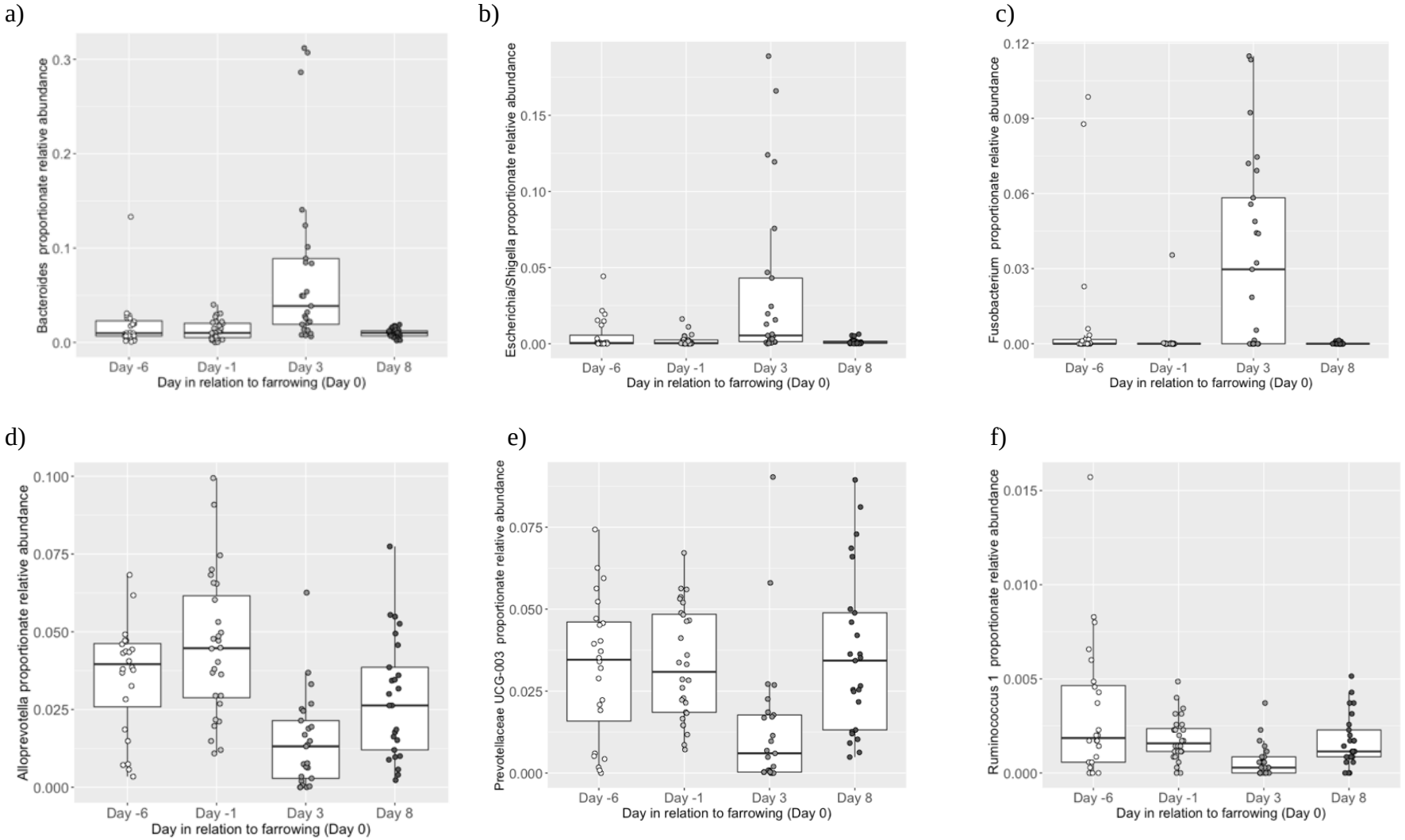


Figure 3.5. The relative genera abundance of *Bacteroides* (a), *Escherichia/Shigella* (b), *Fusobacterium* (c), *Alloprevotella* (d), *Prevotellaceae UCG-003* (e) and *Ruminococcus 1*(f) according to sample time point in relation to farrowing (day 0).

3.4 Discussion

There is currently a lack of research closely monitoring the microbiota changes associated with the periparturient period in sows and whether this is affected by sow parity. To the best of my knowledge, no studies have monitored microbiota changes associated with parity, microbiota changes occurring specifically within the last week of gestation, nor have they ascertained the immediate impact of farrowing on microbiota by comparing samples taken on the last day of gestation to samples collected once the resumption of postpartum bowel movements has occurred. We report longitudinal changes in the microbiota during the periparturient period and an effect of parity on the sow microbiota.

3.4.1 Longitudinal changes in the sow microbiota during the periparturient period

We hypothesised that the microbiota profile of sows will undergo dynamic changes in response to the transition from gestation to lactation. The number of observed OTUs was significantly lower on D-1 and D3 compared with other sampling time points, similar to Cheng *et al.* (2018). Liu *et al.* (2019b) also observed a significant reduction in alpha diversity of sow faeces as gestation progressed, whilst Huang *et al.* (2019b) observed samples collected during the periparturient period to have a lower diversity than non-pregnant sow faeces. This study demonstrated that microbiota richness continues to decline within the last week of gestation. A reduction in microbiota diversity related to progressive gestation stage has been associated with symptoms of metabolic syndrome (Koren *et al.*, 2012) which sows exhibit during late gestation and early lactation (Cheng *et al.*, 2018; Huang *et al.*, 2019b). Metabolic syndrome in sows is characterised by reduced insulin sensitivity (Père *et al.*, 2000; Père and Etienne, 2007) to support the increasing demands for foetal growth (Koren *et al.*, 2012; Père and Etienne, 2019), accompanied by an elevation in levels of faecal pro-inflammatory cytokines, a reduction in faecal IL-10 and an increase in plasma zonulin concentrations (Cheng *et al.*, 2018). The pro-inflammatory status during late gestation is thought to be beneficial for foetal and placental expulsion during parturition (Mor and Cardenas, 2010).

Beta diversity, according to both unweighted and weighted UniFrac distances, was significantly affected by Day, with samples collected on D3 clustering away from the other time points. Cheng *et al.* (2018) used Bray Curtis distances as opposed to UniFrac but reported similar findings for samples collected on D3 of lactation compared with D109 of gestation and D14 of lactation. Furthermore, Liu *et al.* (2019b) reported Landrace gestation samples clustered separately from lactation samples based on Bray Curtis distances, but reported no difference between lactation samples. The results of the present study and Cheng

et al. (2018) suggest that the microbiota community composition is distinct during early lactation in sows.

The predominant phyla associated with the periparturient period were Firmicutes, Bacteroidetes, Spirochaetes, Proteobacteria and Actinobacteria, and Fusobacteria on D3, as reported in previous studies (Cheng *et al.*, 2018; Huang *et al.*, 2019b; Liu *et al.*, 2019b; Shao *et al.*, 2020). Numerous genera segregated according to gestation or lactation abundances. Several butyrate producing genera were significantly reduced in lactation, including *Subdoligranulum* and *Roseburia*. Butyrate is an important energy source for colonocytes/epithelial cells and therefore has an important role in maintaining barrier function. Cheng *et al.* (2018) reported a reduction in butyrate concentration in sow faeces during early lactation. As in this study, Huang *et al.* (2019b) also reported a reduction in *Roseburia* and *Phascolarbacterium* during lactation. *Roseburia* is associated with total antioxidative capacity (Wang *et al.*, 2018). Lactation samples were also characterised by an increase in *Ruminococcaceae UCG-002* and *Ruminococcaceae UCG-010*, as described by Shao *et al.* (2020) in hyperprolific sows. *Christensenellaceae R-7 group* was increased in lactation; this genus has been associated with increased serum triglyceride concentration in humans (Vojinovic *et al.*, 2019) and thus may assist in assimilating nutrients to support lactation. Furthermore, Liu *et al.* (2015) reported Christensenellaceae family abundance to be positively associated with feed intake and energy expenditure, as in lactation.

Several genera abundances were also significantly affected on D3 of lactation in relation to the other sampling days. As reported by Shao *et al.* (2020), there was a significantly lower abundance of *Alloprevotella* and *Prevotellaceae UCG-003* on D3. In contrast, the relative abundance of *Bacteroides*, *Escherichia/Shigella* and *Fusobacterium* was significantly increased, as reported in the literature (Cheng *et al.*, 2018; Shao *et al.*, 2020). *Fusobacterium* abundance is negatively correlated with faecal IL-10 and positively associated with plasma zonulin in sows (Cheng *et al.*, 2018). Liu *et al.* (2019b) reported that *Bacteroides* abundance was negatively correlated with total SCFA concentration of sow faeces. In this study, several SCFA producing bacteria had a significantly lower abundance on D3, including *Prevotella 9*, *Prevotellaceae UCG-003* and *Prevotellaceae NK3B31 group*. Not only does this reduce SCFA availability for sow metabolism to support the energy demands of lactation, but alterations in the concentration of SCFA could have increased GIT pH, creating an environment favourable for *Bacteroides* growth. Further research monitoring pH change of sow faeces during the periparturient period should be conducted to clarify this speculation.

Sows often suffer from constipation around farrowing, Simreń *et al.* (2013) reported a significant increase in *Bacteroides* in patients with constipation predominant-irritable bowel syndrome. There was also a significant increase in *Escherichia/Shigella* abundance on D3, as previously reported (Cheng *et al.,* 2018; Huang *et al.,* 2019b; Shao *et al.,* 2020). Due to their genetic relatedness, 16S rRNA gene sequencing is unable to differentiate *Escherichia coli* from *Escherichia/Shigella* (Khot and Fisher, 2013). *E. coli* are natural components of the sow microbiome, however in a recent study it was demonstrated that giving mice an inflammatory stimulus caused certain strains of *E. coli* to increase the inflammatory response of the host, including IL-6 (Kittana *et al.,* 2018). Cheng *et al.* (2018) observed that, on D3 of lactation, faecal IL-6 was increased, coinciding with an increase in *Escherichia/Shigella* abundance. It was also reported by Gaukroger *et al.* (2020) that *Escherichia/Shigella* and *Bacteroides* relative abundance was highest in piglets at 4 days of age during the first 8 weeks of life, correlating with the peak in the periparturient abundance of these genera.

3.4.2 Differences in the microbiota related to sow parity during the perinatal period

The study hypothesised that greater microbiota dysbiosis during the periparturient period would occur in primiparous compared with multiparous sows. To the best of our knowledge, no studies have compared the microbiota of primiparous to multiparous sows during the periparturient period. In this study, primiparous sows had a lower microbiota richness (number of observed OTUs) and evenness (Shannon diversity index) during the periparturient period compared with multiparous sows. There was a significant interaction between Day and Parity, with primiparous sows having lower microbiota richness at D-6, the last day of gestational housing in straw yards. The increased richness observed in multiparous sows may be associated with their possible higher intake of straw to alleviate any chronic hunger arising from gestational restriction feeding and larger maternal size/gut capacity. A reduction in insulin sensitivity has been associated with lower alpha diversity in sows (Cheng *et al.,* 2018; Huang *et al.,* 2019b). As primiparous sows are required to partition more nutrients to support maternal growth compared with multiparous sows (as they have not yet reached maternal size), in addition to nutrients to support foetal growth and lactation, it is possible that primiparous sows experience a further reduction in insulin sensitivity during the periparturient period. This may explain why alpha diversity is lower in primiparous sows during the periparturient period compared to multiparous sows. Whilst further research is required to determine this, George (1975) reported slower glucose clearance in younger sows. Future research should also record individual feed intakes, inflammatory and metabolic markers

when comparing the microbiota of sows of different parities to determine how the severity of metabolic syndrome is affected by sow parity.

The microbiota community composition of primiparous and multiparous sows was significantly different on D8 according to weighed UniFrac distances; this may arise from the lower microbiota richness and diversity present in primiparous sow faecal samples. Furthermore, microbiota community composition was significantly affected by parity on D-6, -1 and 8 for unweighted UniFrac distances, indicating that low abundance/rare taxa are the main driver of community divergence between parity groupings, especially on D -6 and -1.

Across the periparturient period multiparous sows had a significantly higher abundance of *Bacteroidetes dgA-11 gut group* and *Prevotellaceae UCG-004.* Research monitoring faecal microbiota changes associated with parity in dairy cows also reported *Bacteroidetes dgA-11 gut group* to have a significantly higher abundance in multiparous compared with primiparous cows (Zhang *et al.,* 2019b). Bacteria belonging to the Prevotellaceae family are commonly regarded as propionate producers. *Prevotellaceae UCG-004* has been positively correlated with carbohydrate metabolism and SCFA concentration in pig faeces (Zhang *et al.,* 2019b), suggesting increased microbial fermentation in the hind gut of multiparous compared to primiparous sows.

In conclusion, the study identified longitudinal changes in the periparturient sow microbiota profile. These findings corroborate previous literature, which deduced these microbiota changes to be associated with metabolic syndrome in sows. The significant microbiota changes occurring during the periparturient period highlight the need to utilise time matched samples when determining the longitudinal effects of the sow on progeny microbiota development. The study identified differences in the microbiota profile associated with sow parity, possibly suggesting that primiparous and multiparous sows are differentially affected by metabolic syndrome and perhaps its severity.

Chapter 4. The effect of cross-fostering on neonatal piglet microbiota development and performance

4.1. Introduction

The neonatal period is well recognised as a period of developmental plasticity; perturbations to the microbiota succession during this period, including those caused by housing (Merrifield *et al.*, 2016) and diet (Wang *et al.*, 2019d; Li *et al.*, 2012; Poroyko *et al.*, 2010; Poulsen *et al.*, 2017; Saraf *et al.*, 2017) can result in long term changes to the piglet GIT microbiota and consequently metabolic profile and immune development (Lewis *et al.*, 2012., Inman *et al.*, 2010). Factors affecting microbiota development during the suckling period have been reviewed recently by Nowland *et al.* (2019) and include colostrum, milk quality and the neonatal environment. Under commercial conditions, sources of early GIT microbiota colonisers include the maternal vagina, pen floor, sow faeces, areolar (udder) skin and milk. Early research by Schmidt *et al.* (2011), reported that removing piglets from the sow and rearing them in an isolator from 2 days of age resulted in a significantly different microbiota profile up to 56 days of age. Schmidt *et al.*, (2011) concluded that the continued interaction with the sow and maternal pen environment was required after the first 48 hours of life in order to develop a stable and adult-like microbiota. More recent studies have begun to quantitatively establish the relative importance of these different neonatal microbiota colonising sources. Zhang *et al.* (2018b) sampled sow milk, vagina and faeces pre- and post-farrowing to determine the similarity between the maternal microbiota and the faecal microbiota of piglets pre-weaning. The study identified the microbiota richness of sow milk to be positively correlated with microbiota diversity of piglet faeces and negatively correlated with diarrhoea incidences. Chen *et al.* (2018) compared the pre-weaning piglet faecal microbiota to the pen floor, sow nipple, sow faeces and sow milk on days 1 and 7, and sow vagina on day 1. On day 1, they reported that the piglet microbiota community composition was more similar to the floor, sow nipple and sow milk microbiota, however, as lactation progressed the piglet microbiota became increasingly similar to sow faeces. Similarly, Liu *et al.* (2019a) compared the sow vaginal, nipple, pen floor and creep area microbiota at farrowing, and sow faeces at farrowing -2 days and during lactation, to the small intestine and large intestine mucosal microbiota of piglets until 35 days of age. The community composition of the piglet large intestinal microbiota was more similar to sow milk up to day 7, while after this time point the microbiota of piglets became more similar to sow faeces than other maternal or pen microbiota sources. Furthermore, the microbiota community composition between birth sow and piglet dyads were more similar than between unrelated

sow and piglets, indicative of a litter effect on days 0, 7 and 14. Both studies by Chen *et al.* (2018) and Liu *et al.* (2019a) cover all major sources of microbiota capable of seeding the GIT of neonates, however these studies were conducted with a low number of replicates, especially when early life is considered to be the time of highest inter-piglet microbiota variability, and thus larger studies are required to see if results are consistent.

Cross-fostering is a standard commercial practice, particularly as litter sizes increase. Creating uniform litters based on piglet size reduces competition for resources, resulting in reduced piglet variability at weaning (Douglas *et al.*, 2014a; Huting *et al.*, 2017) and lower mortality (Milligan *et al.*, 2001; Deen and Bilkei, 2004). Cross-fostering most often occurs within the first 24 hours of life, once piglets have had sufficient colostrum from their biological dam. Cross-fostered piglets will be exposed to increased handling, a new pen and additional exposure to nurse sow microbiota sources (faeces, milk and udder), which could alter their microbiota composition. Several studies have been conducted to determine the effect of cross-fostering and the nursing mother on the microbiota of piglets, but the majority of these studies have used different birth and foster sow breeds in the cross-fostering model (Xian *et al.*, 2014; Mu *et al.*, 2019; Bian *et al.*, 2016). In these studies, the GIT microbiota profile of piglets was affected pre-weaning by nursing mother, which Bian *et al.* (2016) attributed to genetic differences in milk composition between pig breeds. To understand the effects on piglets of increasing the diversity of microbial exposure associated with cross-fostering, it is important to study pigs of the same genetics within the cross-fostering model. Maradiaga *et al.* (2018) determined the effects of cross-fostering on GIT and faecal microbiota and mucosal immune gene expression in neonates on day 0 and 21 of age, with cross-fostering occurring after colostrum consumption. On day 0, the genera in piglet faeces were most correlated with colostrum ($r = 0.72$) followed by the vagina ($r = 0.65$) and sow faeces ($r = 0.57$). Cross-fostered piglets had lower microbiota diversity (Chao 1 and Shannon) in the ileum and colonic mucosa pre-weaning compared to piglets who remained on their birth dam. Furthermore, the abundance of *Actinobacillus, Tannerella, Treponema*, *Escherichia/Shigella* and *Campylobacter* were significantly different between cross-fostering groups. This was once again a small study, with 8 replicates per cross-fostering treatment and two sampling time points, missing the period of rapid neonatal microbiota development.

No studies have yet established the effect of cross-fostering within one genotype during the neonatal period on a large scale, or considered the effect of sow parity within the cross-fostering model when studying the neonatal microbiota. Cross-fostering to place LBW piglets on low parity sows is common commercial practice and advised in the UK (AHDB, 2020b), matching the smaller teat size of low parity sows (Balzani *et al.*, 2016; Ocepek *et al.*, 2016) to

the smaller mouth of LBW piglets, and taking account of the increased teat accessibility of the smaller-size lower parity sows (Vasdal and Anderson, 2012). Primiparous born piglets have previously been reported to exhibit lower birthweights (Miller *et al.*, 2012; Craig *et al.*, 2017), reduced weaning and slaughter weight (Carney-Hinkle *et al.*, 2013; Craig *et al.*, 2017) and increased mortality rates (Miller *et al.*, 2012). Furthermore, Huting *et al.* (2019) and Ferrari *et al.* (2014) demonstrated that piglets reared by primiparous sows had lower weaning weights compared with those reared by multiparous sows. Whilst several studies have reported lower milk production by primiparous sows (Hansen *et al.*, 2012; Dourmad *et al.*, 2012; Strathe *et al.*, 2017), Craig *et al.* (2019) reported no difference in colostrum or milk total IgG, fat protein or net energy levels between primiparous and multiparous sows, attributing reduced performance of gilt progeny to lower milk intakes and capacity to digest and absorb milk components. Moreover, primiparous sows have a less diverse microbiota than multiparous sows during the periparturient period (Chapter 3, Gaukroger *et al*., 2021); this could also alter the microbiota colonisation and development of primiparous born and reared piglets.
As the microbiota from the sow nipple and faeces seem to play differing, but equally important, roles in the microbiota development of neonates (Chen *et al.*, 2018), this study focuses on investigating the contribution of sow areolar skin and faecal samples in a longitudinal analysis of piglet microbiota, something which has not previously been done on a large scale. As the sow faecal microbiota changes substantially during the periparturient period (Cheng *et al.*, 2018, Gaukroger *et al.*, 2021) it is important to ensure time-matched samples are included in such an analysis to prevent false conclusions regarding the importance of the sow as a continuous microbiota seeding source throughout the suckling period. By utilising birth sow samples (sow udder and faeces) as well as time-matched faecal samples from the nursing mother in the analysis of the piglet faecal microbiota development, the study can ascertain whether maternal effects on the piglet microbiota are limited to the early neonatal phase or are a continual process throughout lactation, and thus influenced by cross-fostering.

4.1.1 Study aims and hypotheses

The aim of the study was to establish how the development of the microbiota community composition of neonatal piglets is affected by reciprocal cross-fostering and the importance of maternal microbiota sources in the development of the neonatal piglet microbiota. The study hypothesis was that cross-fostering would increase microbiota diversity compared with piglets who remain with their biological dam, and that low birthweight piglets would have a less diverse microbiota due to their previously reported under-developed gastro-intestinal tract. The study also aimed to ascertain how birth or rearing sow parity affected the development of

the neonatal microbiota, hypothesising that piglets born and reared by a multiparous sow would develop a more diverse microbiota, based on the results of Chapter 3.

4.2. Materials and methods

4.2.1 Experimental design

A total of 214 experimental piglets from 30 Large White x Landrace experimental sows (multiparous = genetics from Hermitage Seaborough Ltd, UK and primiparous = genetics from a cross between lines from Hermitage Seaborough Ltd and Rattlerow Farms Ltd, UK) were used in this study. Sows farrowed in 8 consecutive batches and were grouped based on parity as being primiparous (n = 15) or multiparous (2nd parity and above, n = 15, average parity = 3.13 (SD = 1.126)). The study was set up as an incomplete 2 x 2 x 2 factorial design, the factors being: 1) piglet birthweight, with experimental piglets classified as being of low birthweight (LBW; 0.80 - 1.25kg, n = 112) or normal birthweight (NBW; 1.50 - 2.00kg, n = 102); 2) experimental piglets were either cross-fostered (n = 103) or remained with their birth sow (n = 111); 3) fostering was done onto either primiparous or multiparous sows. However, cross-fostering was only done in a reciprocal fashion between one multiparous and one primiparous sow, to generate experimental litters of either LBW or NBW, creating 15 reciprocal litter pairs **(figure 4.1)**. This was done to minimise the variation in microbiota sources within the litter. Significant differences associated with cross-fostering were thus quantifiable when utilising a reciprocal cross-fostering model. Due to the reciprocal nature of the cross-fostering and animal availability, it was not possible to balance experimental litters for parity and birthweight, thus the following experimental litters were created: multiparous LBW n = 9, multiparous NBW n = 6, primiparous NBW n = 9 and primiparous LBW n = 6.

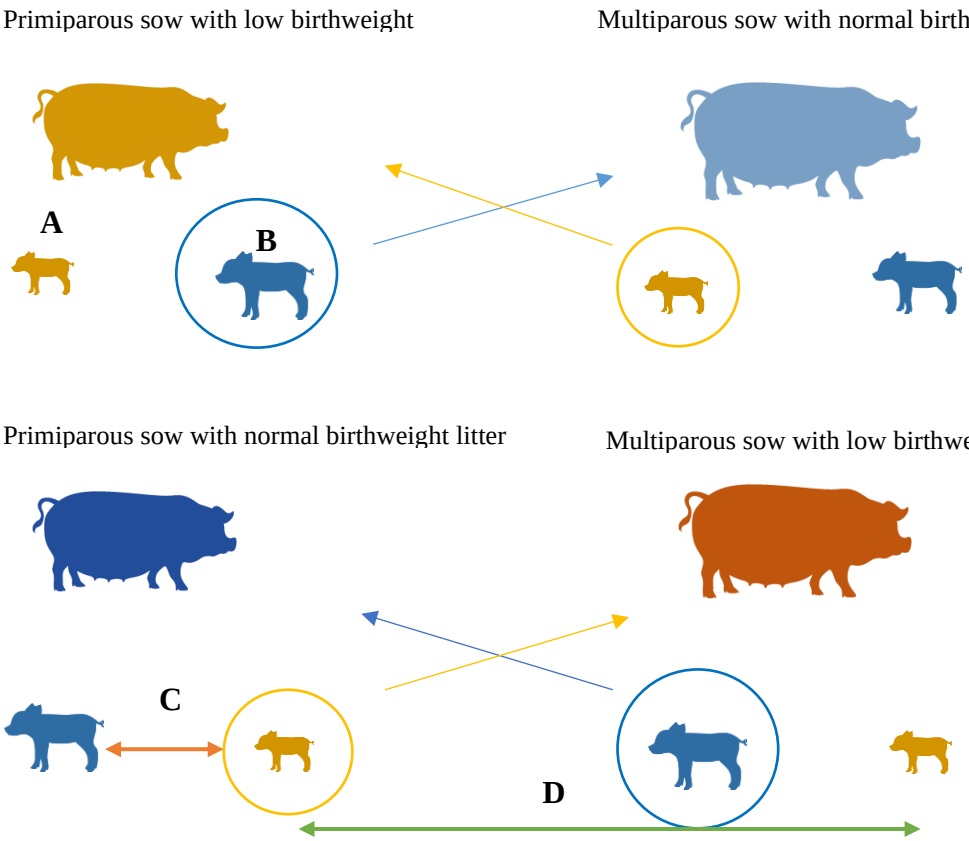


Figure 4.1. Reciprocal cross-fostering illustrating the 2 x 2 x 2 factorial design. The first factor was piglet birthweight (normal birthweight = blue piglets and low birthweight = yellow piglets), the second factor was cross-fostered (yes = circled piglet, no = un-circled piglet) and the third factor sow parity (primiparous = small sows and multiparous = larger sows). Piglet cross-fostering category groupings (used for analysis of microbiota community composition) are indicated by letters A – D and the orange (C) and green (D) arrows. A) distances within siblings who remain on their biological dam ('biological siblings'). B) distances within the group of siblings leaving the litter to be cross-fostered ('cross-fostered'). C) distances between each biological piglet that remains on the litter and every cross-fostered sibling removed from the litter ('cross-fostered siblings'). D) distances between non-biological siblings added to the litter and each piglet that remained in the biological dam litter ('non-siblings).

4.2.2 Animal housing and management

Gestating sows were managed in a 3-week indoor batch farrowing system and housed in straw yards in groups of 5 sows of similar size and parity. Sows were housed in gestation as previously described in Chapter 3. During gestation, sows were fed a home-milled mash gestation diet based on barley and soyabean meal (13.14 MJ DE /kg, 13.82% CP and 0.62% lysine). They received approximately 2-2.50 kg/head at 07:30 daily throughout gestation.

Multiparous sows were moved from solid floored straw yards to a conventional part-slatted farrowing pen with a farrowing crate at approximately 109 days of gestation, with primiparous sows entering at 111 days. The later entry of primiparous sows into the farrowing house was standard practice on the commercial unit, reducing the amount of time primiparous sows spent in farrowing crates in an effort to reduce stress. Prior to entry, the farrowing pen was washed and disinfected (concentration = 0.03% PhenoPharm, East Riding Farm Services, UK) and allowed to dry for a minimum of 7 days. Farrowing crate dimensions were: entire pen 1.80m width x 2.42m length, creep area 1.11m length x 0.80m width and sow crate 0.6m width x 1.77m length to the feed trough. All sows were wormed with Bimectin (5ml primiparous and 8ml multiparous intramuscularly (IM), Bimeda, Llangefni, UK) upon entry to the farrowing house and received a FarrowSure Gold vaccine against porcine parvovirus, erysipelas and leptospirosis on the day before weaning (2ml IM, Zoetis, Surrey, UK), which occurred at ~28 days post-partum. Following housing in the farrowing crates, sows received approximately 0.70 kg of the gestation diet fed twice daily at 07:45 and 15:00 until farrowing. On the day after farrowing sows were transferred to a home-milled mash lactation diet (13.98 MJ DE/kg, 18.50% CP and 0.95% lysine) initially as a 2.0 kg/head/day allowance, which was increased to appetite by 0.5 kg/head/day until a 10 kg/head/day limit was reached. Water was available *ad libitum* through a nipple drinker. Cross-fostering of piglets occurred within the first 24 hours post-partum in order to create uniform litters of piglets based on birthweight; litter size was set according to the number of functional teats. Piglets < 800g and between 1.25 - 1.50 kg were moved to non-experimental sows.

The farrowing house was maintained at 21°C, whilst an enclosed heated creep area (infrared heat lamp) was available to piglets; this contained wood shavings as bedding for the first week of life. Piglets were teeth clipped within the first 12 hours of life, then received an iron injection (1ml IM Gleptosil 200mg iron/ml, CEVA Animal Health Ltd, Amersham, UK) and were tail docked at ~4 days of age. Piglets also received their first stage *Mycoplasma hyponeumoniae* vaccine (1ml IM M+PAC, MSD Animal Health, Walton, UK) at ~7 days of age. Piglets could access water through either a nipple drinker or water trough, whilst creep feed was provided *ad libitum* from 10 days of age (16.50 MJ DE/kg, 22.50% CP and 1.7% SID lysine; Flat Deck 1, A-One Feed Supplements Ltd, Thirsk). The number of piglets weaned per experimental sow was recorded at weaning. Veterinary records for both the sow and her litter were recorded throughout lactation. Following an end-point protocol, sows were treated with a three-day course of penicillin (10ml IM, Pen & Strep, Norbrook, Newry, UK) if they presented thick creamy vaginal discharge, or symptoms of mastitis, metritis, agalactia (n = 5). Piglets were treated for lameness associated with joint swelling (3 days of 1ml IM mix of 0.5ml Loxicom

and 0.5ml Pen & Strep, Norbrook, Newbury, UK) and diarrhoea (1ml Trimacare IM, Norbrook, Newbury, UK). Individual piglets were removed from the experiment to non-experimental sows with supplementary milk if they did not gain > 100g/d over two consecutive days. During the neonatal period 6 piglets were treated for lameness and 12 piglets for diarrhoea; these piglets were retained in the microbiota dataset as samples did not display significant differences in alpha or beta diversity analysis when compared to the 196 antibiotic free piglet cohort. A total of 4 piglets were removed from the trial between days 3 – 8 and a further 16 piglets were removed from the study between days 8 – 27 as they did not meet the weight gain threshold.

4.2.3 Experimental procedures

Sows were faecal sampled (30 ml Sterilin Specimen Container, Starstedt, UK) daily from day 113 of gestation until farrowing, to ensure a farrowing -1 day sample was collected from each experimental sow, and then on days 3 and 8 post-partum. The faeces produced on day -1 or residue from this faecal sample will be present in the farrowing pen as the sow gives birth. The farrowing -1 day sample is likely to be the only faeces neonates will come into contact with until after cross-fostering, as resumption of sow bowel movements does not occur until 2-3 days post-partum. Therefore, the day -1 sow faecal sample was utilised as a time matched day 1 sample with piglets day 1 faeces and thus referred to as such in subsequent text. Udder swabs (Sterilin plain flocked swab, Thermo Scientific, UK) were taken from un-suckled teats and around the base of the teat, to mimic the teat searching, nosing and suckling behaviour of piglets, from a subset of 20 experimental sows after the first 1- 2 piglets were born. It was not possible to collect udder swabs from all experimental sows as some farrowed between 22:00-06:00, therefore a swab from an un-suckled teat could not be taken with certainty.

Experimental sows were selected to form a reciprocal litter pairing primarily based on similarity in farrowing time to a sow of the opposing parity grouping, and the presence of both LBW and NBW piglets ($n \geq 2$). Furthermore, particularly in the case of multiparous sows, the quality of the udder and teats was also assessed, as all experimental piglets had to be reared without access to supplementary milk, irrespective of birthweight, due to the influence of milk replacer on the microbiota profile (Poroyko *et al.*, 2010; Li *et al.*, 2012; Poulsen *et al.*, 2017).

All piglets were weighed and sexed within 6 - 12 hours of life; those who fell into either the LBW or NBW classification and were viable (no splay legs, lethargy or physical defects) were selected for the study and ear tagged for identification purposes (Dentag, Toptag, UK). Piglets were reciprocally cross-fostered between 9 - 24 hours of age, to ensure piglets had sufficient colostrum intake from their birth mother, to form experimental litters of the same

birthweight class (LBW or NBW; **figure 4.1**). Piglets were rectally swabbed during the cross-fostering process to collect a day 1 microbiota sample and then again on days 3 and 8 of age; they were weighed at the same time to limit handling stress. Swabbing induced defecation in some piglets, particularly if they had suckled and had slept for at least 30 minutes prior to sampling. These additional faeces were collected, and a small proportion added to the swabs during DNA extraction, to increase the potential microbial DNA yield from samples. All faecal swabs and samples were frozen at -80°C within 2 hours of sampling and stored at this temperature until DNA extraction was performed, snap freezing of microbiota samples in liquid nitrogen was not feasible due to the pig unit set up. Piglet liveweights were also recorded on weaning – 1 day (~day 27). Subsequently, piglet average daily gain (ADG) for birth – day 3, day 3 – day 8 and day 8 – weaning -1 day, along with pre-weaning ADG (day 1 – 27) were calculated for each piglet.

4.2.4 16S rRNA gene sequencing

Bacterial DNA was extracted from < 250mg of faeces using the DNeasy PowerSoil HTP 96 kit (Qiagen, UK), following manufactures instructions. The V4 region of the 16S rRNA gene was amplified by PCR and library generation, quality control steps and sequencing procedure were conducted in accordance with the Kozich *et al.* (2013) standard operating procedure as described in Chapter 3. Sequencing was performed on the Illumina MiSeq (Illumina, USA) using the 2 × 250 bp paired-end read protocol at NU-OMICS DNA sequencing facility. The read pairs were demultiplexed and reads were merged using USEARCH v7.0.1090 (Edgar, 2010). Merging allowed zero mismatches and a minimum overlap of 50 bases. Furthermore, merged reads were trimmed at the first base with a $q \leq 5$. A quality filter was applied to the resulting merged reads and those containing above 0.5% expected errors were discarded. Sequences were stepwise clustered into OTUs at a similarity cut-off value of 97% using the UPARSE algorithm (Edgar, 2013). Chimeras were removed using USEARCH v7.0.1090 (Edgar, 2010) and UCHIME v4.2 (Edgar *et al.*, 2011). To determine taxonomies, OTUs were mapped to a version of the SILVA Database (Quast *et al.*, 3013) containing only the 16S V4 region using USEARCH v7.0.1090 (Edgar, 2010). Abundances were recovered by mapping the merged reads to the UPARSE OTUs. A custom script constructed an OTU table from the output files generated in the previous two steps for downstream analyses of taxonomic relative abundance, alpha diversity, and beta diversity. A total of 18,183,147 sequencing reads were obtained from an initial 732 sow and piglet samples run on the Illumina MiSeq. Sequences were rarefied to 4000 reads per sample. After rarefaction 725 samples were retained and the full rarefied OTU file utilised for alpha and beta diversity analysis. For the

genera relative abundance analysis taxa genera abundance was initially filtered to $\geq$ 0.01%, retaining 17 phyla, 27 classes, 35 orders, 60 families and 159 genera for subsequent genera relative abundance analysis. In addition, a total of 12 PCR negative, 12 PCR positive and 20 DNA extraction kit negatives were also sequenced, after rarefaction 1 PCR kit negative and 2 DNA extraction kit negatives and all 12 PCR positives controls were retained. These samples did not display a microbiota profile similar to piglet or sow samples and were removed from the dataset (**Appendix 4a).**

4.2.5 Statistical analysis

All statistical analyses were conducted in R (v 3.6.2). The experiment was analysed as a 2 x 2 x 2 factorial, with the factors defined above, including piglet birthweight class, sow parity and whether piglets were cross-fostered, with the addition of time point for longitudinal analyses. Individual piglets formed the experimental unit in all the analyses. For longitudinal performance and alpha diversity models, piglet ID nested within birth sow ID formed the random effects in all models to account for repeated measures (multiple microbiota samples from one piglet over different time points, thus precautions needed to be taken to account for individual and potential litter effects on the development of the microbiota profile over time). performance and alpha diversity models were built in a backwards fashion, interactive terms were removed from models where no significant interactions between fixed effects existed. An example of a longitudinal observed OTUs statistical model where there were no significant interactions between fixed effects was as follows: *Observed OTUs = Days of age + Birthweight class (LBW/NBW) + Birth sow parity (Primi/Multi) + Cross-fostered (Y/N) + (1| Sow ID/ Piglet ID).* All performance data and alpha diversity values were tested for normality by the Shapiro Wilk's normality test. Model diagnostic plots were used to assess model fit, these included fitted values vs residual values and qqnorm plots with a qqline added to the plot. Models were accepted when diagnostic plots did not show signs of violating model assumptions and presented a heteroscedastic fit of the fitted vs residual values and qqnorm values aligned closely to the qqline, thus model residuals observed to be normally distributed. P values below 0.05 were considered statistically significant for all models. The *car* (v 3.0 – 8) and *emmeans* package (v 1.4.8) were used for post-hoc pairwise comparisons of significant fixed and interactive effects. The *multcomp* package (v 1.4 - 13) was used to generate compact letter displays of all pairwise comparisons, whereby least square mean values with different superscript lowercase letters denote significantly differences ($P < 0.05$) between pairwise comparisons.

A total of 5 sows were treated with antibiotics post-partum. Preliminary alpha, beta and taxa abundance analysis demonstrated that sow antibiotic treatment did not significantly affect the sow faecal microbiota profile, therefore these sows were retained in the dataset. Meanwhile, a total of 18 piglets of the 214 piglet cohort were treated with antibiotics before 8 days of age, for lameness or diarrhoea. These piglets were retained for statistical analysis due to the unbalanced study design and low piglet availability for each of the cross-fostered and non-cross-fostered groups per litter replicate, in order to increase statistical power.

4.2.5.1 Piglet performance

Liveweights (LWs) were not normally distributed and displayed positive skew (*skewness* function 'moments' package v 0.14), thus a log10 transformation was applied to the LW data. A general linear model (glmer) ('lme4' package v 1.1 – 23) method = REML was performed, testing all interactions between piglet age, BiW class, cross-fostering group and birth sow parity. Interactive terms were removed from the model in the absence of significance. Model diagnostic plots revealed model residuals to be normally distributed.

Piglet ADG between successive time points was not normally distributed but did not display skewness, thus a glmer model was performed, testing the aforementioned interactions between fixed effects, in a backwards fashion. There was no significant interaction between fixed effects and piglet age in the repeated measures ADG model, therefore an overall pre-weaning ADG model (weight gain from birth to day 27) was performed testing the 2 x 2 x 2 factorial experimental design. Pre-weaning ADG was normally distributed and so a linear mixed effects model (lmer) ('lme4' package v 1.1 – 23) was performed. Birth sow ID formed the random effect in the pre-weaning ADG model. Model diagnostic plots displayed normality for the repeated measures and pre-weaning (birth – day 27) ADG models.

4.2.5.2 Alpha diversity

Piglet observed OTUs and Shannon diversity index were calculated using the 'vegan' package (v 2.5) in order to analyse microbiota diversity longitudinally (day 1, day 3 and day 8 data). Interactions between all fixed effects were tested (time point, BiW class, cross-fostering group, birth sow parity). Longitudinal diversity indices were not normally distributed and so glmer models ('lme4' package v 1.1-23) were used to analyse all alpha diversity data. Observed OTUs were square root transformed, and Shannon diversity index was box-cox transformed for the longitudinal modelling, lambda = 1.4. Following the respective transformations, model diagnostic plots displayed normality.

4.2.5.3 Beta diversity

Weighted and unweighted UniFrac distances, calculated using the 'rbiom' package (v 1.0.2.9002), were used to assess the similarity of the microbiota community composition in all analyses; UniFrac distances were selected in order calculate dissimilarity between microbiota communities based on their phylogenetic tree (Lozupone and Knight, 2005). Weighted UniFrac distances also enable microbiota community compositions to be assessed based on taxa relative abundances between samples (Lozupone *et al.*, 2007). A PERMANOVA was performed using the *Adonis* function in the 'vegan' package, with 999 Monte Carlo permutations on both UniFrac distances to assess the effect of piglet age/ time post-partum on the microbiota community composition of piglet faeces and sow microbiota sources. Repeated measures cannot be accounted for in PERMANOVA models, thus cross-sectional models were conducted on days 1, 3 and 8 to analyse factors affecting the piglet microbiota community composition. As cross-fostering had not occurred at the time of sampling on day 1, the interaction between BiW class and birth sow parity were the fixed effect in the day 1 PERMANOVA. The cross-sectional PERMANOVA analysis on days 3 and 8 tested the interaction between BiW class, cross-fostering group and birth sow parity.

4.2.5.4 The influence of the sow on the development of the piglet microbiota and the effect of cross-fostering

From the weighed UniFrac distance matrix, to account for differences in relative abundance within the community composition, distances associated with individual reciprocal cross-foster sow pairings (sow, udder and piglet distances related to all piglet-sow, piglet-udder and piglet-piglet combinations) were isolated and used to create new matrices corresponding to day 1, day 3 and day 8, respectively. Several distance analyses were performed between siblings and non-siblings (following cross-fostering), birth sow faeces and piglets (progeny who remained and those who were cross-fostered on to another litter), rearing sow faeces and piglets (progeny who remained and those who were cross-fostered into the litter) and between birth sow areolar skin and piglet faeces. A summary and description of the distances can be seen in **table 4.1**. The aim of the experiment was to understand how influential the sow is on neonatal piglet microbiota development and the effect of cross-fostering, and so this was the focus of the subsequent analyses described below. The analysis was conducted at the 'cross-fostering group' level per litter, rather than for individual piglets, therefore factors such as piglet birthweight and birth sow parity were not included in the beta diversity analysis. Birth sow ID formed the random effect in all models. Weighted UniFrac distances were tested for normality according to the Shapiro-Wilk's normality test and subsequently modelled as either

a lmer *method = REML* (normally distributed) or a glmer (not normally distributed) model ('lme4' package v 1.1 – 23). Model diagnostic plots (qqnorm and fitted vs residual values) were inspected for each model and deemed normally distributed, therefore no transformations to the raw distance measures were applied.

Table 4.1. A description of sow – piglet and piglet – piglet cross-fostering groupings used for weighted UniFrac distance comparisons.

Sow - piglet cross-fostering groups	
Cross-fostering grouping	**Description of group classification**
Birth sow faeces farrowing	Distances between the day 1* sow faecal microbiota and her biological piglets (at a specified time point)
Birth sow piglet	Distances between the sow faecal microbiota and her biological piglets using time-matched samples
Birth sow udder farrowing	Distances between the day 1 sow udder microbiota and her biological piglets (at a specified time point)
Cross-fostered birth sow piglet	Distances between the sow faecal microbiota and her biological piglets that were cross-fostered on to another litter using time-matched samples
Cross-fostered birth sow faeces farrowing	Distances between the day 1 sow faecal microbiota and her biological piglets that were cross-fostered on to another litter (at a specified time point)
Cross-fostered sow piglet	Distances between a sow and non-biological piglets cross-fostered into the litter using time-matched samples
Cross-fostered birth sow udder farrowing	Distances between the day 1 sow udder microbiota and biological piglets cross-fostered on to another litter (at a specified time point)
Piglet - piglet cross-fostering groups	
Cross-fostering grouping	**Description of group classification**
Biological group	Distances within a sibling group which remain on their birth dam
Cross-fostered group	Distances within a sibling group cross-fostered together on to another sow
Non-sibling cross-fostered	Distances between each birth piglet remaining on a sow and all non-sibling piglets cross-fostered into the litter
Siblings cross-fostered	Distances between each birth piglet remaining on a sow and all siblings who leave the litter and are cross-fostered onto another sow

*Day 1 sow faeces refers to farrowing -1 day sow faecal sample (see main text)

To determine the most important source (sow faeces or udder microbiota) for seeding the piglet microbiota on day 1, weighted UniFrac distances between piglets and their biological dam faeces, and between piglets and their biological dam udder, were calculated and averaged

across the experimental piglets for each experimental litter. As piglets had not yet been cross-fostered at the time of sampling on day 1 and were a composite mix of birthweights, sow sample type (faeces or udder) formed the fixed effect in the analysis, in order to determine which microbiota source was most influential to neonatal microbiota development.

To determine whether the piglet microbiota became more similar to the sow faecal microbiota over time, compared to day 1 sow udder and faecal samples, weighted UniFrac distances between sow faeces and time-matched piglet faecal samples from 'biological' piglets ('Biological sow piglet' distances) only were analysed, to avoid any confounding effects of cross-fostering. Longitudinal weighted UniFrac distances were not normally distributed and so a glmer model was performed. Sample time point (day 1 udder, day 1 sow faeces, day 3 faeces and day 8 faeces) formed the fixed effect in the model.

To assess whether seeding of the piglet GIT microbiota by the sows continues outside of the first 24 hours, weighted UniFrac distances between cross-fostered only piglets and their rearing sow faeces ('Cross-fostered sow piglet') as well as their biological dam faeces ('Cross-fostered biological sow piglet') were compared on day 3 and day 8, using time-matched sow and piglet faecal samples. Day 3 and day 8 weighted UniFrac distances were normally distributed and so a lmer model (lme4 v 1.1-23 package) was performed with *method = REML*. Sample time point (day 3 and day 8), cross-fostering grouping (biological or foster sow) and their interaction were the fixed effects in the model.

The final sow – piglet beta diversity model assessed how the weighted UniFrac distances of both 'biological' and 'cross-fostered' piglets to day 1 sow udder ('biological udder farrowing' and 'cross-fostered udder farrowing' grouping categories) and faecal ('biological faeces farrowing' and 'cross-fostered faeces farrowing' grouping categories) samples changed over time in order to establish if there was a longitudinal seeding effect of day 1 samples on the piglet microbiota development, and if so from which sow source. The model also compared time matched birth sow and piglet faecal sample weighted UniFrac distances ('biological sow piglet' and 'cross-fostered biological sow piglet' grouping categories), and in the case of cross-fostered piglets the weighted UniFrac distances to rearing sow time-matched faecal samples ('cross-fostered sow piglet' grouping category). Distance comparison groupings formed the fixed effect in the model and birth sow ID formed the random effect. Longitudinal weighted UniFrac distances were not normally distributed and so a glmer model was performed. Model diagnostic plots were normally distributed thus no transformations to the raw distance values were performed.

4.2.5.5 Cross-fostering effects on the piglet microbiota community composition

To determine the effect of cross-fostering on the neonatal microbiota and detect whether a birth-litter specific microbiota community composition existed, cross-sectional analysis was first performed to analyse the effect of cross-fostering group (within 'biological' and 'cross-fostered' piglet groupings, and between 'non-siblings cross-fostered' and 'siblings cross-fostered' groupings) on weighted UniFrac distance for each time point (**table 4.1**). Cross-fostering group was the fixed effect in the model, and birth sow ID formed the random effect in all cross-sectional models. The data were not normally distributed for day 1 and day 8 distances and so glmer models were performed, whilst a lmer model was run for day 3 distances (lme4 v 1.1-23 package). Diagnostic plots for each cross-sectional model were inspected and appeared to be normally distributed, thus no transformation to cross-fostering weighted UniFrac distances were applied. To determine whether distances between cross-fostering groups increased over time, thus loss of a litter specific microbiota over time, a longitudinal model was also performed. Cross-fostering grouping and time point (day 1, 3 and 8) formed the fixed effects in the longitudinal model. Longitudinal weighted UniFrac distances were not normally distributed, thus a glmer model was performed.

4.2.5.4 Quantitative determination of neonatal piglet microbiota origin

SourceTracker (Knights, 2011) was used to quantitively determine how much the day 1 birth sow faecal and udder microbiota contributed to the development of the piglet microbiota over time, and the potential continual seeding of the piglet gut microbiota from time-matched sow faecal samples. Sow udder and faecal samples were categorised as 'source' samples, whilst piglet faecal samples were determined as 'sink'. The analysis was conducted cross-sectionally, with day 1 sow udder and faeces added as additional 'source' samples for the day 3 and day 8 analysis.

4.3. Results

All mean values reported are least square means, calculated by the *emmeans* package (v 1.4.8).

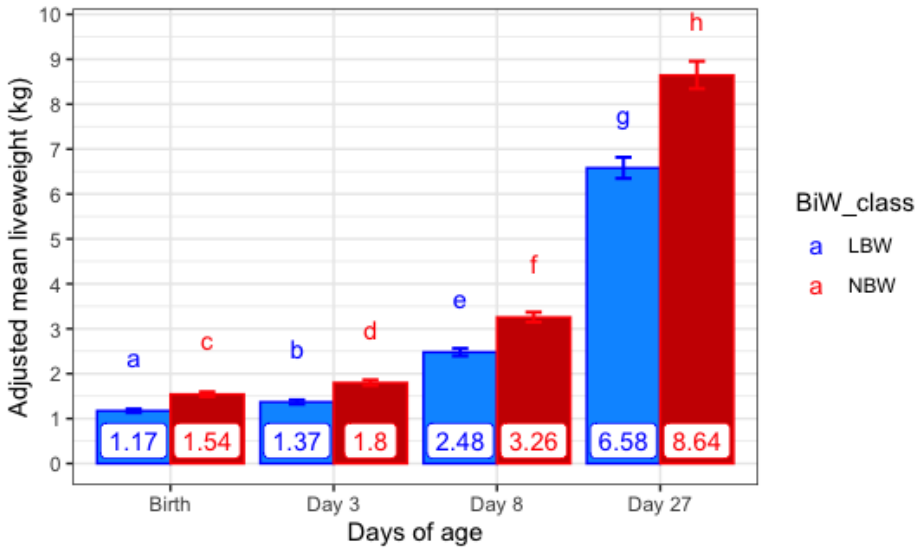
4.3.1 Piglet performance

Piglet LW significantly increased between each time point ($P < 0.001$). LBW piglets were significantly lighter than NBW piglets ($P < 0.001$) at each time point (**figure 4.2a**). There was no significant effect of cross-fostering group ($P > 0.05$) or birth sow parity ($P > 0.05$) on piglet LW. There was also no significant interaction between piglet age and BiW class, cross-

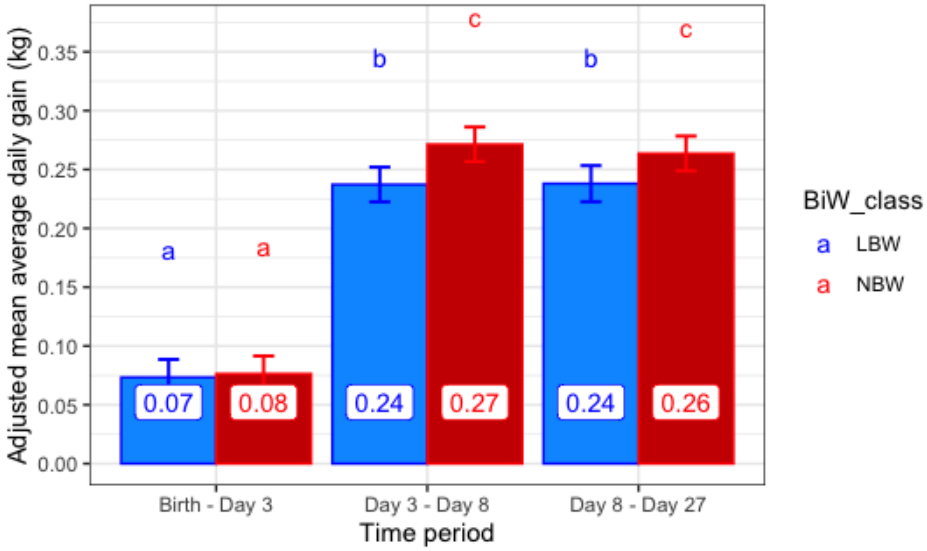
fostering group and birth sow parity, respectively (P > 0.05). However, there was a significant interaction between cross-fostering group and birth sow parity (P < 0.001), with primiparous progeny which were not cross-fostered having significantly lower LWs across all piglet ages than multiparous progeny which were not cross-fostered. There were no other significant interactions between fixed effects.

Similarly to the LW analysis, there was a significant increase in ADG between successive time periods (P < 0.001). In relation to the overall pre-weaning ADG (birth – day 27), LBW piglets grew significantly slower than NBW piglets (P < 0.001; 0.22 kg/d + 0.005 SE vs 0.25 kg/d + 0.005 SE, respectively). Moreover, there was a significant interaction between time point and BiW class (P < 0.001); LBW piglets had significantly lower ADG between day 3 – 8 and day 8 - 27 compared with NBW piglets (**figure 4.2b**). There was a significant effect of birth sow parity (P < 0.05), with multiparous progeny having a faster ADG (0.20 kg/d $\pm$ 0.004 SE) than primiparous progeny (0.19 kg/d $\pm$ 0.04 SE). There was also a significant interaction between cross-fostering group and birth sow parity (P < 0.001), with primiparous progeny which were not cross-fostered and multiparous progeny which were cross-fostered having a significantly lower ADG across all time points compared with multiparous progeny which were not cross-fostered (**figure 4.2c**), but there was no interaction with a specific time point (**figure 4.2d**). Furthermore, there was a significant interaction between time point, BiW class, cross-fostering group, and birth sow parity (P < 0.05; **table 4.2**) Primiparous LBW progeny which were not cross-fostered had a significantly lower ADG between days 8 – 27 (0.22 kg/d $\pm$ 0.011 SE) compared with multiparous NBW progeny which were not cross-fostered (0.29 kg/d $\pm$ 0.011 SE).

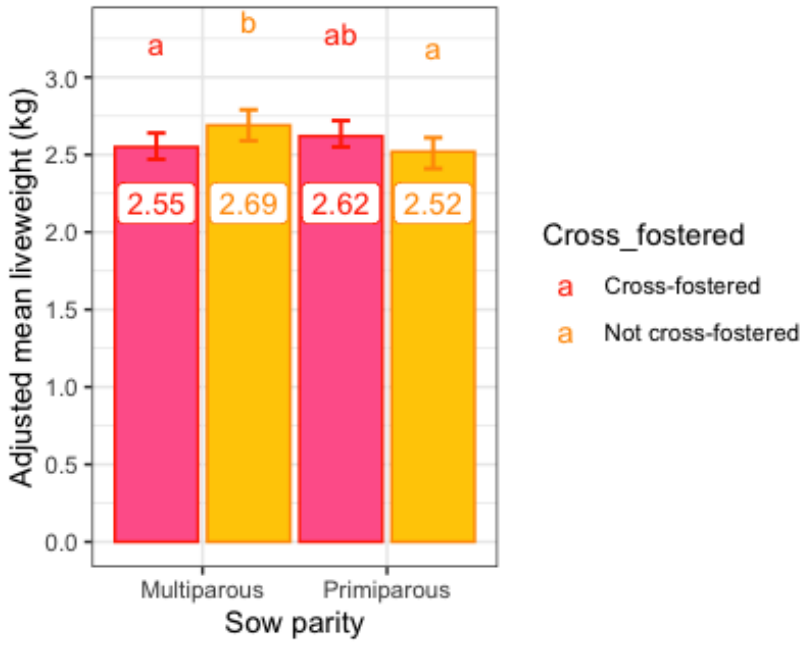
a)



b)


Adjusted mean average daily gain (kg)
0.35
0.30
0.25
0.20
0.15
0.10
0.05
0.00
a
a
b
c
b
c
0.07
0.08
0.24
0.27
0.24
0.26
Birth - Day 3
Day 3 - Day 8
Day 8 - Day 27
Time period
BiW_class
a LBW
a NBW

c)


Adjusted mean liveweight (kg)
3.0
2.5
2.0
1.5
1.0
0.5
0.0
a
b
ab
a
2.55
2.69
2.62
2.52
Multiparous
Primiparous
Sow parity
Cross_fostered
a Cross-fostered
a Not cross-fostered

d)

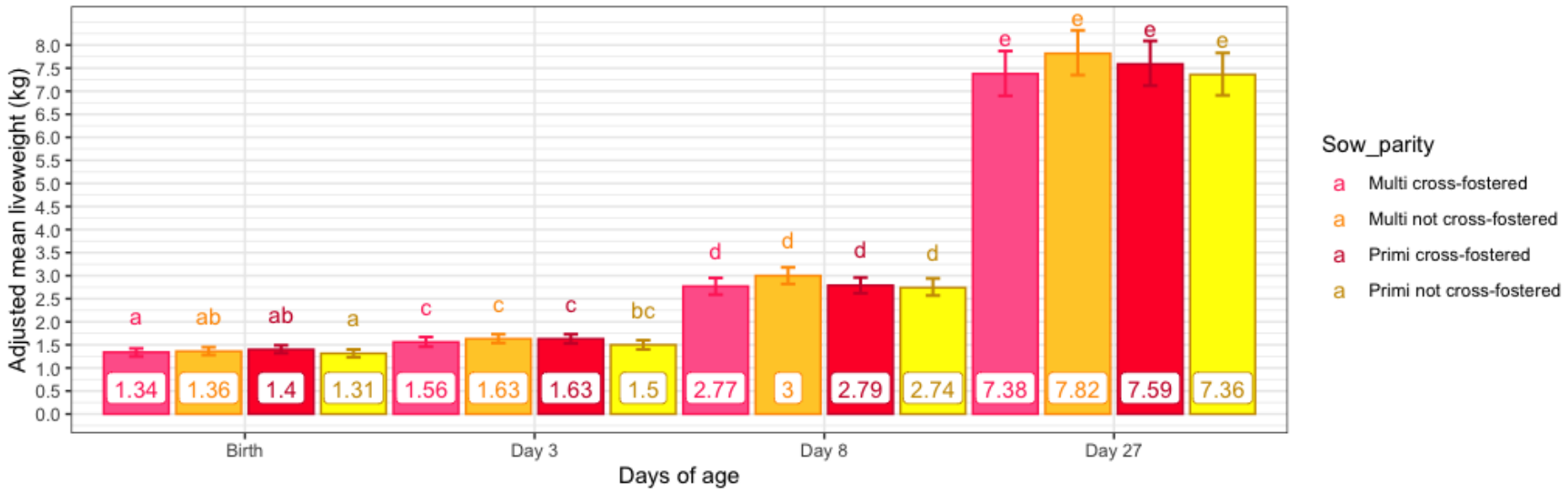


Figure 4.2. Changes in mean liveweight (a) and average daily gain (b) according to piglet birthweight class (BiW class; LBW = low birthweight (0.80 – 1.25 kg), NBW = normal birthweight (1.5 – 2.00 kg)). Changes in pre-weaning average daily gain birth – day 27 (c) and liveweight at each time point (d) between multiparous and primiparous progeny according to whether they have been cross-fostered (c). Adjusted means and confidence intervals are presented. Different superscript letters (a – h) denote significant difference between groups ($P < 0.05$).

Table 4.2. The adjusted mean average daily gain (ADG; kg) and standard error (SE) for the interaction between time-period, birthweight class (low = 0.80 – 1.25kg; normal = 1.50-1.25kg), cross-fostering group and birth sow parity. ADG values with a different superscript (a-e) demonstrate a significant difference in ADG.

Time-period	Birthweight class	Birth sow parity	Cross-fostered	ADG	SE
Birth - day 3	Low	Primiparous	Not cross-fostered	0.055^{a}	0.0110
			Cross-fostered	0.069^{a}	0.0100
		Multiparous	Not cross-fostered	0.091^{a}	0.0099
			Cross-fostered	0.079^{a}	0.0130
	Normal	Primiparous	Not cross-fostered	0.067^{a}	0.0108
			Cross-fostered	0.074^{a}	0.0120
		Multiparous	Not cross-fostered	0.087^{a}	0.0104
			Cross-fostered	0.077^{a}	0.0097
Day 3 - day 8	Low	Primiparous	Not cross-fostered	0.234^{bc}	0.0108
			Cross-fostered	0.229^{b}	0.0099
		Multiparous	Not cross-fostered	0.238^{bcd}	0.0103
			Cross-fostered	0.247^{bcde}	0.0130
	Normal	Primiparous	Not cross-fostered	0.261^{bcde}	0.0110
			Cross-fostered	0.287^{cde}	0.0124
		Multiparous	Not cross-fostered	0.300^{e}	0.0105
			Cross-fostered	0.236^{bc}	0.0102
Day 8 - day 27	Low	Primiparous	Not cross-fostered	0.221^{b}	0.0112
			Cross-fostered	0.246^{bcd}	0.0103
		Multiparous	Not cross-fostered	0.247^{bcde}	0.0120
			Cross-fostered	0.238^{bcd}	0.0134
	Normal	Primiparous	Not cross-fostered	0.253^{bcde}	0.0110
			Cross-fostered	0.261^{bcde}	0.0124
		Multiparous	Not cross-fostered	0.291^{de}	0.0105
			Cross-fostered	0.250^{bcde}	0.0106

4.3.2 Alpha diversity

The number of observed OTUs and Shannon diversity index significantly increased over the neonatal period ($P < 0.001$ for both models) (**figure 4.3a, b**). Whilst there were no other significant fixed effects on the number of observed OTUs ($P > 0.05$), multiparous progeny (3.78 $\pm$ 0.080 SE) had a significantly higher ($P < 0.05$) Shannon diversity index than primiparous progeny (3.53 $\pm$ 0.082 SE). There were no significant interactions between fixed effects for the number of observed OTUs or Shannon diversity index ($P > 0.05$).

a) b)

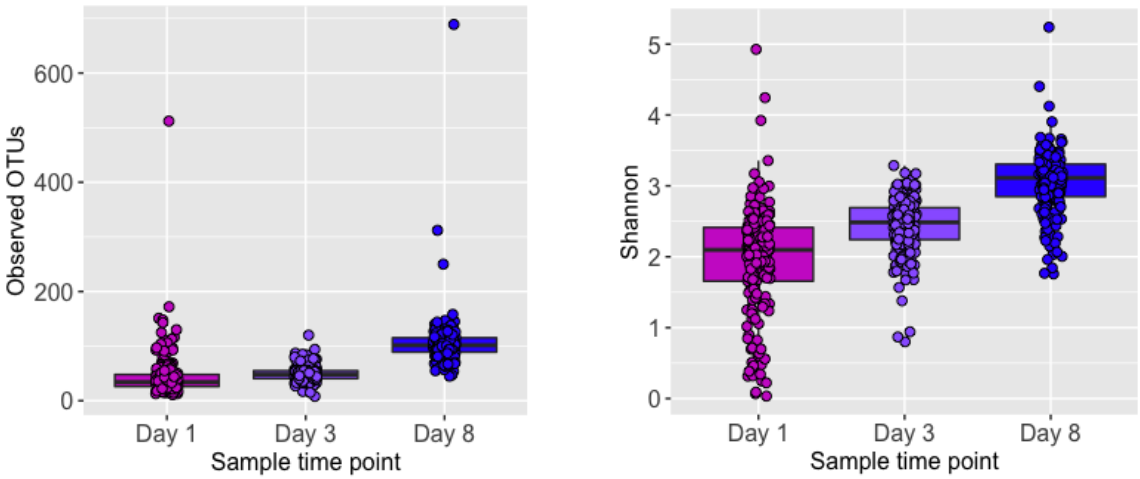
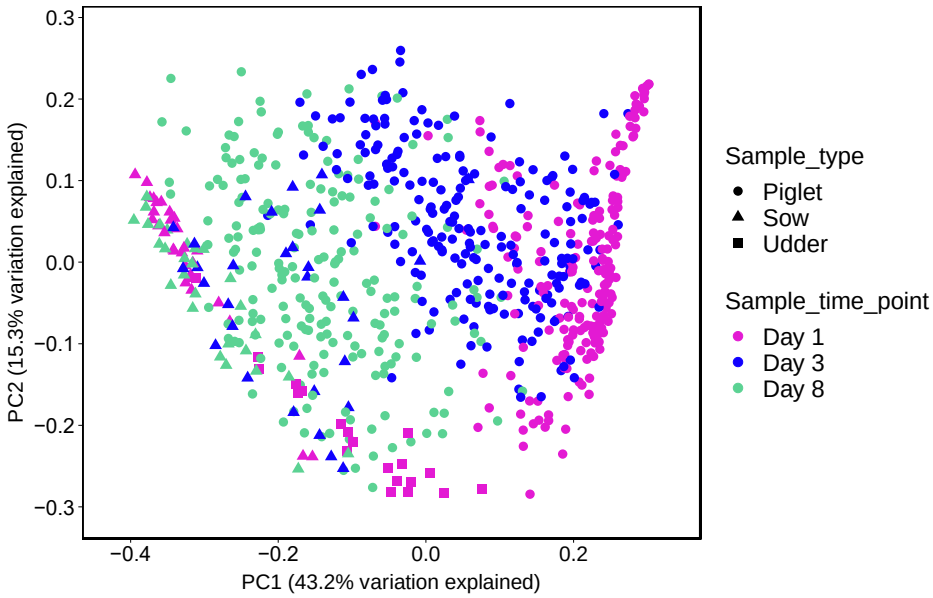


Figure 4.3. The number of observed OTUs (a) and Shannon diversity index (b) according to piglet age.

4.3.3 Beta diversity

All results are displayed as principal coordinate analysis plots. There was a significant effect of time point on the microbiota community composition of both sow and piglet faeces ($P < 0.001$; **figure 4.4a**). In comparison to the piglet samples, the community composition of sow faeces was less impacted by time point as demonstrated by the general overlap of sow microbiota samples regardless of day. However, on day 3 the distances between sow samples were greater, demonstrating reduced similarity between sow samples on day 3 compared with between day 1 and day 8 faecal samples (**figure 4.4b**).

a)



b)

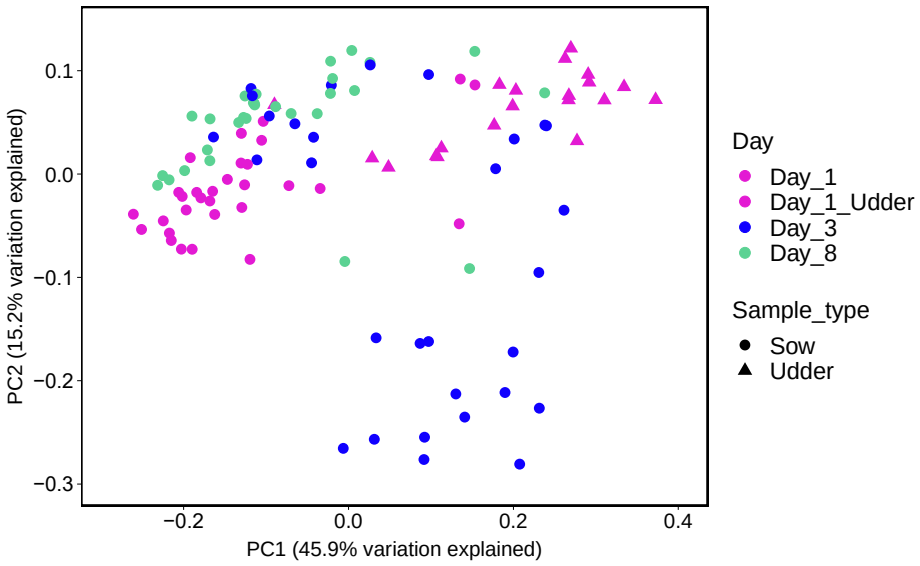


Figure 4.4. Principal coordinate analysis (PCoA) plot of the weighted UniFrac distances between piglet and sow faeces on day 1, 3 and 8, and sow udder samples on day 1 (a) and sow only samples (b). Each point represents an individual sample, points more closely clustered together indicate a more similar microbiota community composition.

On day 1, there was no significant effect of BiW class or birth sow parity, and no significant interaction ($P > 0.05$) in relation to weighted UniFrac distances. There was also no significant fixed effect of BiW in relation to unweighted UniFrac distances ($P > 0.05$), although there was a significant effect of birth sow parity ($P < 0.01$) (**figure 4.5**). However, there was no significant interaction between BiW class and birth sow parity on unweighted UniFrac distances.

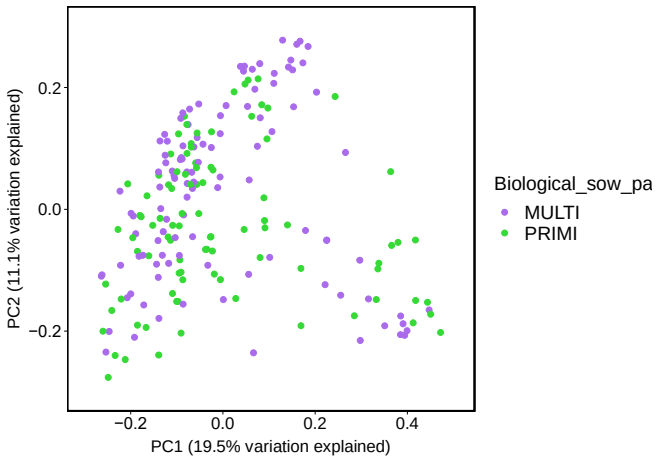


Figure 4.5. Unweighted UniFrac distances between multiparous and primiparous progeny on day 1.

On day 3 there was no significant fixed effect of BiW class or cross-fostering (P > 0.05), although there was a significant effect of birth sow parity (P < 0.01; **figure 4.6a**) for weighted UniFrac distances. There was also a significant interaction between BiW class and cross-fostering (P < 0.05) for weighted UniFrac distances, attributed to differences in the community composition between NBW cross-fostered and not-cross-fostered piglets, whilst no differences between LBW cross-fostering groups were observed. No further interactions between fixed effects were reported (P > 0.05). However, there were significant differences in the microbiota community composition between BiW classes (P < 0.01; **figure 4.6b**), birth sow parity (P < 0.01; **figure 4.6c)** and in response to cross-fostering (P < 0.01; figure **4.6d**) on day 3 based on unweighted UniFrac distances.

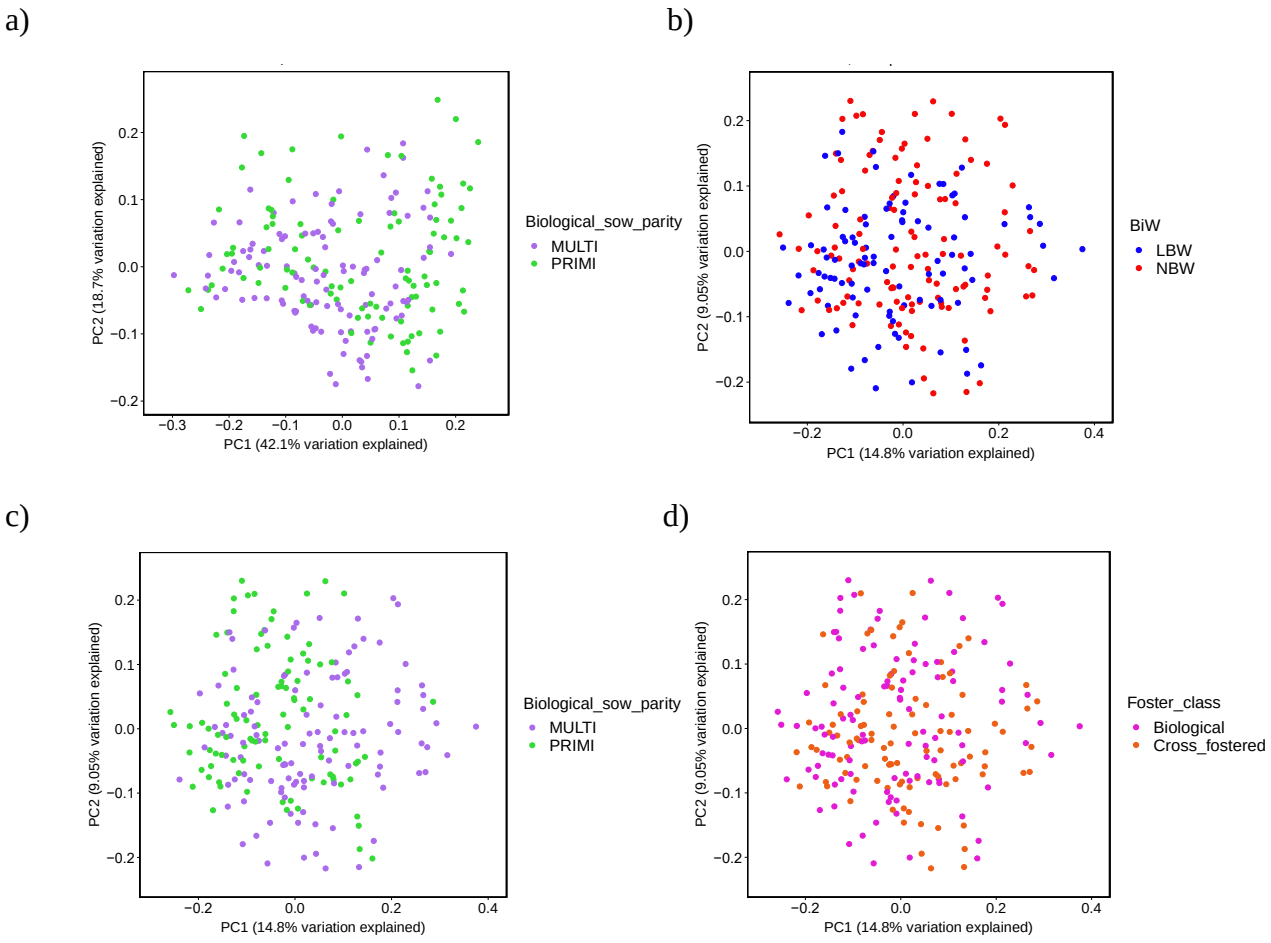


Figure 4.6. Principle coordinates analysis of the weighted UniFrac distances on day 3 between progeny from multiparous (MULTI) and primiparous (PRIMI) sows (a). Principle coordinates analysis of the day 3 unweighted UniFrac between BiW classes (LBW = low birthweight (0.80 – 1.25kg), NBW = normal birthweight (1.50 – 2.00kg)) (b), between multiparous and primiparous progeny irrespective of cross-fostering (c) and between piglets

that had been cross-fostered (Cross_fostered) and those who has not (Biological) irrespective of birth sow parity (d).

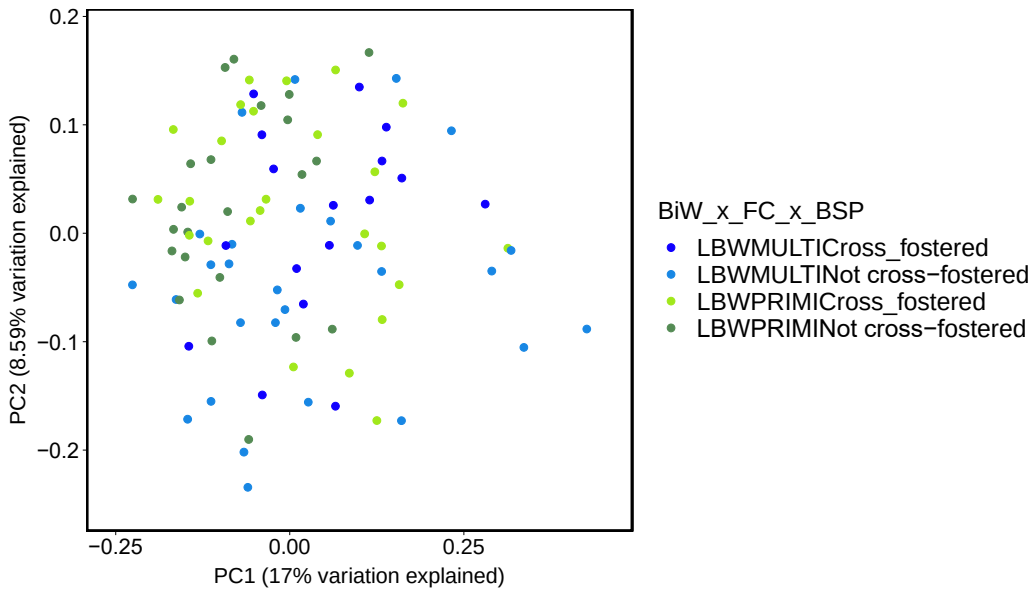
On day 3 there was also a significant interaction between BiW class and cross-fostering group ($P < 0.01$), with unweighted UniFrac distances again significantly different between NBW cross-fostered piglets and those which were not cross-fostered, while no differences between LBW cross-fostering groups were seen. Furthermore, there was a significant interaction between BiW class, birth sow parity and cross-fostering group ($P < 0.01$; **table 4.2**). Within each BiW and parity class, there were significant differences between cross-fostered piglets reared together ($P < 0.05$), indicating the microbiota composition to be significantly different and to have not converged with litter mates on D3. However, there was no significant difference in unweighted UniFrac distances between LBW piglets from multiparous sows which had not been cross-fostered compared with LBW piglets from primiparous sows which had been cross-fostered ($P > 0.05$). Suggesting that for LBW from primiparous sows progeny the rearing sow parity alters the community composition on day 3, although the reverse was not true for LBW progeny from multiparous sows, whereby the unweighted UniFrac distances were significantly different ($P < 0.05$; **table 4.2**). However, based on a PCoA plot of the unweighted UniFrac distances, each BiW class seems to cluster more closely based on birth sow parity, irrespective of cross-fostering status (**figure 4.7a, b**).

Table 4.3. Day 3 pairwise comparisons for three-way interaction between birthweight class (LBW = low birthweight class (0.80 – 1.25kg, NBW = normal birthweight 1.50 – 2.00kg), birth sow parity and cross-fostering group in relation to unweighted UniFrac distances. P values for pairwise comparisons were false discovery rate (FDR) adjusted for multiplicity.

Interactive term	Comparison interactive term	FDR adjusted P value
LBW multiparous cross-fostered	LBW multiparous not-cross-fostered	0.049
	LBW primiparous cross-fostered	0.007
	LBW primiparous not-cross-fostered	**0.003***
	NBW multiparous cross-fostered	0.049
	NBW multiparous not-cross-fostered	0.128
	NBW primiparous cross-fostered	0.033
	NBW primiparous not-cross-fostered	0.003
LBW multiparous not-cross-fostered	LBW primiparous cross-fostered	**0.114**
	LBW primiparous not-cross-fostered	0.003
	NBW multiparous cross-fostered	0.389
	NBW multiparous not-cross-fostered	0.035
	NBW primiparous cross-fostered	0.046
	NBW primiparous not-cross-fostered	0.003
LBW primiparous cross-fostered	LBW primiparous not-cross-fostered	0.021
	NBW multiparous cross-fostered	0.035
	NBW multiparous not-cross-fostered	0.003
	NBW primiparous cross-fostered	0.055
	NBW primiparous not-cross-fostered	0.005
LBW primiparous not-cross-fostered	NBW multiparous cross-fostered	0.003
	NBW multiparous not-cross-fostered	0.003
	NBW primiparous cross-fostered	0.050
	NBW primiparous not-cross-fostered	0.003
NBW multiparous cross-fostered	NBW multiparous not-cross-fostered	0.015
	NBW primiparous cross-fostered	0.035
	NBW primiparous not-cross-fostered	**0.003**
NBW multiparous not-cross-fostered	NBW primiparous cross-fostered	**0.016**
	NBW primiparous not-cross-fostered	0.005
NBW primiparous cross-fostered	NBW primiparous not-cross-fostered	0.058

* P-values in bold and underlined interactive terms are those values discussed in the results

a)



b)

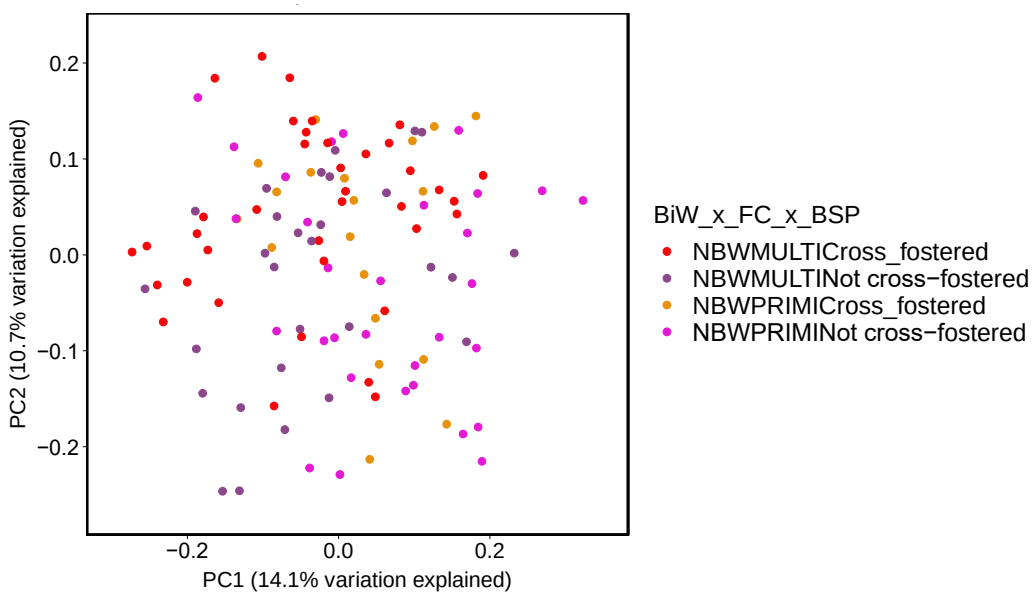


Figure 4.7. The unweighted UniFrac distances displaying the interaction between birthweight class (BiW), birth sow parity (BSP) and cross-fostering group (XF) on day 3. For ease of interpretation, plots are segregated for low birthweight piglets (LBW; 0.80 – 1.25kg) (a) and normal birthweight piglets (NBW; 1.50 – 2.00kg) (b). Samples are coloured based on birth sow parity (MULTI = multiparous, PRIMI = primiparous) and cross-fostering group (Cross_fostered or Not cross_fostered).

On day 8, there were no significant fixed effects of BiW class, birth sow parity or cross-fostering group ($P > 0.05$), with respect to weighted UniFrac distances. There was also no significant interaction between any fixed effects ($P < 0.05$). However, there was a significant difference in the community composition in relation to BiW class ($P < 0.05$) and birth sow

parity (P < 0.05) associated with unweighted UniFrac distances (**figure 4.8a, b**), although there was no significant effect of cross-fostering group (P > 0.05).

a) b)

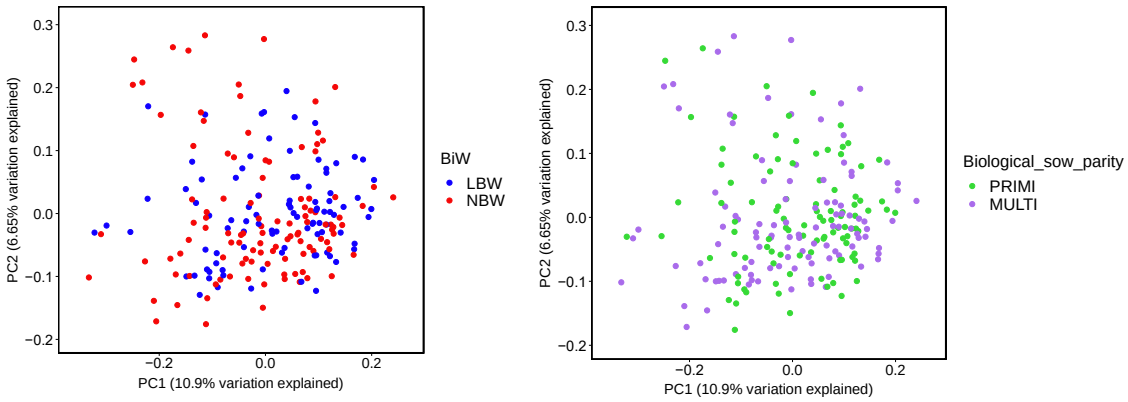


Figure 4.8. Unweighted UniFrac distances on day 8 between BiW classes (a) (LBW = low birthweight (0.80 – 1.25kg), NBW = normal birthweight (1.50 – 2.00kg)) and birth sow parity (b) (MULTI = multiparous, PRIMI = primiparous).

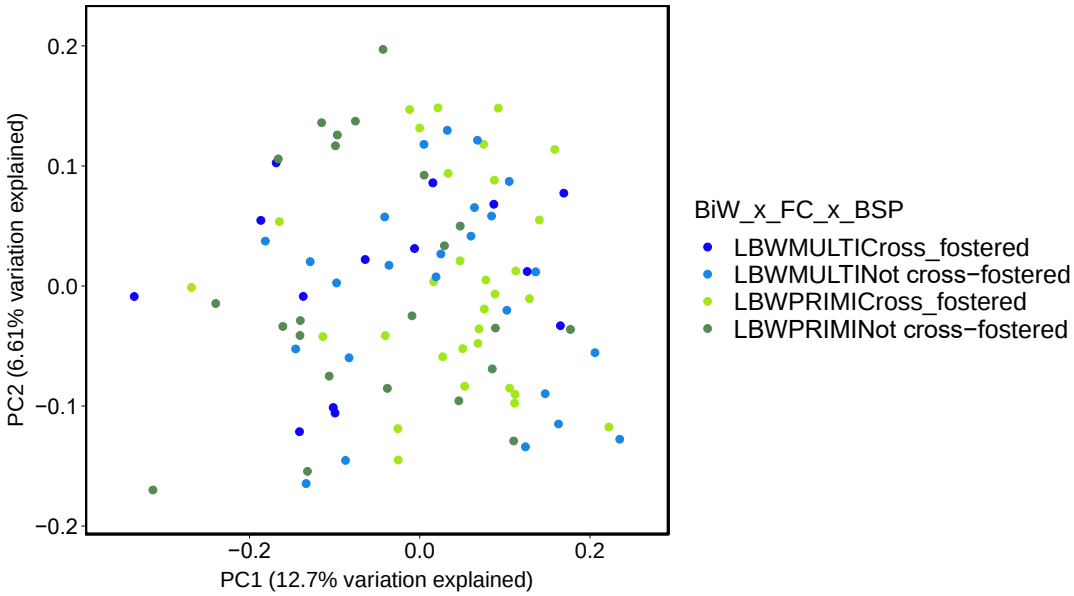
Moreover, there was a significant interaction between BiW class, birth sow parity and cross-fostering group (P < 0.01) for unweighted UniFrac distances on day 8 (**table 4.3**). Within each BiW class, there was no significant difference (P > 0.05) in unweighted UniFrac distances between progeny from multiparous sows which had been cross-fostered and progeny from primiparous sows which had not been cross-fostered, or between piglets from multiparous sow which had not been cross-fostered and piglets from primiparous sow which had been cross-fostered, suggesting microbiota convergency between piglets reared together. In comparison, there was a significant difference (P < 0.05) for each BiW class, respectively, between primiparous cross-fostered and not-cross-fostered progeny and between multiparous cross-fostered and not-cross-fostered progeny. Therefore, suggesting the action of cross-fostering induced significant changes in the microbiota community composition at 8 days of age, whereby the microbiota community composition between non-siblings reared together appears to converge. Therefore, when plotted as a PCoA, within each BiW class, piglets cluster according to rearing sow parity (**figure 4.9a, b**).

Table 4.4. Day 8 pairwise comparisons for three-way interaction between birthweight class (LBW = low birthweight class (0.80 – 1.25kg, NBW = normal birthweight 1.50 – 2.00kg), birth sow parity and cross-fostering group in relation to unweighted UniFrac distances. P values for pairwise comparisons were false discovery rate (FDR) adjusted for multiplicity.

Interactive term	Comparison interactive term	FDR adjusted P value
LBW multiparous cross-fostered	LBW multiparous not-cross-fostered	**0.059***
	LBW primiparous cross-fostered	0.020
	LBW primiparous not-cross-fostered	**0.706**
	NBW multiparous cross-fostered	0.044
	NBW multiparous not-cross-fostered	0.364
	NBW primiparous cross-fostered	0.266
	NBW primiparous not-cross-fostered	0.034
LBW multiparous not-cross-fostered	LBW primiparous cross-fostered	**0.793**
	LBW primiparous not-cross-fostered	0.034
	NBW multiparous cross-fostered	0.082
	NBW multiparous not-cross-fostered	0.044
	NBW primiparous cross-fostered	0.034
	NBW primiparous not-cross-fostered	0.028
LBW primiparous cross-fostered	LBW primiparous not-cross-fostered	**0.020**
	NBW multiparous cross-fostered	0.019
	NBW multiparous not-cross-fostered	0.019
	NBW primiparous cross-fostered	0.019
	NBW primiparous not-cross-fostered	0.034
LBW primiparous not-cross-fostered	NBW multiparous cross-fostered	0.020
	NBW multiparous not-cross-fostered	0.292
	NBW primiparous cross-fostered	0.460
	NBW primiparous not-cross-fostered	0.040
NBW multiparous cross-fostered	NBW multiparous not-cross-fostered	**0.034**
	NBW primiparous cross-fostered	0.059
	NBW primiparous not-cross-fostered	**0.161**
NBW multiparous not-cross-fostered	NBW primiparous cross-fostered	**0.328**
	NBW primiparous not-cross-fostered	0.200
NBW primiparous cross-fostered	NBW primiparous not-cross-fostered	**0.044**

* P-values in bold and underlined interactive terms are those values discussed in the results

a)



b)

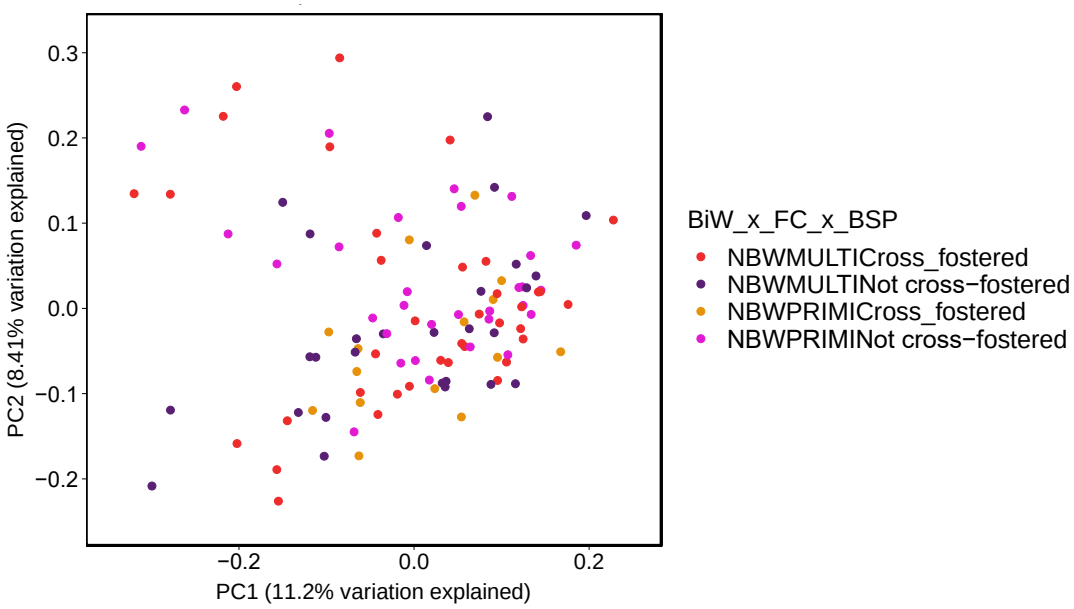


Figure 4.9. Unweighted UniFrac distances demonstrating the interaction between birthweight class (BiW), birth sow parity (BSP) and cross-fostering group (XF) on day 8. For ease of interpretation, plots are segregated for low birthweight piglets (LBW; 0.80 – 1.25kg) (a) and normal birthweight piglets (NBW; 1.50 – 2.00kg) (b). Samples are coloured based on birth sow parity (MULTI = multiparous, PRIMI = primiparous) and cross-fostering group (Cross_fostered or Not cross-fostered).

4.3.4 The influence of the sow on the development of the piglet microbiota and the effect of cross-fostering

The piglet faecal microbiota on day 1 were more similar to sow udder microbiota than sow faeces (P value < 0.001) with lower weighted UniFrac distances, (**figure 4.10**).

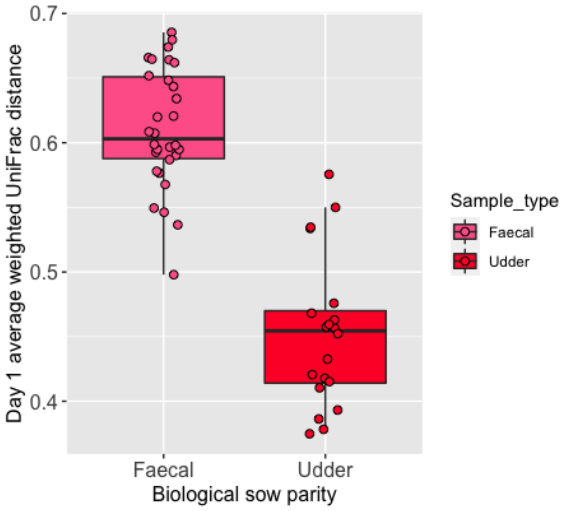


Figure 4.10. Weighted UniFrac distances between day 1 piglet faeces and day 1 birth sow faeces or udder microbiota community composition.

For piglets who were not cross-fostered, longitudinal analysis of time-matched sow – piglet sample weighted UniFrac distances ('Biological sow piglet' distances) revealed there was a significant effect of time (P < 0.001). Day 1 piglet – sow faeces distances were significantly greater than day 1 piglet faeces – sow udder, day 3 piglet – sow faeces and day 8 piglet – sow faeces distances, demonstrating lower similarity between day 1 piglet – sow faecal microbiota communities (**figure 4.11**).

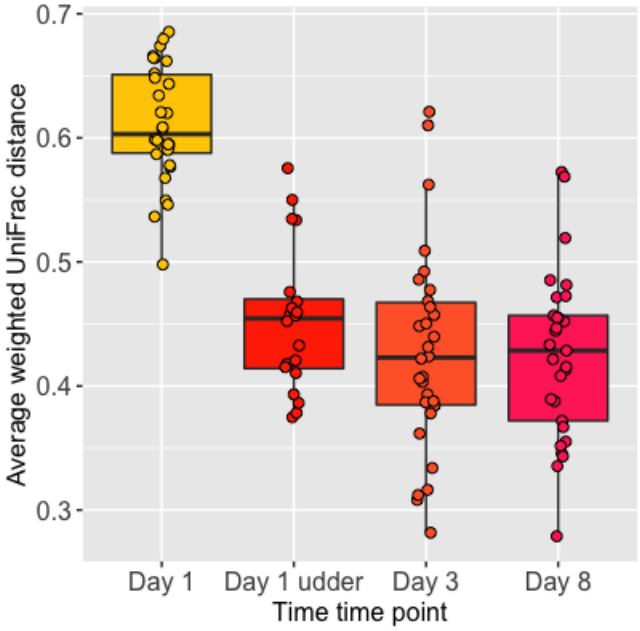


Figure 4.11. Weighted UniFrac distances between time-matched piglet faecal samples and sow faecal samples, in addition to day one piglet faeces – sow udder distances. All distances relate to 'biological' piglets who remained with their birth sow throughout the experiment.

The weighted UniFrac distances between cross-fostered piglets and sow faeces ('Cross-fostered sow piglet' distances and 'Cross-fostered birth sow piglet' distances) were not significantly affected by time point (day 3 or day 8) ($P > 0.05$). Moreover, weighted UniFrac distances were not significantly different ($P > 0.05$) between cross-fostered piglets and either their birth sow ('Cross-fostered birth sow piglet' distances) or their foster sow ('Cross-fostered sow piglet' distances) using time-matched sow faecal samples. Furthermore, there were no significant interactions between time point and cross-fostered piglets' faeces – birth or rearing sow faeces distances ($P > 0.05$) (**figure 4.12**). Therefore, cross-fostered piglets did not develop a faecal microbiota more like their rearing sow than their birth sow over time.

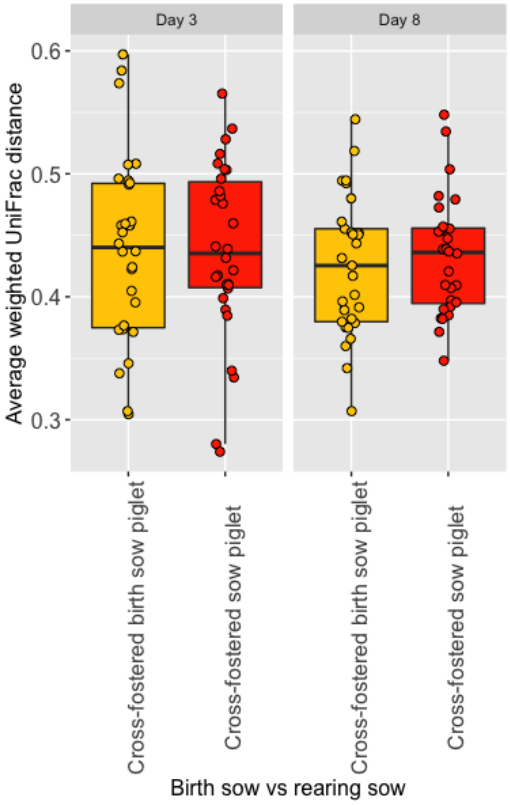


Figure 4.12. Weighted UniFrac distances on days 3 and 8 of age between time matched cross-fostered piglet faeces and birth sow faeces (Cross-fostered birth sow piglet) or rearing sow faeces (Cross-fostered sow piglet).

The final sow – piglet weighted UniFrac distance analysis combined the day 1 piglet – birth sow udder and faeces distances with the time-matched piglet and sow (birth and rearing sow for cross-fostered piglets) weighted UniFrac distances, as well as day 3 and day 8 piglet vs day 1 birth sow udder and faeces distances, thus combining all the sow - piglet distance categories described in **table 4.1**. There was a significant effect of distance category (P value < 0.001). Day 1 piglet and sow faecal samples had the most dissimilar microbiota community composition ('Day 1 Birth sow faeces farrowing'), as demonstrated by the greatest weighted UniFrac distances between piglet and sow microbiota samples. Day 1 birth sow vs day 3 piglet weighted UniFrac distances (biological 'Day 1 vs Day 3 Birth sow faeces farrowing' and cross-fostered 'Day 1 vs Day 3 Cross-fostered birth sow faeces farrowing') were significantly lower than day 1 piglet – birth sow faeces distances ('Day 1 Birth sow faeces farrowing'), but significantly greater than all other distance comparisons. There were no significant differences between remaining weighted UniFrac distances. Therefore, piglets shared the greatest community composition similarity with time-matched day 1 birth sow udder sample, time-matched day 3 and day 8 sow faecal samples (birth and rearing sow faecal samples for cross-fostered piglets) and day 1 birth sow udder samples on day 3 and day 8

(**Figure 4.13)**. Thus, the udder microbiota community composition at farrowing is important for seeding the piglets at birth and shaping the neonatal microbiota, whilst time-matched sow faecal samples become more important for shaping the microbiota community composition from day 3.

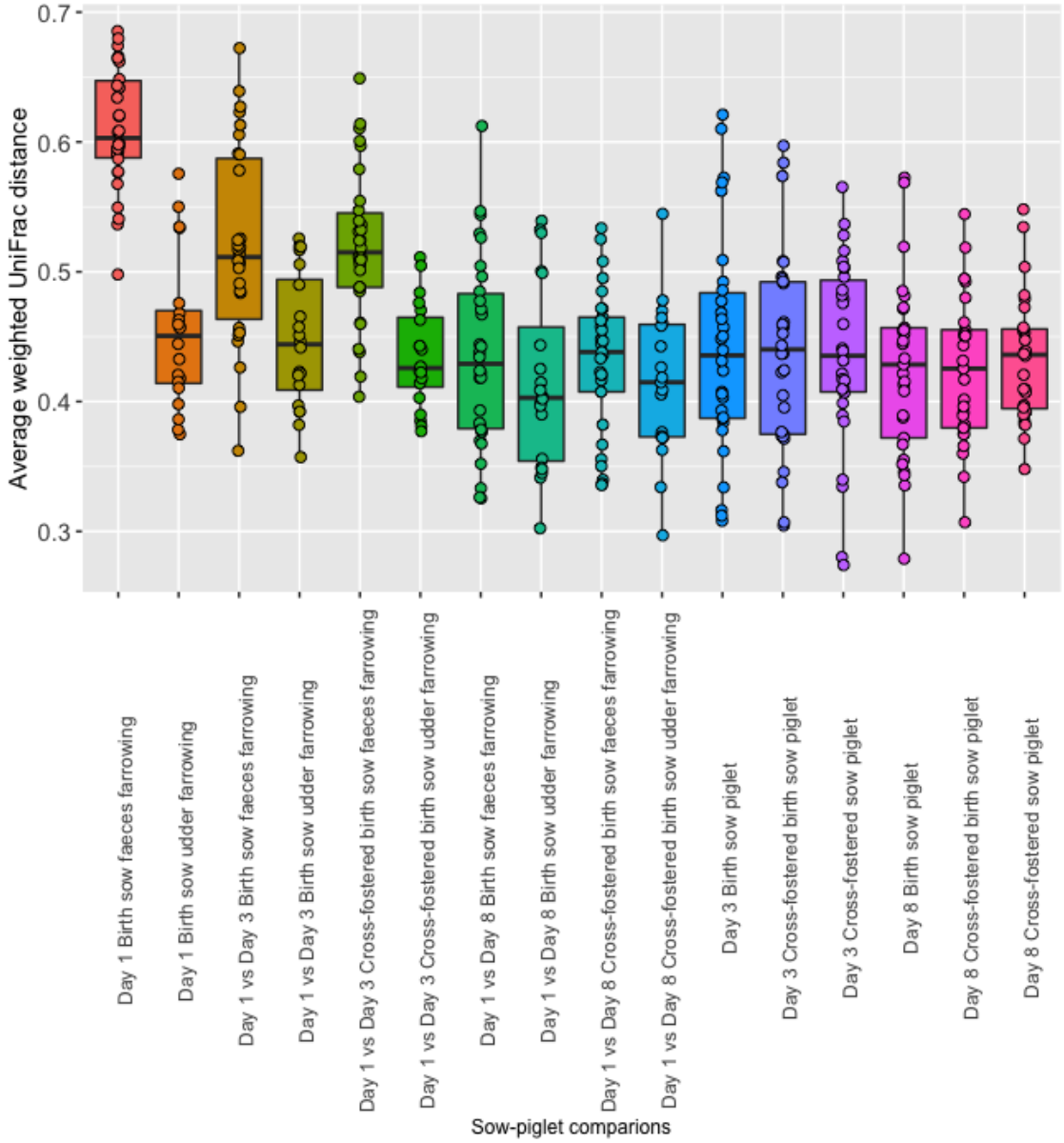


Figure 4.13. Weighted UniFrac distances between biological and cross-fostered piglets and their birth and rearing sow faeces (cross-fostered piglets only) from day 1 to day 8 using time-matched samples, as well as between time-matched piglet and birth sow udder samples on day 1, and between day 3 and day 8 piglet samples to their day 1 birth sow udder or faecal samples. When only one day is specified on the x axis this indicates time matched samples, whilst 'Day 1 vs Day 3/8' indicates a day 1 sow faecal or udder sample compared to a day 3 or day 8 piglet faecal sample. Meanwhile, 'sow' stated on its own denotes the rearing sow

comparison for cross-fostered piglet comparisons, whilst 'birth sow' indicates comparisons to original birth mother microbiota samples.

4.3.5 Cross-fostering effects on the piglet microbiota community composition

Piglets were classified into 4 groups ('Biological', 'Cross-fostered', Cross-fostered siblings' and 'Cross-fostered non-siblings') and weighted UniFrac distances calculated for each cross-fostering group on day 1, day 3 and day 8. There was a significant effect of cross-fostering group on day 1 ($P = 0.05$) and day 3 ($P < 0.001$), with 'Cross-fostered non-siblings' having significantly larger weighted UniFrac distances than the other cross-fostering groups, thus a more different microbiota between non-siblings reared together. However, there was no significant effect of cross-fostering group on day 8 ($P > 0.05$) (**table 4.4**). Therefore, a litter specific piglet microbiota exists on day 1 and is retained after cross-fostering on day 3. However, by day 8 this litter specific microbiota was lost, with the microbiota community composition of cross-fostered non-siblings equally as similar as that between siblings.

Table 4.5. Cross-sectional weighted UniFrac distances for each cross-fostering group on days 1, 3 and 8 of life. Values are presented as adjusted means $\pm$ SE.

Time point	Cross-fostering group				P value
	Biological	Cross-fostered	Cross-fostered siblings	Cross-fostered non-siblings	
Day 1	$0.199^{a} \pm 0.0165$	$0.229^{a} \pm 0.0169$	$0.231^{a} \pm 0.0163$	$0.249^{b} \pm 0.0163$	0.05
Day 3	$0.216^{a} \pm 0.0091$	$0.213^{a} \pm 0.0094$	$0.225^{a} \pm 0.0091$	$0.258^{b} \pm 0.0091$	< 0.001
Day 8	0.296 ± 0.0112	0.284 ± 0.0118	0.305 ± 0.0114	0.279 ± 0.0114	> 0.05

[a,b]Different superscripts denote significant differences between cross-fostering groups.

Longitudinally, there was a significant effect of time point ($P < 0.001$), with overall piglet weighted UniFrac distances significantly higher on day 8 (0.292 ± 0.0072 SE) compared with day 1 and day 3 (0.227 ± 0.0072 SE and 0.227 ± 0.0071 SE, respectively). Across all time points, there was only a trend for cross-fostering group ($P = 0.053$), associated with lower weighted UniFrac distances for 'Biological' vs 'Cross-fostered non-siblings' groups. Furthermore, there was no significant interaction between time point and cross-fostering group ($P > 0.05$), with only a trend for lower weighted UniFrac distances between 'Biological' vs 'Cross-fostered non-siblings' on day 1 ($P = 0.091$). Thus, the similarity of the microbiota community composition between piglets, irrespective of cross-fostering, is reduced on day 8, as illustrated by higher weighted UniFrac distances.

4.3.6 Taxonomic origins of the piglet microbiota from maternal sources

Based on the results of the SourceTracker analysis (**Figure 4.14**), the microbiota of piglets on day 1 originated 4.5% from the udder and 0.1% from sow faeces, with the remaining 95.4% from unknown sources. On day 3, 1.2% of the piglet faecal microbiota originated from day 3 sow faeces, whilst 0.1% originated from sow faeces on day 1 and a further 1.1% from the sow udder on day 1, with the remaining 97.6% originating from unknown sources within the neonatal environment. The percentage of the day 8 piglet faecal microbiota originating from the sow faeces on day 1 increased to 1.2%, whilst the percentage originating from the day 1 sow udder and day 8 sow faeces remained stable at 1.3% and 1.2%, respectively. Similarly to day 3, the remaining 96.3% of the microbiota on day 8 originated from unknown sources.

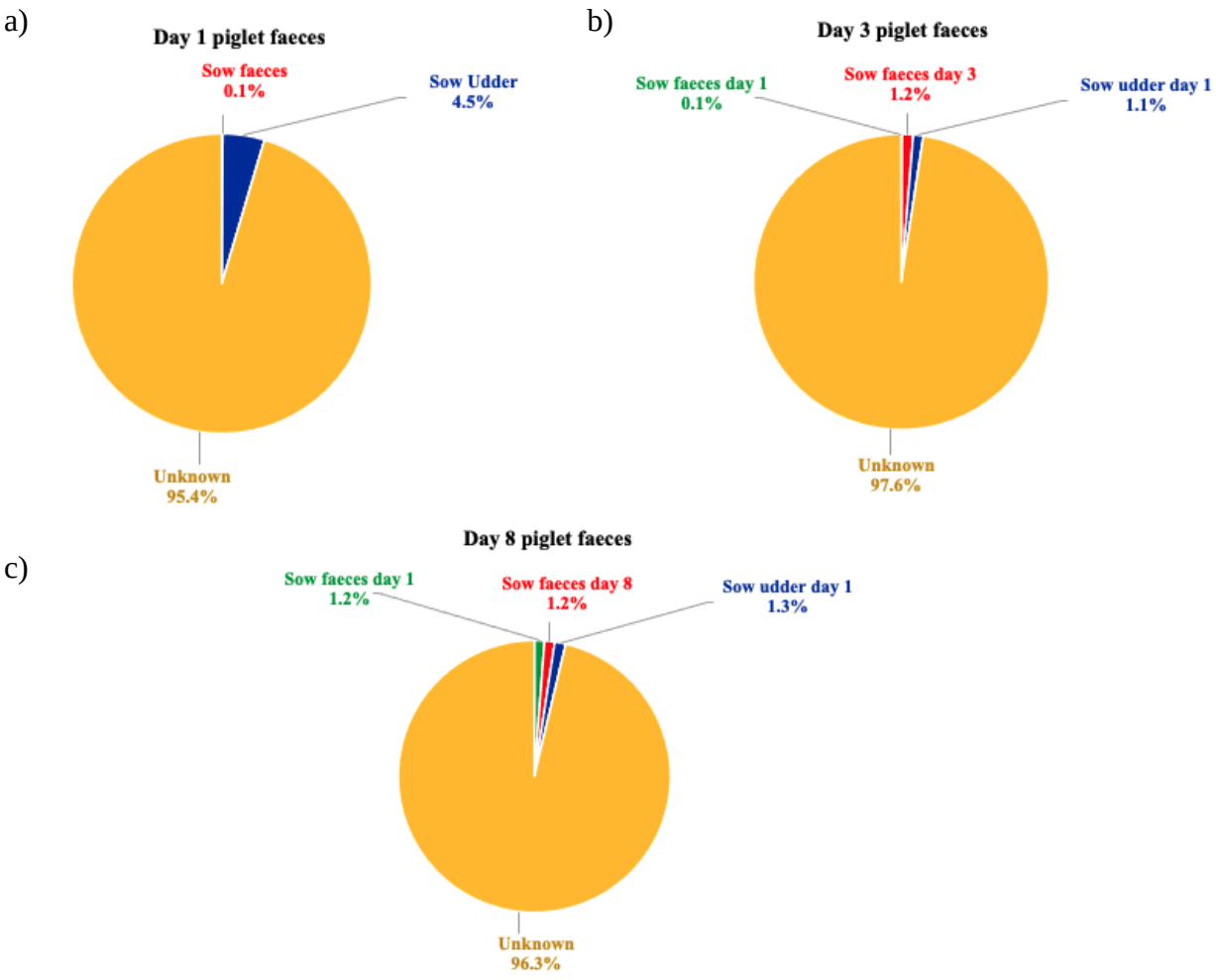


Figure 4.14. The proportion of the neonatal piglet microbiota on days 1 (a), 3 (b) and 8 (c) of life which originate from the sow faeces or areolar skin (at farrowing) or from time-matched sow faeces, based on the SourceTracker results.

4.4 Discussion

The study hypothesised that during the immediate neonatal period the piglet faecal microbiota would more closely resemble the sow udder than faeces, but would become more similar to

sow faeces over time; the study results support this hypothesis. The study also hypothesised that a litter specific microbiota profile would exist during the neonatal period; this was observed on day 1 and 3 but diminished by day 8 as the microbiota community composition of non-siblings converged. The study further hypothesised that cross-fostered piglets would exhibit increased microbiota diversity; this hypothesis was rejected. Moreover, the study hypothesised that progeny from multiparous sows would have a more diverse microbiota as a result of multiparous sows producing faeces of higher microbiota diversity during the periparturient period, as shown in Chapter 3, and this hypothesis was accepted to some extent, with higher Shannon diversity indices reported for progeny from multiparous sows.

4.4.1 The effect of BiW class, birth sow parity and cross-fostering on pre-weaning piglet performance

Low birthweight piglets had significantly lower LW on days 3, 8 and 27 of age, and lower ADG between day 3 – day 8 and day 8 – day 27 and pre-weaning. The negative effects of LBW on pre-weaning performance reported in this study are consistent with literature findings (Douglas *et al.*, 2014a; Huting *et al.*, 2017, 2018, 2019; Gaukroger *et al.*, 2020). The poorer pre-weaning performance associated with LBW piglets has been discussed in detail in Chapter 1 and Chapter 2.

Progeny from primiparous sows which were not cross-fostered had lower LWs across all experimental time points compared with multiparous progeny which were not cross-fostered. Progeny from primiparous sows also had significantly lower ADG across all ADG time periods. Furthermore, there was a significant interaction between birth sow parity and cross-fostering group, progeny from primiparous sows which were not cross-fostered and progeny from multiparous sows which were cross-fostered (and thus nursed by primiparous sows) had significantly lower ADG compared with progeny from multiparous sows which were not cross-fostered across all of the ADG periods and for the pre-weaning (birth - day 27) period as a whole. Lower pre-weaning and weaning weights in primiparous sow progeny have previously been reported by Carney-Hinkle *et al.* (2013) and Craig *et al.* (2017). Primiparous sow progeny have reportedly lower small intestinal weight: length at birth and weaning (Craig *et al.*, 2017, 2019; Cottrell *et al.*, 2017) and increased GIT permeability (FD4) at birth and weaning (Cottrell *et al.*, 2017; Wijesiriwardana *et al.*, 2019), with studies concluding the GIT maturation and barrier function of primiparous sow progeny to be underdeveloped at birth and weaning. Furthermore, Craig *et al.* (2017, 2019) reported primiparous sow progeny had lower serum IgG at 24 hours of age, despite no significant difference in the colostrum IgG

concentration of primiparous and multiparous sows, attributing lower serum IgG concentrations to lower colostrum intake or impaired absorption of IgG by primiparous sow progeny. These combined physiological characteristics of progeny from primiparous sows could explain the poorer pre-weaning performance reported in this study and in the literature.

In the present study, an interaction between cross-fostering and sow parity was found. Amongst piglets which were not cross-fostered, both piglet LW and ADG on day 3 and birth – day 3, respectively, were significantly lower in progeny from primiparous sows than in progeny from multiparous sows. Piglet LW and ADG were significantly higher in multiparous sow progeny which were not cross-fostered than multiparous sow progeny which were cross-fostered and primiparous sow progeny which were not cross-fostered. Similarly, Huting *et al.* (2019) and Ferrari *et al.* (2014) reported lower weaning weights of primiparous sow reared piglets. Furthermore, the ADG of primiparous sow progeny has been previously shown to be significantly increased when fostered onto multiparous sows in early life (Piñeiro *et al.*, 2019). Although numerical increases in ADG were present at all time points in primiparous sow progeny which were cross-fostered compared with primiparous sow progeny which were not cross-fostered, these differences were not significant in the present study. Primiparous sows exhibit higher levels of oxidative stress (Roy *et al.*, 2016), must partition a greater proportion of nutrients to support maternal growth, have a smaller udder size and reduced digestion efficiency (Clowes *et al.*, 1998; Pluske *et al.*, 1998; Zak *et al.*, 1998) compared with multiparous sows. A combination of these factors may explain the reported lower milk yield in primiparous sows (Beyer *et al.*, 2007; Devillers *et al.*, 2007; Dourmad *et al.*, 2012; Hansen *et al.*, 2012; Strathe *et al.*, 2017) and consequently the poorer performance of primiparous sow reared piglets in the present study and the literature.

4.4.2 The effect of BiW class, sow parity and cross-fostering on the neonatal microbiota diversity and community composition of piglets

Alpha diversity (observed OTUs and Shannon diversity index) significantly increased from day 1 to day 8. It is well established that alpha diversity increases with age, especially during the neonatal period (Chen *et al.*, 2018; Liu *et al.*, 2019a; Gaukroger *et al.*, 2020 (Chapter 2)). This pre-weaning increase in microbiota diversity with age is associated with the rapid maturation of the neonatal GIT, increased expression of exploratory and rooting behaviours, coprophagy and the introduction of solid feed. There was no significant difference in the number of observed OTUs or Shannon diversity index between BiW classes, as previously reported in early life (Li *et al.*, 2018; Gaukroger *et al.*, 2020 (Chapter 2)). Longitudinally,

there was no fixed effect of cross-fostering on either the number of observed OTUs or Shannon diversity index. Similarly, Maradiaga *et al.* (2018) observed no difference in the number of observed OTUs in piglet faeces on day 0 and 21 of age associated with cross-fostering, although the study only had a small number of replicates per treatment (n = 8) and sampling at day 0 would not allow sufficient time for any cross-fostering effects to be observed in the faecal microbiota. Whilst Bian *et al.* (2016) also reported no significant effect of cross-fostering on alpha diversity measures, as part of a reciprocal cross-fostering model with two distinct breeds, experimental piglets were cross-fostered before they had suckled any colostrum and thus had not had access to the same level of maternal microbiota sources (milk and udder skin) as the experimental piglets in the present study. Longitudinally, primiparous-born piglets also had a lower Shannon diversity index than multiparous born piglets. This may be due to lower periparturient Shannon diversity index values in primiparous sow faeces subsequently altering the maternal seeding of the piglet GIT (Chapter 3, Gaukroger *et al.*, 2021).

PERMANOVA analysis demonstrated the neonatal microbiota community composition of piglets to progressively develop with age, in agreement with previous research (Chapter 2; Bian *et al.*, 2016; Li *et al.*, 2018). Cross-section PERMANOVA analysis reported the microbiota community composition to be different between LBW and NBW piglets on day 3 and 8 in relation to unweighted UniFrac distances, suggesting that differences between BiW class exist between the low abundant/rare taxa, as differences were not identified with weighted UniFrac distances. Similarly, Zhang *et al.* (2019a) reported significant differences in the jejunum microbiota community composition between LBW and NBW at 7 days of age. Cross-sectionally there was also a significant difference in unweighted UniFrac distances between primiparous and multiparous sow progeny on days 1, 3 and 8. Indicating that predominately lower abundance taxa induce differences in the community composition of neonatal piglets. This may be explained by differences in the GIT maturation of progeny from primiparous sows, altering their ability to digest milk components (Cottrell *et al.*, 2017; Craig *et al.*, 2019) and thus the availability of fermentable milk components for the microbiota. Furthermore, there was a significant interaction between BiW class, birth sow parity and cross-fostering group on days 3 and 8 for unweighted UniFrac distances. Whilst differences in the microbiota community composition existed between NBW and LBW piglets, within each BiW class the community composition of piglets clustered more closely to birth sow parity on day 3. However, on day 8 the microbiota community composition of piglets clustered

according to rearing sow parity, suggesting a rearing litter specific microbiota by 8 days of age.

4.4.3 The influence of the sow and cross-fostering on the development of the piglet microbiota

Piglet faecal weighted UniFrac distances from time-matched sow faecal samples and day 1 sow faecal and udder samples were compared to establish how the microbiota community composition develops over time and which maternal microbiota sources are most important in shaping the neonatal microbiota. The microbiota community composition of day 1 piglet faeces was more closely related to the areolar skin than faeces. Teat searching and suckling are innate behaviours of neonatal piglets, thus interaction with the areolar skin will represent a large proportion of the time when they are not sleeping, and consequently their microbial interactions. Chen *et al.* (2018) reported that the day 1 piglet faecal microbiota community composition was more similar to the floor, sow nipple and sow milk microbiota than sow faeces, in agreement with the results of this study. Furthermore, there was no significant difference in the weighted UniFrac distances between the day 1 sow udder samples and piglet faecal samples at each time point up to day 8, suggesting that the day 1 udder microbiota community composition shared with piglet faeces is retained during the neonatal period.
The significantly larger weighted UniFrac distance between day 1 sow faeces and day 3 piglet faeces compared with day 3 time-matched sow – piglet distance demonstrates the continuous seeding/colonising of the neonatal GIT by maternal microbiota sources, in particular the importance of the sow faeces. The sow faecal microbiota undergoes dysbiosis during the periparturient period, the community composition on day 3 is distinct from sow faeces taken immediately prior to farrowing (Cheng *et al.*, 2018; Chapter 3, Gaukroger *et al.*, 2021). The day 8 sow faecal community composition more closely resembles day 1 sow faeces than day 3 (Chapter 3), possibly reflecting the changed defecation patterns/microbiota dysbiosis immediately post-farrowing, and consequently may explain the lack of significant difference in weighted UniFrac distances between day 1 sow faeces and day 8 piglet faeces compared with day 8 time-matched sow – piglet faeces. There was no significant difference between day 3 and day 8 sow - piglet time-matched weighted UniFrac distances. Thus, despite changes in the community composition of the sow microbiota between day 3 and 8 (Chapter 3), piglet faeces share equal similarity in community composition between the time points, suggesting that the piglet microbiota community composition mirrors the changes in sow microbiota community composition. Time-matched day 8 piglet – sow distances were numerically, but not significantly, smaller than on day 3, whilst the weighted UniFrac distances between sow

faeces on day 1 and piglet faeces significantly decreased with each time point, demonstrating increased similarity between sow and piglet faecal community composition as the neonatal period progressed. Similarly, Chen *et al.* (2018) reported that the piglet faecal microbiota community composition became more similar to sow faeces as lactation progressed. Furthermore, Liu *et al.* (2019a) reported that the cross-sectional microbiota community composition (unweighted UniFrac distances) of the piglet large intestine was more similar to sow milk microbiota from days 0 – 7 of age, but increasingly similar to the sow faecal microbiota between days 7 – 35 of age when compared with all other maternal and pen microbiota sources (sow vagina and areolar skin, pen and creep area floor). No significant differences between the weighted UniFrac distances of cross-fostered piglets to birth sow or rearing sow faecal samples were reported on days 3 and 8. This demonstrates that inter-sow variability in faecal community composition does not alter the community composition of cross-fostered piglets when using beta diversity distances which take into consideration relative abundance of taxa. Liu *et al.* (2019a) reported that the milk, vaginal and environmental microbiota resulted in significantly lower unweighted UniFrac distances between related sow – piglet dyads than un-related dyads when analysing the large intestinal microbiota, however this was not observed when comparing sow faeces, as in this study. Moreover, in contrast to this study, Daft *et al.* (2015) reported that cross-fostering was an effective method to induce a permanent shift in the microbiota of mouse pups when taking place within the first 48 hours, attributing changes in colonisation due to the foster rather than birth mother.

The results of the Source Tracker analysis in the present study corroborate the beta diversity findings, quantitatively determining the impact of maternal microbiota sources on neonatal microbiota development. The sow udder microbiota contributed 4.5% of the microbiota population on day 1 but contributed ~1.2% on days 3 and 8. Consistent with the present study, Chen *et al.* (2018) also reported that the sow nipple contributed 4.3% of the piglet microbiota on day 1 but this contribution declined to 0% on days 3 and 8 and 0.1% on day 21 of age. Conversely, Liu *et al.*(2019a) reported that the sow areolar skin contributed a negligible but consistent percentage (values not reported in paper) from day 0 – 35 of age to the large intestinal microbiota of piglets, supporting the observed consistent weighted UniFrac distances observed in this study between day 1 udder samples and piglet faeces over time. Day 1 faeces accounted for 0.1% of the piglet faecal microbiota on days 1 and 3 and 1.2% on day 8, whilst time-matched day 3 and day 8 sow faeces contributed 1.2%. Chen *et al.* (2018) only reported sow faeces to contribute 1.1% on day 21, whilst Liu *et al.* (2019a) reported sow faeces to contribute 7% on day 7 increasing to 20% at day 35 to the piglet large intestinal

microbiota, supporting the notion that the piglet microbiota becomes increasingly like the sow with age. Differences in the Source Tracker results between studies may be attributed to differences in the hygiene protocols used on each experimental farm or differences in piglet microbiota sampling site (large intestine vs faeces). Despite negligible contributions to the microbiota identified by Source Tracker, correlation network analysis by Chen *et al.* (2018) revealed the highest number of positive associations between OTUs to occur when comparing piglet and sow faeces on day 1, compared with other maternal and environmental microbiota sources (pen floor, vagina and milk), indicating sow faeces to contain OTUs capable of promoting colonisation of the neonatal GIT. However, on day 7 the highest correlation of OTUs was with the sow nipple microbiota, with Chen *et al.* (2018) suggesting this change was due to the nipple area being extensively orally explored by piglets.

In the present study over 95% of the microbiota was explained by unknown sources during the neonatal period, as only the sow udder and faecal samples were used as possible microbiota sources in the Source Tracker analysis. Both Chen *et al.* (2018) and Liu *et al.* (2019a) utilised a greater variety of maternal and environmental sources in their Source Tracker analysis and reported that, particularly on day 1, the piglet microbiota originated from the pen floor, sow vagina, udder and milk. However, in both studies, after day 3 a large proportion of the microbiota could not be explained by maternal or pen microbiota sources (64.7% - 98.7% between days 3 – 21 (Chen *et al.*, 2018) and 65 – 75% between days 7 – 35 days (Liu *et al.*, 2019a)). This change in microbiota source could be related to the increased GIT maturity of piglets, providing a selective pressure for colonisers, as well as increased exploratory, coprophagy behaviour and creep feed intake altering substrates for microbial fermentation in the GIT. Based on the literature, it appears that whilst the sow vagina, milk and pen floor provide the first microbiota sources that colonise the neonatal GIT, the colonisation by these sources is only transient. The results of the present study and the literature suggest that the sow udder and faeces seem important maternal microbiota sources to promote colonisation of the neonatal GIT over time, even when contributing only a small percentage to the microbiota of neonatal piglets.

4.4.4 Cross-fostering effects on the piglet microbiota community composition

The piglet microbiota is thought to be highly stochastic in early life (Thompson *et al.*, 2008), but in the present study we demonstrate that a birth-litter-specific microbiota exists on day 1 and 3, with weighted UniFrac distances significantly smaller between biological siblings, even after cross-fostering, than non-siblings reared in the same litter. Due to the reciprocal model, the study was able to quantify and differentiate litter-specific microbiota effects from

the effect of cross-fostering. The distance within 'Biological' siblings and 'Cross-fostered siblings' from the same litter was not significantly different longitudinally, indicating that the action of cross-fostering had not significantly altered the weighted UniFrac distance between siblings during the neonatal period. As a result, the significantly greater distances between non-siblings on day 1 (before fostering) and 3 (when reared together after fostering) was attributable to non-siblings having a litter-specific microbiota developed within the first 24 hours of life. However, by day 8 this litter-specific microbiota was lost, with no significant difference between the weighted UniFrac distances of the cross-fostering groups, coinciding with increased similarity between the piglet and sow microbiota. This was supported by the findings of the PERMANOVA analysis. Thompson *et al.* (2008), who removed piglets from the sow at 3 days of age and co-housed piglets in unrelated pairs, reported a co-housing effect on the faecal microbiota occurring between 2 - 4 weeks of age when compared with siblings remaining on the sow and non-co-housed piglets. No co-housing effect was reported before two weeks of age, with piglet variability highest during the first week of life. Conversely, the present study reports a co-housing effect occurring by 8 days of age, and weighted UniFrac distances to increase between piglets longitudinally (indicating greater inter-piglet variability), irrespective of cross-fostering. Bian *et al.* (2016) also reported increased weighted UniFrac distances between piglets with age pre-weaning. The loss of the litter-specific microbiota by day 8 is likely due to the transient and diminishing influence of the milk, pen floor and vaginal microbiota and the increased influence of sow faecal and unknown environmental microbiota sources (potentially including increasing amounts of littermate faeces) on the colonisation of the microbiota, as previously discussed.

In conclusion, LBW and primiparous-born piglets exhibited poorer pre-weaning performance. Primiparous progeny had significantly lower microbiota evenness, in addition to a different microbiota community composition during the neonatal period. Cross-fostering did not significantly affect neonatal microbiota community composition, the development of which was mainly driven by age. The study demonstrated that a birth-litter-specific microbiota community composition existed for the first 3 days of life. The piglet microbiota on day 1 was more closely related to that of the sow udder than her faeces, with a proportion of the day 1 sow udder microbiota retained throughout the neonatal period. However, the similarity of the piglet microbiota to sow faeces increased between days 1 to 8, coinciding with the loss of birth litter specificity by day 8.

Chapter 5. Early life administration of an autogenous *Enterococcus faecium* strain to alter microbiota development and promote piglet performance

5.1 Introduction

The neonatal microbiota is shaped by the postnatal environment (Thompson *et al.*, 2008; Chen *et al.*, 2018), and by nutritional influences (Poulsen *et al.*, 2017). Modulation of the gut microbiota in early life is subsequently associated with piglet health (Dou *et al.*, 2017), performance (Chapter 2; Gaukroger *et al.*, 2020) and immune function (Inman *et al.*, 2010; Schokker at al., 2014, 2015).

Weaning presents a multitude of challenges to piglets, resulting in changes to gastro-intestinal tract (GIT) physiology, immune function and microbiota (Gresse *et al.*, 2017; Pluske *et al.*, 2018; Guevarra *et al.*, 2019). Direct-fed microbials (DFM), sometimes called probiotics, can be given during the weaning period to relieve aspects of microbiota dysbiosis, reduce post-weaning diarrhoea and, in some incidences, improve piglet performance (Patil *et al.*, 2015; Yang *et al.*, 2015; Dubreuil, 2017; Hou *et al.*, 2015; Taras *et al.*, 2006; Pajarillo *et al.*, 2015; Jiang *et al.*, 2015). The use of probiotics, has recently been reviewed by Liao and Nyachoti (2017) in relation to pig nutrition, categorising their effects to fall within 5 modes of action: 1) modulation of the microbiota by competitive exclusion or microbial inhibition, 2) altering immune system functionality, 3) reducing incidences of diarrhoea and antitoxic effects, 4) improving nutrient digestion by fermentation or enzyme secretion, 5) other modes of action including, but not limited to, quorum sensing and reducing antioxidant stress. In contrast to the use of probiotics in weaned piglets, the use of DFM delivered directly to suckling piglets (Hou *et al.*, 2015; Hayakawa *et al.*, 2016; Liu *et al.*, 2017; Wang *et al.*, 2019c,e; Xin *et al.*, 2020), rather than indirectly through sow nutrition, have been explored more recently as the potential benefits of modulating the early life microbiota on long term piglet health and performance have gained increased research interest. Whilst it presents a less labour-intensive option, indirect delivery of probiotics via the sow faeces does not ensure even colonisation between piglets or survival of the selected probiotic strain when exposed to aerobic conditions upon excretion. Early life studies in which piglets have received an oral probiotic have sometimes demonstrated improvements in ADG and intestinal morphology, reduced diarrhoea and immunomodulatory effects for the duration of probiotic treatment (Liu *et al.*, 2014; Hou *et al.*, 2015; Hayakawa *et al.*, 2016; Kiros *et al.*, 2019; Wang *et al.*, 2019c,e; Wang *et al.*, 2020), as well as short terms effects up to 1 week after termination of the probiotic treatment (Liu *et al.*, 2017; Wang *et al.*, 2019c; Xin *et al.*, 2020). These studies were conducted with a

range of previously identified probiotic species including *Lactobacillus* species, namely *L. reuteri, L. rhamnosus, L. fermentum, L. johnsonii, L. plantarum,* as well as *Saccharomyces cerevisiae, E. faecium, Bacillus subtilis, B. mesentericus* and *Clostridium butyricum*. However, studies which look at the effect of probiotics on the neonatal microbiota profile using 16S rRNA gene sequencing are scarce (Wang *et al.*, 2019c; Kiros *et al.*, 2019).

Enterococcus faecium is a species of bacteria commonly found in the GIT of piglets. *E. faecium* probiotics have been cited for their therapeutic ability to reduce diarrhoea in piglets pre- and post-weaning (Taras *et al.*, 2006; Zeyner and Boldt, 2006; Wang *et al.*, 2016b; Peng *et al.*, 2019). Their mode of action is thought to relate to *E. faecium* probiotic strains preventing *Escherichia coli*, specifically enterotoxigenic *E. coli* (ETEC), from adhering to the intestinal mucosa (Zeyner and Boldt, 2006; Bednorz *et al.*, 2013b) and damaging intestinal barrier function (Lodemann *et al.*, 2015; Klingspor *et al.*, 2015). Furthermore, *E. faecium* probiotics increased villus height and crypt depth under ETEC challenge in pre-weaned piglets (Peng *et al.*, 2019). *E. faecium* is a lactic acid bacterium (LAB). Lactic acid will lower the pH of the GIT but can also be used as a metabolizable substrate by other commensal bacteria, thus helping to improve gut health in a multi-factorial way.
The immunomodulatory effects of *E. faecium* probiotics have also been widely reported *in vitro* (Kreuzer-Redmer *et al.*, 2016) and *in vivo* (Scharek-Tedin *et al.*, 2009; Huang *et al.*, 2012; Twardziok *et al.* 2014; Siepert *et al.*, 2014; Peng *et al.*, 2019) and is thought to characterise the second main mode of action of probiotic *E. faecium* strains, after diarrhoea reduction. The main effect of *E. faecium* probiotics appears to be down-regulation of the immune system resulting in immunosuppression. It has been reported in several studies that *E. faecium* reduces the expression of IL-8, IL-10, CTLA4 and CD86 gene expression in Peyer's patches (Twardziok *et al.* 2014; Siepert *et al.*, 2014; Huang *et al.*, 2012).

The microbiota of pigs is affected by the farm/rearing environment (Vigors *et al.*, 2020a; McCormack *et al.*, 2019a) and genotype (Xiao *et al.*, 2016; Xiao *et al.*, 2017; Bergamaschi *et al.*, 2020a). Thus, the appropriate DFM to promote piglet health and performance may vary from farm to farm, especially if farm health status is vastly different. DFM from the home farm are therefore attractive, as the species in question should already be able to colonize the GIT of recipient piglets. To date, only Wang *et al.* (2016b) have isolated an autogenous *E. faecium* strain and used it as a DFM for neonatal piglets to determine the effect this has on performance and the neonatal microbiota using 16S rRNA gene sequencing. Moreover, despite increasing evidence that the microbiota profile of piglets is significantly affected by

birthweight (Chapter 1, Gaukroger *et al.*, 2020; Li *et al* 2018, 2019, Zhang *et al.*, 2019a), it is yet to be established whether piglet birthweight modulates the effect of DFM treatment in early life on the neonatal piglet microbiota and performance.

5.1.1 Study aims and hypotheses

The overall aim of this study was to determine the effect on the performance and faecal microbiota community composition of LBW (low birthweight) and NBW (normal birthweight) pigs after administration of an autogenous *E. faecium* inoculum for the first 7 days of life. The study firstly hypothesised that piglets receiving the *E. faecium* treatment for the first 7 days of life will have a superior pre- and post-weaning performance compared with placebo-dosed controls. Secondly, it was hypothesised that *E. faecium* treated piglets will display an altered microbiota profile at the end of the 7-day *E. faecium* treatment. Thirdly, it was hypothesised that there would be an interaction between birthweight and experimental *E. faecium* treatment on the microbiota profile of piglets. The experimental *E. faecium* strain was originally isolated from a high performing LBW piglet, thus adapted to colonise the physiological conditions of the LBW piglet GIT which are assumed to differ from NBW, thus resulting in a different microbiota profile in response to experimental *E. faecium* treatment.

5.2 Materials and methods

5.2.1 Animal housing and management

A total of 185 experimental piglets from 18 Large White x Landrace sows (average parity = 2.94, SD = 1.924; parity 1-3 = genetics from Hermitage Seaborough Ltd x Rattlerow farms Ltd, UK and parity 4-6 = Hermitage Seaborough Ltd, UK) were used in this study from three consecutive farrowing batches. Experimental sow housing and management during gestation and lactation, and piglet management during the suckling period, were as previously descripted in Chapter 3 and Chapter 4, respectively. Cross-fostering of piglets occurred within the first 24 hours post-partum in order to create uniform litters of piglets based on birthweight. Experimental litter size was set according to the number of functional teats, which ranged from 11 – 13 piglets per litter. Due to limited piglet availability and the number of sows available per batch, where functional teat capacity per experimental litter exceeded the number of experimental pigs, additional non-experimental piglets of similar birthweight were used to ensure all teats were suckled. Experimental piglets were identified by small individual ear tags (Dentag, Toptag, UK) which were replaced by small flag tags (FLEXO, Dalton Tags, UK) on the day before weaning during day 27 experimental procedures. Piglets could access water through either a nipple drinker or water trough, whilst creep feed was

provided *ad libitum* from 10 days of age in a creep feed hopper (Flat Deck 1, 16.50 MJ DE/kg, 22.50% CP and 1.7% SID lysine, A-One Feed Supplements Ltd, Thirsk, UK). Piglets were vaccinated against *Mycoplasma hyponeumoniae* (2ml IM M+PAC, MSD Animal Health, Walton, UK) and porcine circovirus type 2 (1ml IM Ingelvac CircoFLEX; Boehringer Ingelheim, Duluth, USA) on the day before weaning.

At weaning (28 days after batch expected farrowing date), experimental piglets were housed in fully slatted, temperature controlled, flat deck accommodation. Room temperature and humidity were recorded daily. Initially room temperature was set to 26°C and reduced by 0.2°C/day to a minimum of 22°C, which was sustained for the rest of the weaner period. Experimental pigs were housed according to rearing litter and experimental *E. faecium* treatment (see below), resulting in 3 - 6 piglets per pen (pen diameter 1.10m x 1.84m). Pens were filled adjacently based on experimental *E. faecium* treatment and birthweight class to reduce within-room environment differences between experimental treatment groups. Each pen was furnished with one nipple drinker, a multi-space feed trough and three toys. During the experiment, piglets received a two-stage weaner starter diet regime *ad libitum* (Stage 1: 16.50 MJ DE/kg, 22.50% CP and 1.7% SID lysine (Flat Deck 1). Stage 2: 16.00 MJ DE/kg, 21.00% CP and 1.55% SID lysine (Flat Deck 150; A-One Feeds Supplements Ltd, Thirsk, UK). Neither post-weaning diets, nor creep feed contained antibiotics or pharmacological levels of zinc oxide. The stage 1 diet was given for 4 days post-weaning, this was then changed to a 50:50 mix of stage 1 and stage 2 diets for a further 3 days. Piglets then received the stage 2 diet for the remaining 4 days of the experiment.

The number of piglets weaned per experimental sow was recorded at weaning. Veterinary treatments for experimental animals were recorded throughout lactation and post-weaning. Seven piglets were removed from the experiment for welfare reasons as they were gaining less than the cut off of 100g/d over two consecutive days and were subsequently reared by non-experimental sows with supplementary milk. A further 3 piglets died during the first week of life, thus the data analysed in this experiment is associated with the remaining 174 experimental piglets.

5.2.2 Experimental design

The experiment was designed as a 2 x 2 factorial. The first factor was piglet birthweight, with experimental piglets classified as either low birthweight (LBW; 0.80 – 1.25kg, n = 97) or normal birthweight (NBW = 1.50 – 2.00kg, n = 77). The second factor in the experimental design was experimental *E. faecium* treatment, with piglets receiving oral dosing with either 1ml *E. faecium* inoculum (Treatment; ~5 x 10^8 *E. faecium* colony forming units (CFU) /ml

phosphate buffer saline (PBS), n = 87) or 1ml PBS (Control; n = 87) for the first 7 days of life. Piglets were cross-fostered shortly after birth to establish litters with either LBW, or NBW piglets. Experimental *E. faecium* treatment was then applied within-litter such that approximately half of the experimental piglets per litter were Treatment and the other half Control piglets. The decision was taken to do this rather than a whole litter being assigned to one treatment, as the risk of contamination between Treatment and Control littermates was perceived to be less crucial to control than between litter variation of the microbiota. Precautions were taken to limit the chance of contamination between litter mates of different treatments.

5.2.3 E. faecium isolation, culture and inoculum preparation

5.2.3.1 Culture and species identification

In order to create an autogenous inoculum, early life faecal samples from the Chapter 4 experiment were used. Two day 3 samples originating from LBW piglets with an above average pre-weaning average daily gain (> 0.226kg/d) were selected for culture and defrosted from -80ºC storage. The following laboratory work was conducted in an anaerobic chamber (Coy Lab Products, USA, 36 - 37ºC, O_2 = 44ppm and H_2 = 2.4%). Approximately 250mg of the selected faecal sample was added to 1ml PBS and vortexed, generating the neat solution. 10µl, 100µl and, in case of lawn formation, 100µl of -2 dilution (1:100) were plated on BSM (Bifidus selective media; BSM broth, Sigma Aldrich, UK) agar. After overnight incubation, 3 separate/distinct colonies were present on each of the plates for both piglet samples. A single colony for each colony type was streaked on BSM, MRS and BHI (Brain-heart infusion) agar plates (Sigma Aldrich, UK) to see if colony growth was affected by agar media, showing that each colony type grew on all media. For each piglet, 2 colonies for each colony type across the 4 dilutions were selected and spiked into 5ml of BHI broth (n = 24), or MRS broth (n = 24). The BHI and MRS spiked broths were vortexed and then incubated and shaken at 100rpm (SSM1, Stuart mini orbital shaker, UK) overnight. 500µl of cultured broth was added to 500µl of 50% glycerol in 1.2ml cryogenic tubes (Corning, Corning Incorporated, New York) mixed by pipetting, removed from the anaerobic chamber and frozen to -80ºC. Each broth was streaked on to BSM agar to check the glycerol stocks were not contaminated. Glycerol stocks were then part-thawed and a 10µl spot from each stock was transferred to a BSM agar plate to test for culture viability after freezing. Based on the spot plate, 19 BSM and 18 MRS glycerol stocks showed good viability after freezing. 10µl of each viable stock was streaked on the respective agar to grow individual colonies following overnight incubation. For each colony type (n = 3), one of the BHI and BSM plates (n = 4 replicates per

colony type and piglet) was selected and used to inoculate 5ml of BHI broth (n = 6; 3 colony types and 2 piglet replicates). Broths were left to incubate for 24 hours and shaken at 100rpm, before being removed from the anaerobic chamber. 1.5ml from each cultured broth was added to a 2ml Ependorf tube and spun down for 3 minutes at 9000 x g and the supernatant was discarded. The residual bacterial cells and MRS broth were centrifuged for a further 3 minutes at 9000 x g. Once the residual MRS broth had been discarded, 750µl PowerBead solution from the DNeasy PowerLyzer PowerSoil kit (Qiagen, USA) was used to resuspend the bacterial cells and transfer them by pipetting to a bead beating tube. DNA from each culture type was then extracted following the manufacturer's instructions.

The DNA from each of the 3 colonies was amplified by PCR (T100 Thermo Cycler, Bio-Rad, UK), see **Appendix 5a** for PCR master mix and PCR cycle and primers. 5µl of the PCR product for each colony was then added to 2µl of ExoSAP-IT PCR clean-up reagent (Thermo Fisher Scientific, UK) in PCR tubes, subjected to another round of PCR (**Appendix 5b**) and visualised by 1% agarose gel electrophoresis to confirm no DNA contamination had occurred during the PCR process. The concentration of 1ul of ExoSAP-IT PCR product was determined using the NanoDrop One/One^{C} Microvolume UV-Vis Spectrophotometer (Thermo Fisher Scientific, UK). Based on the concentration, 1µl ExoSAP-IT PCR product was diluted with 14µl sterile distilled water and sent for Sanger Sequencing at Eurofins. The resulting Sanger sequences were run through BLAST (Basic Local Alignment Search Tool, NCBI, USA) to identify the species for each culture. Across the 3 cultures and 2 piglet replicates per culture (total samples n = 6) that were Sanger Sequenced, the species identified were *Clostridium perfringens*, *Enterococcus faecium*, *Enterococcus faecalis*, *Enterococcus cecorum and Enterococcus gallinarum*. *E. faecium* is a commonly used probiotic given to piglets, thus the *E. faecium* species isolated from the LBW piglet with the greatest pre-weaning growth rate (0.294 kg/d vs 0.259 kg/d) of the two replicates was used to create the experimental *E. faecium* treatment.

5.2.3.2 E. faecium growth curve preparation

The subsequent lab work was conducted outside the anaerobic chamber in sterile conditions, as *E. faecium* is a facultative anaerobe. 10µl of *E. faecium* glycerol stock was used to streak a BHI agar plate and incubated at 37ºC (Labnet Mini Incubator, Labnet International Inc, UK). A single *E. faecium* colony was picked and added to 30ml of BHI broth (50ml Greiner centrifuge tubes, Sigma-Aldrich, UK), in triplicate, incubated at 37ºC and shaken at 170 rpm overnight (Max Q6000, Thermo Scientific, UK). For each of the 3 cultured BHI replicates, a

dilution series was created in 1.5ml Eppendorf tubes to create a 1/8 dilution. The 1/2 and 1/8 dilution were then used to generate a 1:10 serial dilution down to the -8; 100µl of the -6 to -8 and -5 to -6 of the 1/2 and 1/8 dilutions, respectively, were spread on BHI plates and incubated overnight at 37ºC (Labnet Mini Incubator, Labnet International Inc, UK). The plate counts for the -6 dilution of the 1/2 dilution and the -5 dilution of the 1/8 dilution were recorded for each of the 3 replicates.

The remaining broth was used to generate the values for the growth curve using spectrophotometer readings (U-1800, Digilab Hitach, Japan; wavelength = 600 and results given in ABS). A blank reading was firstly taken for BHI broth to calibrate the spectrophotometer, followed by neat cultured BHI broth. Serial solutions from the neat BHI cuvette down to the 1/16 were then generated and spectrophotometer readings taken for each dilution, for each of the three replicates. The plate counts and spectrophotometer readings were used to calculate the growth curve, and subsequently CFU/ml (**Appendix 5c**). To calculate future CFU/ml, spectrophotometer readings of neat PBS washed inoculum were used to calculate CFU/ml against the growth curve **(Appendix 5c)**.

5.2.3.3 Experimental Enterococcus faecium inoculum preparation

The experimental *E. faecium* inoculum was made on a per batch basis. Preparation of the inoculum started 1 week prior to experimental batch farrowing due date, in order to minimise freezing time (48 hours – 10 days for the last day of experimental treatment) and to fit around the experimental pig data collection schedule. Moreover, longitudinal effects of freezing on CFU count had not been fully established, although over the course of the study longitudinal declines in CFU counts after the initial freezing loss were demonstrated to be minimal (**Appendix 5d**). To create the experimental inoculum, 10µl of the *E. faecium* glycerol stock was streaked on to BHI agar, in triplicate, and incubated overnight at 37ºC (Labnet Mini Incubator, Labnet International Inc, UK) in order to create single colonies which could be used to spike broths. One control BHI agar plate was also incubated to check for any contamination during the *E. faecium* spreading process.

For each batch, 1.5l of BHI broth was autoclaved and 30ml decanted into 50ml centrifuge tubes (50ml Greiner centrifuge tubes, Sigma-Aldrich, UK), then incubated at 37ºC overnight (GalaxyB, Scientific Laboratory Supplies, UK) to check broths were not contaminated prior to spiking. A single *E. faecium* colony from the BHI agar was then picked using a 1µl loop and added to each 50ml centrifuge tube of BHI broth. The spiked broths were then incubated

for 24 hours (to ensure exponential growth was reached) at 37ºC and shaken at 170rpm (Max Q6000, Thermo Scientific, UK). In addition, 2 x control BHI broths were incubated with the rest of the spiked broths to check for contamination during the spiking process. After the 24 hour incubation, the spiked broths were spun down for 10 minutes at 3260rpm and 4ºC to minimise further *E. faecium* growth. Residual BHI broth was discarded. The *E. faecium* pellet was then resuspended in 1ml PBS, followed by another 19ml PBS and vortexed for 5 seconds to wash the *E. faecium* cells. The suspension was then centrifuged for 10 minutes at 3260 rpm and 4ºC. Residual PBS and BHI were discarded. The washed *E. faecium* cells were then resuspended in 3-5ml PBS depending on pellet size; the resuspension from all the tubes were combined in a 500ml sterile beaker over a flame.

The spectrophotometer (U-1800, Digilab Hitach, Japan) wavelength was set to 600 and blanked with 1ml PBS, a reading was then taken in ABS for 1ml 1:10 dilution of the resuspension and inputted into the growth curve; following correction for the 1:10 dilution, the neat *E. faecium* resuspension was determined. Using batch 1 as an example, batch 1 *E. faecium* inoculum preparation contained 4.61 x 10^{10} CFU/ml. The aim was to provide experimental treatment pigs with ~5 x 10^{8} CFU/ml, taking into consideration *E. faecium* death during freezing (determined to be on average 68.35% in preparatory work (**Appendix 5d**), two potential formulation doses which may deliver ~5 x 10^{8} CFU/ml viable *E. faecium* after freezing were formulated. 275ml of 1 x 10^{10} CFU/ml *E. faecium* inoculum was created by mixing 59.65ml *E. faecium* suspension with 215.35ml of PBS, and 275ml of 5 x 10^{9} CFU/ml by mixing 29.825ml *E. faecium* suspension with 245.18ml PBS. Under a hood, (Envair, UK), approximately 50ml at a time of each *E. faecium* inoculum concentration was decanted into sterile reagent reservoirs (50ml; Heathrow Scientific, UK) and 1ml of inoculum was drawn into a 3ml sterile syringe (Fisher Scientific, UK) and sealed with Parafilm (Heathrow Scientific, UK) until all of the inoculum in the beaker had been used, respectively, for the 1x10^{10}CFU/ml and 5x10^{9}CFU/ml *E. faecium*. Once 50 syringes had been drawn, they were bagged and frozen immediately at -80ºC to limit the time for cells to divide whilst making the syringes. For the control piglets, 275 x 1ml of PBS were drawn in 3ml syringes and sealed with Parafilm and then frozen at -80ºC. Batch 2 and 3 *E. faecium* treatment syringes were generated in the same way; however, the formulated concentration differed to batch 1 (**table 5.1**).

To determine whether the fresh *E. faecium* syringe contained the desired amount of *E. faecium* formulated, 5 syringes collected at random throughout the syringe preparation

process (1 syringe per bag of 50 made) were refrigerated until all syringes were drawn in the batch. From a 1:1 dilution series with PBS, the 1/2 and 1/8 dilutions of *E. faecium* syringes were diluted 1:10 down to the -8. Using BHI agar, 100µl of the -4 to -7 dilutions were spread and incubated for 24 hours (Labnet Mini Incubator, Labnet International Inc, UK). CFU/ml counts for each replicate were subsequently calculated. After 24 hours of freezing, 4 syringe replicates per batch were defrosted and the process repeated in order to calculate defrosted CFU/ml counts and thus determine the final concentration of the experimental *E. faecium* treatment syringes per batch, in relation to the target defrosted 5 x 10^8 CFU/ml. The target formulation, fresh and defrosted viable counts for each batch are shown in **table 5.1**.

Table 5.1. Summary of formulated CFU/ml counts of *E. faecium* and the subsequent fresh and defrosted counts determined through plate counts on BHI agar, along with the percentage loss of viable *E. faecium* following a 24 hour period of freezing at -80ºC (n = 5 fresh, n = 4 defrosted per batch[1]).

Batch	Formulated CFU/ml	Fresh CFU/ml	Defrosted CFU/ml	Percentage loss
1	$1x10^{10}$	$2x10^9$	$7.1x10^8$	64.5%
1*	$5x10^9$	$6.6x10^8$	$4.19x10^8$	36.5%
2	$6.5x10^9$	$7.1x10^8$	$4.51x10^8$	36.5%
3	$7x10^9$	$3.3x10^{10}$	$4.68x10^8$	98.6%

[1]Each syringe replicate used to calculate fresh CFU/ml was selected per 50 syringes generated to account for possible *E. faecium* division and thus increased CFU/ml of the later syringes drawn per batch, as whilst the BHI broth had been removed the hood was maintained at approximately 19ºC. The syringes selected for defrosted CFU/ml calculations were selected at random.

*Defrosted counts were closest to the target of 5 x 10^8 CFU/ml and so this batch of syringes was used as the experimental *E. faecium* treatment for batch 1 and to base formulation calculations for batch 2 and 3.

5.2.4 Experimental procedures

Approximately 6 – 12 hours after farrowing, piglets were weighed, sexed and experimental piglets ear tagged. Experimental litters were formed within the first 24 hours of life (day 0); where possible, siblings and piglets of similar ages (in hours) were used to form each experimental litter to minimise litter disruption. Between days 1 – 7 of life, experimental piglets were boxed into the creep area one litter at a time and received either 1ml *E. faecium* (~5 x 10^8 CFU/ml; Treatment) or 1ml PBS (Control) between 0830 – 1000 h. Approximately 1 hour prior to inoculum treatment, the *E. faecium* and PBS syringes were removed from -80ºC storage and thawed. The *E. faecium* inoculum treatment (Treatment or Control) was delivered via the syringe at the back of the tongue slowly enough to enable piglets to swallow the inoculum.

Piglet liveweight was recorded at birth/ processing and then on days 3, 5, 8, 13, 19, 27, 32, 35 and 39 of age. Faecal samples were collected by rectal swab (Sterilin plain flocked swab, Thermo Scientific, UK) and, where possible, a small amount of faecal sample (7ml sterile polypropylene bijou container, Star Lab, UK) was also collected at the same time as weighing (with an additional day 1 faecal sample collected). For the purpose of this experiment, the faecal microbiota from the day 8 sample was analysed, as we hypothesised that any effect of *E. faecium* on the microbiota would be most pronounced on completion of the 7 days of experimental *E. faecium* treatment. Faecal samples were placed on dry ice immediately after sampling and then transferred to -80ºC storage until DNA could be extracted.
Pen (Birthweight class * Treatment * Rearing litter) feed intake was recorded via a weigh back system for the 11 day post-weaning experimental period. Total pen feed intake was used to calculate average daily feed intake per piglet and average post-weaning piglet FCR within each pen.

5.2.5 DNA extraction and 16S rRNA gene sequencing

DNA was extracted and sequenced, and bioinformatics procedures were conducted following the methodologies described in Chapter 3. All DNA extractions were performed in a hood (TriPass2, CAS, UK).
A total of 2,562,888 sequencing reads were obtained from an initial 164 piglet faecal samples and 24 controls (12 x DNA extraction kit negative, 6 x PCR negative and 6 x PCR positive controls) run on the Illumina MiSeq. Sequences were rarefied to 3350 reads per sample. Any of the controls retained after rarefaction were assessed for microbiota similarity to piglet faecal samples by inspection of a weighted UniFrac Principal Coordinate Analysis (PCoA) plot (**Appendix 5e**). Controls were deemed to be significantly different from piglets, thus removed from further analysis. After rarefaction, 157 piglet faecal samples were retained and relative abundances were filtered to $\geq$ 0.01%, retaining 14 phyla, 19 classes, 35 orders, 59 families and 125 genera.

5.2.6 E. faecium genome sequencing

Although running Sanger Sequenced DNA through BLAST (Basic Local Alignment Search Tool, NCBI, USA) was able to identify *E. faecium* at the genus level, it was not able to identify the strain. To obtain further information on the *E. faecium* bacterial culture used in the inoculum treatment the genome had to be sequenced and analysed.
The *E. faecium* glycerol culture (10µl) was added to 10ml BHI broth (1.5% agar; BHI broth, Sigma-Aldrich,UK) for 24 hours at 37ºC and 170rpm, the centrifuged for 10 minutes at 4ºC

and 3260rpm to form a pellet. The residual BHI broth was then discarded and 750µl of the PowerBead solution was added to resuspend the *E. faecium* pellet. The suspension was then pipetted into the PowerBead tube and *E. faecium* DNA was extracted according the Qiagen DNeasy PowerLyzer PowerSoil kit manufactures instruction, along with a kit negative. The DNA concentration was determined using a nanodrop One/One^{c} Microvolume UV-Vis Spectrophotometer (Thermo Fisher Scientific, UK). The kit negative contained 1.3ng DNA/µl, whilst the *E. faecium* sample contained 243.5ng/µl of high purity DNA (1.83 A260/A280 and 2.33 A260/A230 readings). *E. faecium* DNA (30µl) was then sent to the NU-OMICS DNA facility for genome sequencing on the Illumina MiSeq. The paired end FASTQ files generated by the MiSeq run were trimmed using BB Decontaminating Using Kmers (BBDuk) for the quality control process (Joint Genome Institute, 2020). The sequences were assembled using SPades v 3.14.1 (Nurk *et al.*, 2013). The contig FASTA file generated by SPades was then inputted to the mlst tool (Jolley and Maiden, 2010) to obtain an MLST profile which was, in turn, then inputted into the PubMLST database (Jolley and Maiden, 2010) which outputted any matches, PubMLST scheme names and sequence type. The assembled genome was also run through proGENOME v 2.1 (Mende *et al.*, 2020) against the representative genome contigs database in order to identify the taxonomy associated with the genome contigs.

5.2.7 Statistical analyses

All statistical analyses were conducted in R version 3.6.2. All performance data and alpha diversity values were tested for normality by the Shapiro Wilk's normality test. Model diagnostic plots (qqnorm and fitted vs residual values) were inspected for normality to assess model fit for all statistical models. The 'emmeans' (v 1.4.8), 'car' (v 3.0 – 8) and 'multcomp' (v 1.4 – 13) packages were used for all post-hoc comparisons of significant fixed and interactive effects for all performance and alpha diversity models, and to generate compact letter displays, whereby least square means with different lowercase superscript letters denote significant differences ($P < 0.05$).

5.2.7.1 Piglet performance

The fixed effects in the piglet performance models were experimental *E. faecium* treatment (Treatment or Control), BiW class (LBW or NBW) and Time point for LW/Time period for ADG. Fixed effects were tested with all possible interactions. Sow ID formed the random effect to account for any rearing litter effect in the model, and piglet ID was nested in sow ID to account for longitudinal repeated measures. Piglet LW and ADG (measured between

successive time points) were deemed to be not normally distributed. Subsequently, data were analysed using generalised linear mixed effect models (glmer; 'lme4' package v1.1 - 23). The longitudinal LW fitted vs residuals plot demonstrated a funnel effect in the plot, with the data positively skewed (*skewness* function, 'moments' package v 0.14), and so a log10 transformation was applied to the LW values. Following the log transformation, the diagnostic plots displayed normality. Similarly, the longitudinal ADG diagnostic plots were suboptimal and raw data positively skewed. As some negative values were reported between day 27 – 32, the *powerTransform* function with *family = bcnpower* was utilised to determine the best lambda (0.58) and gamma (0.16) values to use in the box cox transformation (*bcnPower* function) of the longitudinal ADG data. Following the box cox transformation, the model diagnostic plots displayed normality.

Weaning pen average formed the experimental unit in the post-weaning (day 28 – 39) analysis of piglet ADFI and FCR. ADFI data were normally distributed (Shapiro Wilk's $P > 0.05$), whilst pen average FCR data were not (Shapiro Wilk's $P < 0.05$), thus lmer and glmer models were run, respectively. Fixed effects in each model were *E. faecium* treatment (*E. faecium* or control) and BiW class (LBW or NBW), with the interaction specified in the models and rearing sow ID as the random effect. Model diagnostic plots were normally distributed therefore no transformations were applied to the FCR data.

The proportion of piglets treated for diarrhoea for each week from birth (piglet age) to the end of the trial at 39 days of age was calculated with respect to BiW class and experimental *E. faecium* treatment groupings. Longitudinal proportional data were analysed by beta regression ('betareg' package v 3.1 – 3); BiW class, experimental *E. faecium* treatment and piglet age formed the fixed effects in the model and the interactions between fixed effects were tested. The model diagnostic fitted vs residuals plot was inspected and appeared normally distributed.

5.2.7.2 Microbiota analysis

5.2.7.2.1 Alpha diversity

The number of observed OTUs and Shannon diversity index were the two measures of alpha diversity used in this experiment. Alpha diversity values were calculated using the 'vegan' package (v 2.5). Neither of the alpha diversity measures were normally distributed, therefore glmer ('lme4' package v1.1 - 23) models were used to analyse these data. Piglet ID formed the experimental unit in the analysis and rearing sow ID was the random effect in both models. The fixed effects in both models tested the main effects and interaction of BiW class and experimental *E. faecium* treatment. Observed OTUs were count data and so the model

family was set to Poisson, whilst the Shannon diversity index model family was left as the default gaussian, due to the continuous nature of the data. Model diagnostic plots were inspected and considered to be normally distributed.

5.2.7.2.1 Beta diversity

Weighted and unweighted UniFrac distances were used as beta diversity measures to assess the similarity of the microbiota community composition between piglet faecal samples. Both weighted and unweighted UniFrac distances were used to account for any differences when taking into consideration relative abundance of taxa or giving equal weighting to low abundance taxa, respectively (Lozupone and Knight, 2005; Lozupone *et al.*, 2007). UniFrac distances were calculated using the 'rbiom' package (v 1.0.2.9002). A PERMANOVA was performed using the *Adonis* function in the 'vegan' package (v2.5 - 5), with 999 Monte Carlo permutations, testing the interaction between BiW class and experimental *E. faecium* treatment.

5.2.7.1.2 Genera abundance

Relative genera abundances were calculated from the rarefied OTU table. Genera were only retained when mean genus abundance was $\geq$ 0.01% across all samples and the genus was present in $\geq$ 10% samples. MaAsLin2 (Microbiome Multivariable Association with Linear Models) is an R package (v 1.0.0) capable of simultaneously analysing genera relative abundances and identifying significant differences between levels of fixed effect in a mixed effect model format. The MaAsLin2 package requires the following packages as pre-requisites: pscl (v 1.5.5), pbapply (v 1.4 – 2), dplyr (v 1.0.0), vegan (v 2.5 - 6), chemometrics (v 1.4.2), ggplot2 (v 3.3.2), pheatmap (v 1.0.12), cplm (v 0.7 – 8), logging (v 0.10 – 108), data.table (v 1.12.8), lmerTest (v 3.1 – 2), edgeR (v 3.28.1) and metagenomeSeq (v 1.28.2). Fixed effects in the MaAsLin2 model were BiW class and experimental *E. faecium* treatment, with rearing sow ID as the random effect. Genera abundances were arcsine square root transformed (transform = "AST") to enable the model analysis method to function under the default setting, linear model ("LM"). The q-value threshold was set to 0.05 (max_significance = 0.05). MaAsLin2 cannot formally test an interaction between two fixed effects and so an interactive BiW class and *E. faecium* treatment variable was created and run in a second MaAsLin2 model. All P values were FDR (false discovery rate) adjusted.
Enterococcus prevalence (0 = absent, 1 = present) was calculated based on OTU table counts. *Enterococcus* prevalence was modelled by logistic regression using a glm ('nlme' v 3.1 –

148), *family = binomial.* BiW class and experimental *E. faecium* treatment and their interaction formed the fixed effects in the model.

5.3 Results

5.3.1 E. faecium strain identification

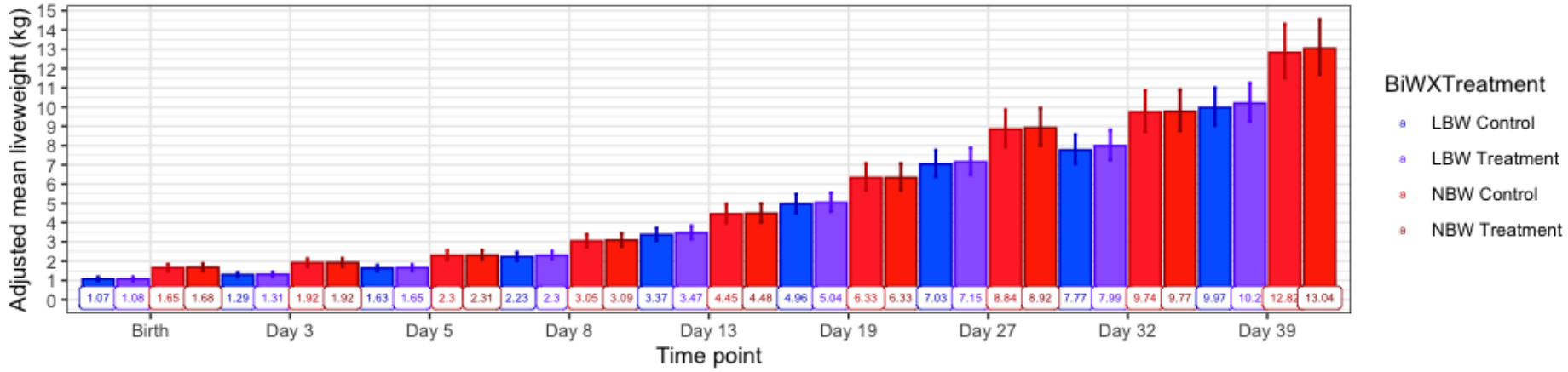
The proGENOME analysis produced matches to all the target genes used and confirmed that the bacterium was *E. faecium* although there was a single match to *Acinetobacter,* indicating a low level of contamination. As the bacterium was cultured from a single colony and all plates had uniform morphology this is likely to have been introduced during the sequencing process and is unlikely to indicate contamination of the glycerol stock. The MLTS profile for the sample was atpA(5), ddl(3), gdh(6), purK(6), gyd(6) pstS(1),adk(1). This had one gene loci difference to two other *E. faecium* strains in the database (*E. faecium* sequence type 511 and 610) which originated from an environmental sample and human sample. This result indicates that the isolate is unique and has not been characterised before.

5.3.2 Piglet performance

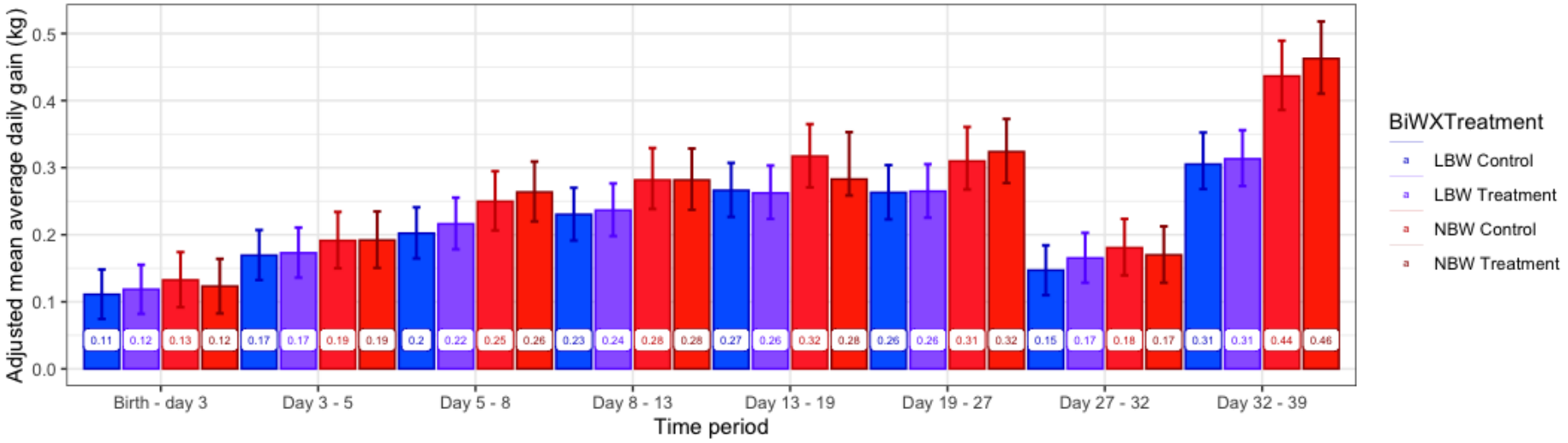
There was a significant effect of sample time point ($P < 0.001$), with liveweight increasing significantly over time. There was a significant effect of BiW class ($P < 0.001$), with NBW piglets weighing significantly more than LBW piglets across the 39-day experimental period. There was also no significant fixed effect ($P > 0.05$) of experimental *E. faecium* treatment on piglet liveweight. There was no significant interaction between piglet BiW class and sample time point, between experimental *E. faecium* treatment and sample time point or between BiW class, experimental *E. faecium* treatment and time point on piglet liveweight ($P > 0.05$) (**figure 5.1a**).

ADG between successive time points was significantly different over time ($P < 0.001$). ADG gradually increased over time, with the exception of ADG between day 27 – 32 (immediately post weaning) which was lower than all other time periods except birth – day 3 and day 3 – 5. There was a significant effect of BiW class ($P < 0.001$), across the 39-day experimental period NBW piglets had a higher ADG than LBW piglets. There was no significant fixed or interactive effect of experimental *E. faecium* treatment on piglet ADG ($P > 0.05$; **figure 5.1b**.) Meanwhile, there was a significant interaction between time period and BiW class ($P < 0.001$). NBW piglets displayed a significantly higher ADG value between days 5 – 8, 8 – 13, 19 – 27 and 32 – 39, but no significant difference was detected between birth – day 3, days 3 – 5 and 13 – 19 of age between BiW classes (**figure 5.1).**

a)


Adjusted mean liveweight (kg)
0 1 2 3 4 5 6 7 8 9 10 11 12 13 14 15
1.07 1.08 1.65 1.68 1.29 1.31 1.92 1.92 1.63 1.65 2.3 2.31 2.23 2.3 3.05 3.09 3.37 3.47 4.45 4.48 4.96 5.04 6.33 6.33 7.03 7.15 8.84 8.92 7.77 7.99 9.74 9.77 9.97 10.2 12.82 13.04
Birth
Day 3
Day 5
Day 8
Day 13
Day 19
Day 27
Day 32
Day 39
Time point
BiWXTreatment
LBW Control
LBW Treatment
NBW Control
NBW Treatment

b)


Adjusted mean average daily gain (kg)
0.0 0.1 0.2 0.3 0.4 0.5
0.11 0.12 0.13 0.12 0.17 0.17 0.19 0.19 0.2 0.22 0.25 0.26 0.23 0.24 0.28 0.28 0.27 0.26 0.32 0.28 0.26 0.26 0.31 0.32 0.15 0.17 0.18 0.17 0.31 0.31 0.44 0.46
Birth - day 3
Day 3 - 5
Day 5 - 8
Day 8 - 13
Day 13 - 19
Day 19 - 27
Day 27 - 32
Day 32 - 39
Time period
BiWXTreatment
LBW Control
LBW Treatment
NBW Control
NBW Treatment

c)

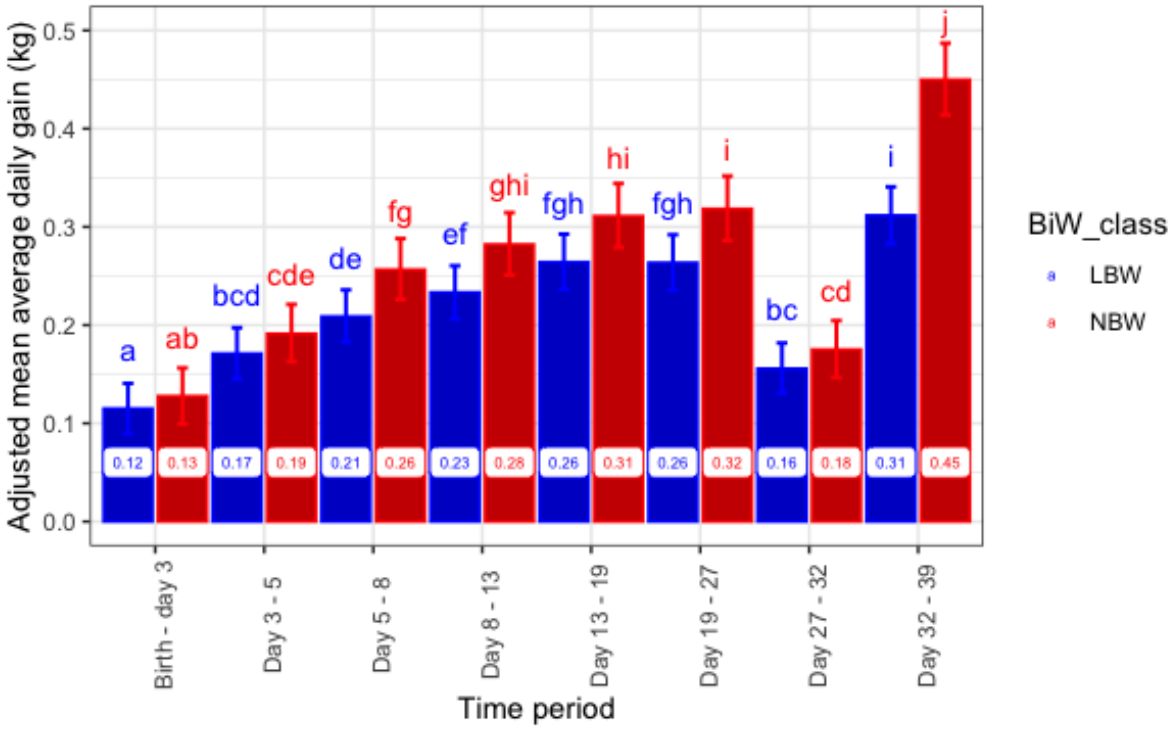


Figure 5.1. Longitudinal adjusted means and 95% confidence intervals using back transformed liveweight (a) and raw average daily (ADG; b) values according to the interaction between birthweight class (LBW = low birthweight, NBW = normal birthweight) and experimental *E. faecium* treatment (Treatment or Control) and time period, whilst (c) demonstrated the interaction between piglet birthweight class and time period only in relation to ADG. There were no significant interactions between experimental *E. faecium* treatment and birthweight class on piglet performance at any time point. Consequently, superscript compact letter displays identifying significant differences ($P < 0.05$) have not been added for (a, b), but have been added to (c).

At the pen level, during the experimental post-weaning period, NBW pigs ate significantly more than LBW piglets ($P < 0.01$). However, there was no significant main or interactive effect of experimental *E. faecium* treatment ($P > 0.05$). Furthermore, there was no significant main or interactive effect of BiW or experimental *E. faecium* treatment on average pen FCR ($P > 0.05$). Adjusted mean values for the post-weaning FI and FCR data can be seen in **table 5.2**.

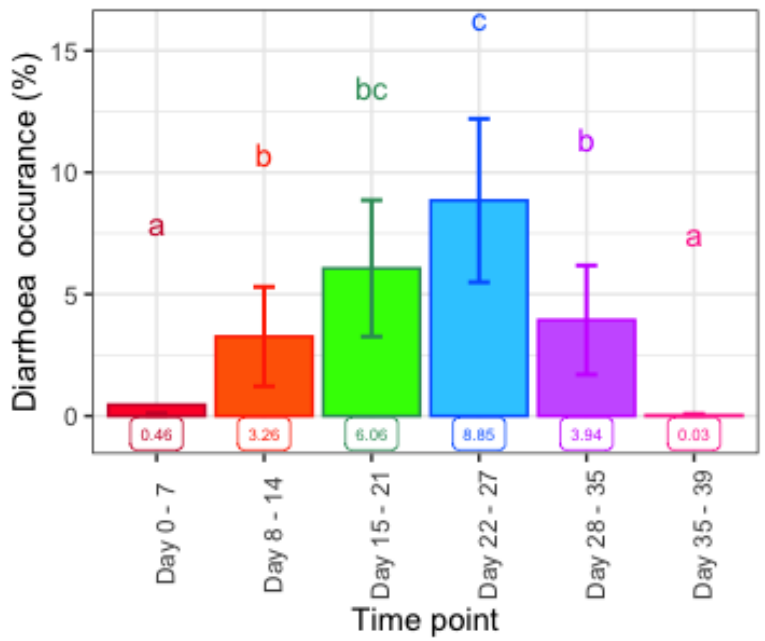
Table 5.2. Mean pen post-weaning (28 – 39 days of age) feed intake (FI) and feed conversion ratio (FCR) based on birthweight class (LBW = low birthweight, NBW = normal birthweight) and experimental *E. faecium* treatment (Treatment = 1ml 5 x108 CFU *E. faecium*, Control = 1ml PBS). Data are expressed as adjusted means ± SEM.

Post-weaning performance measure	Birthweight class			*E. faecium* treatment			P value		
	LBW	NBW	SEM	Treatment	Control	SEM	BiW class	*E. faecium* treatment	BiW class * *E. faecium* treatment
Pen mean FI (kg/hd/day)	0.29[a]	0.35[b]	0.018	0.32	0.32	0.012	0.0033	0.9209	0.2789
Pen mean FCR	1.21	1.08	0.092	1.14	1.15	0.053	0.1864	0.8903	0.8652

[a,b] Different superscripts demonstrate a significant difference between fixed effect levels ($P < 0.05$).

Diarrhoea incidence was low across the experimental period time points. However, there was a significant effect of piglet age ($P < 0.001$) with respect to diarrhoea occurrence. Diarrhoea occurrence was significantly higher between days 15 - 21 and days 22 – 27 (than on days 0 - 7 and days 35 – 39 (**figure 5.2b**). There was no significant effect of BiW class on the proportion of piglets who developed diarrhoea ($P > 0.05$). There was a significant effect of experimental *E. faecium* treatment ($P < 0.001$), with *E. faecium* Treatment piglets having lower diarrhoea incidence (2.4% ± 0.39 SE) than Control piglets (5.2% ± 0.57 SE). Furthermore, there was a significant interaction between experimental *E. faecium* treatment and piglet age ($P < 0.001$), with *E. faecium* Treatment piglets have lower diarrhoea occurrence between days 28 – 35 of age compared with Control piglets (**figure 5.2b**) There was no significant interaction between BiW class and time point in relation to diarrhoea occurrence, or significant interaction between BiW class, experimental *E. faecium* treatment and time point ($P > 0.05$).

a)



b)

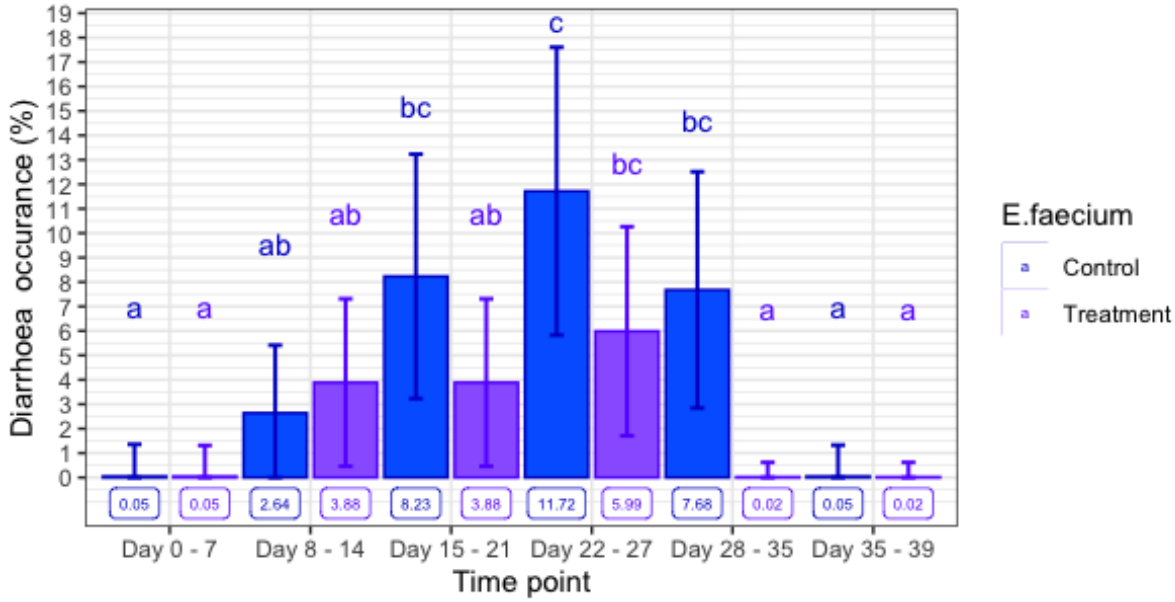


Figure 5.2. Diarrhoea occurrence across the 39-day experimental period (a). Diarrhoea occurrence associated with each successive week of the 39-day experimental period with respect to experimental *E. faecium* group. Different superscripts letters (a – c) denote significant differences ($P < 0.05$). Adjusted mean values and confidence intervals are plotted.

5.3.3 Microbiota analysis

At the phylum level, the faecal microbiota of day 8 piglets was dominated by Firmicutes (40.9% ± 0.69 SD), Bacteroidetes (29.8 % ± 2.26 SD) and Proteobacteria (13.8% ± 2.06 SD) (**figure 5.3a**), whilst at the genera level the microbiota was dominated by *Bacteroides* (19.0% ± 2.79 SD), *Escherichia/Shigella* (13.8% ± 2.08), *Lactobacillus* (5.0% ± 0.65 SD), *Limosilactobacillus* (5.7% ± 1.45 SD), *Lachnoclostridium* (3.6% ± 0.52 SD),

Oscillospiraceae UCG-002 (3.2% ± 0.47 SD), *Prevotella* (2.7% ± 1.72 SD), *Prevotellaceae NK3B31 group* (2.6% ± 1.45 SD) and *Fusobacterium* (2.1% ± 0.62 SD) (**figure 5.3b)**.

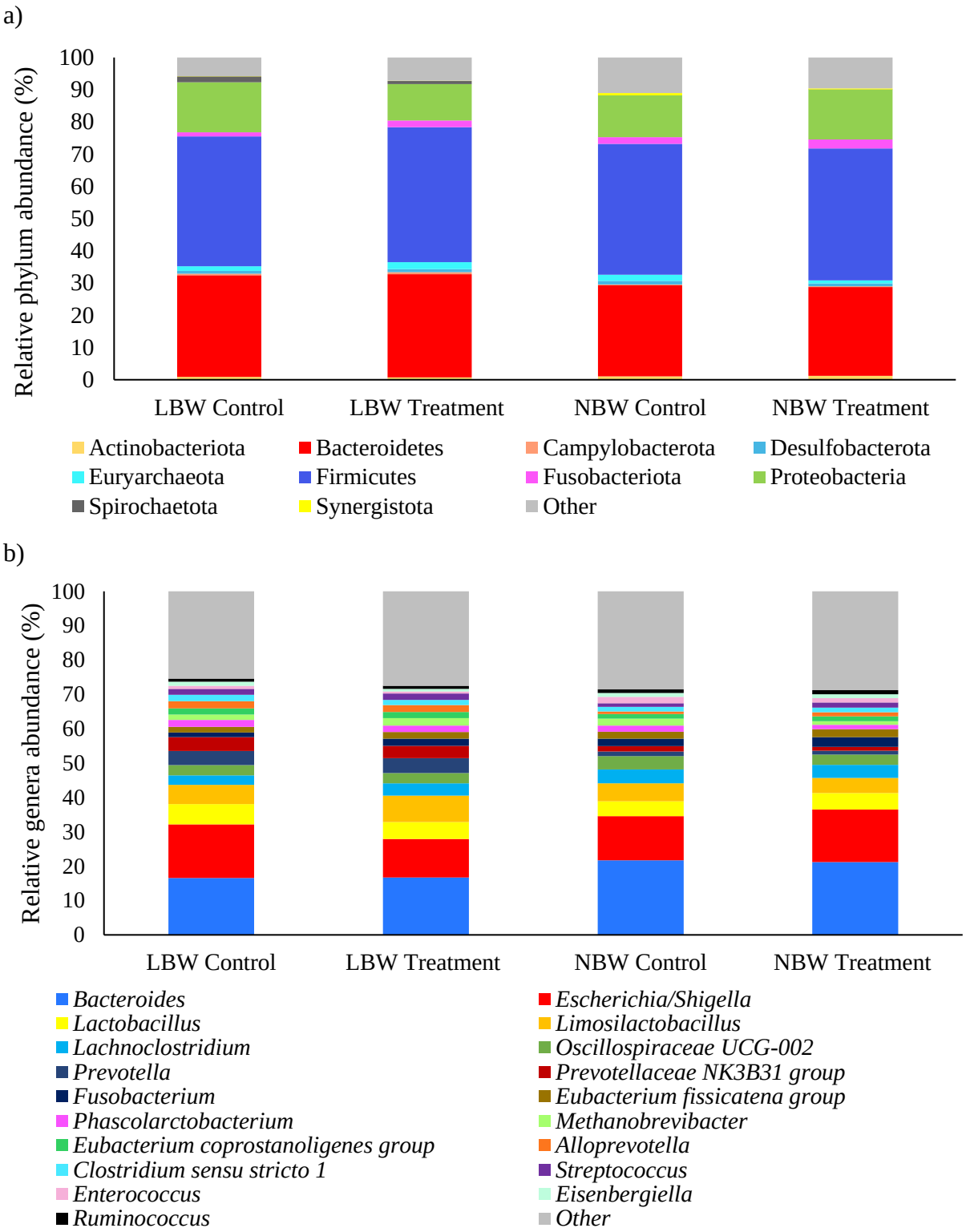


Figure 5.3. The top 11 phyla (a) and 20 genera (b) present in piglet faeces on day 8.

5.3.3.1 Alpha diversity

There was a significant effect of BiW class on the number of observed OTUs ($P < 0.05$; LBW = 109.9 ± 2.67 SE vs NBW = 99.8 ± 2.72 SE) and Shannon diversity index ($P < 05$; LBW = 3.17 ± 0.041 SE vs NBW 3.03 ± 0.042 SE) (**figure 5.4ab)**. There was no significant effect of experimental *E. faecium* treatment on either the number of observed OTUs ($P > 0.05$) or Shannon diversity index ($P > 0.05$) (**figure 5.4cd)**. Furthermore, there was no significant interaction between BiW class and experimental *E. faecium* treatment on the number of observed OTUs or Shannon diversity index ($P > 0.05$).

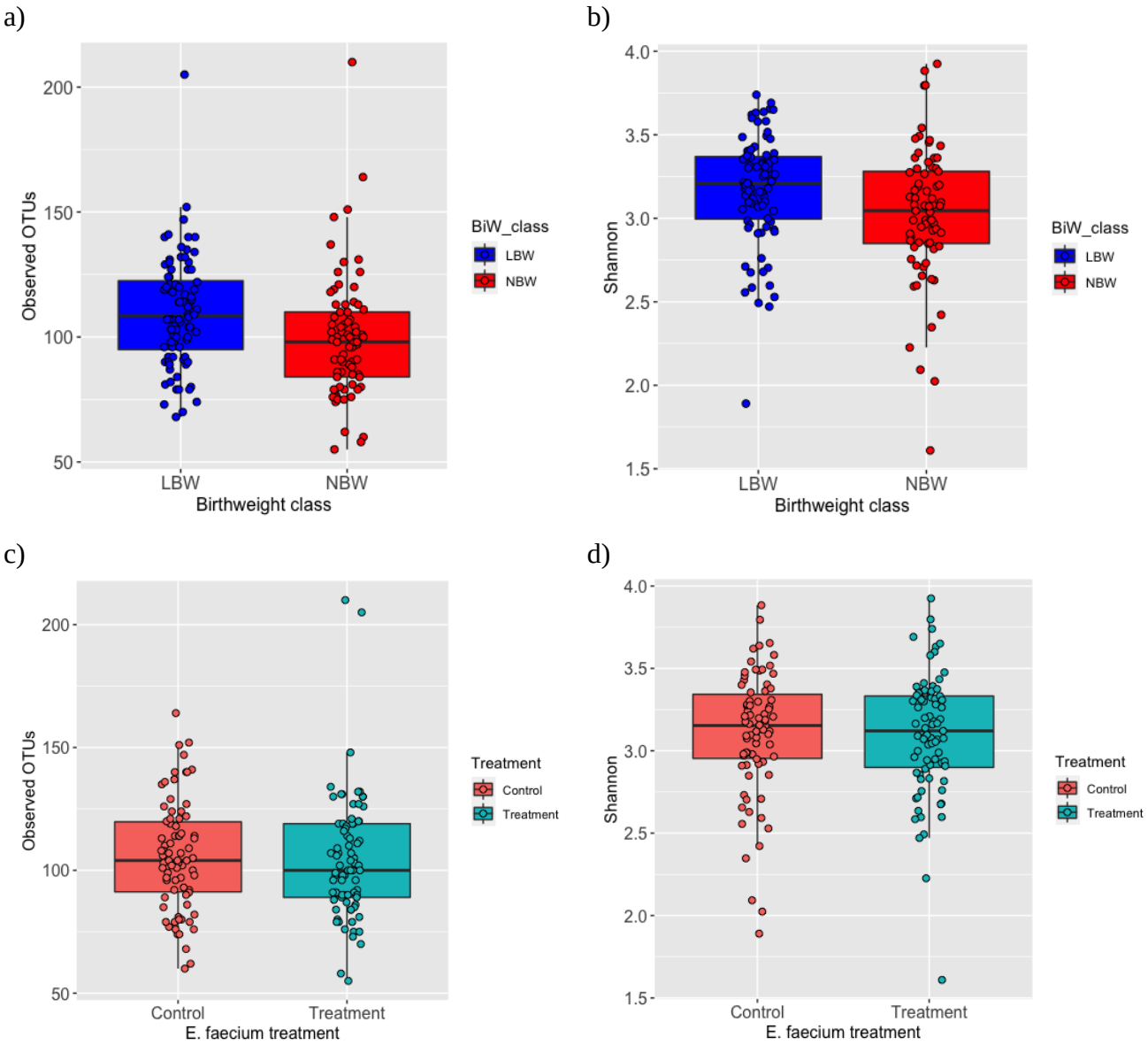


Figure 5.4. The number of observed OTUs (a, c) and Shannon diversity index (b, d) according to birthweight (BiW) class (LBW = low BiW, NBW = normal BiW) and *E. faecium* treatment.

5.3.3.2 Beta diversity

The weighted and unweighted UniFrac distances were significantly different between BiW classes ($P < 0.01$) (**figure 5.5a and b)**. There was no significant effect of experimental *E. faecium* treatment on either the weighted or unweighted UniFrac distances ($P > 0.05$) (**figure 5.5c and d**). Furthermore, there was no significant interaction between BiW class and experimental *E. faecium* treatment for either weighted or unweighted UniFrac distances ($P > 0.05$).

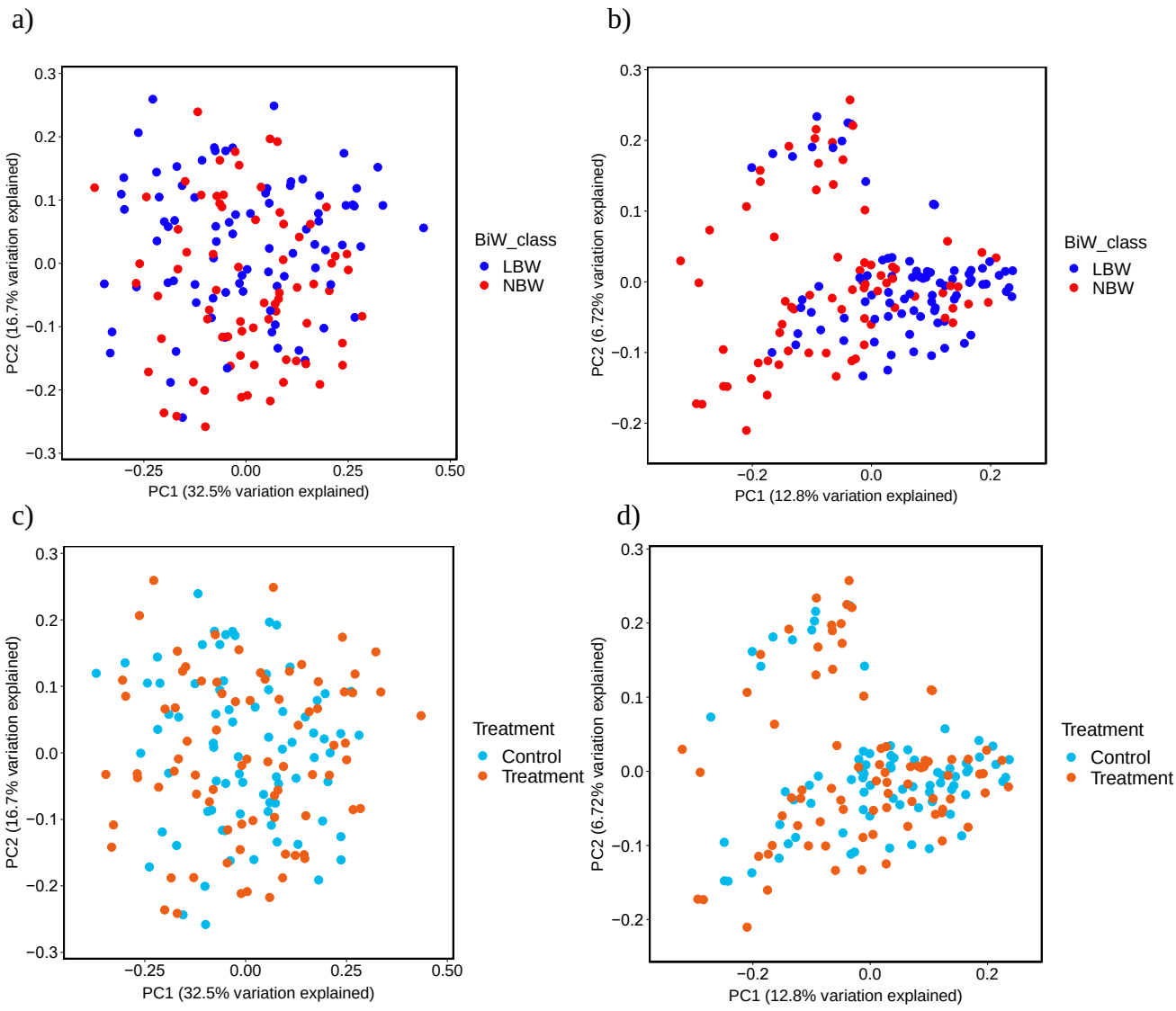


Figure 5.5. Principal Component Analysis plots of weighted (a, c) and unweighted (b, d) UniFrac distances between piglets grouped by birthweight (BiW; a, b) class (LBW = low BiW, NBW = normal BiW) and *E. faecium* treatment (c, d).

5.3.3.3 Genera abundance

There was a significant effect of BiW class on genera relative abundance, with LBW piglets having a significantly higher abundance of *Alloprevotella* (FDR adjusted P < 0.05) and *Christensenellaceae R-7 group* (FDR adjusted P < 0.05) (**figure 5.6a and b)**. There was no significant effect of experimental *E. faecium* treatment on genera abundance (FDR adjusted P > 0.05). Furthermore, there was no significant interaction between BiW class and experimental *E. faecium* treatment on genera abundance (FDR adjusted P > 0.05). Whilst there was no significant difference in the relative abundance of *Enterococcus* between experimental *E. faecium* treatment and BiW class groups (**figure 5.7a**), *E. faecium* Treatment piglets had a significantly higher prevalence of *Enterococcus* (P < 0.001), with *Enterococcus* detected in more *E. faecium* treated piglets. There was no significant effect of BiW class on *Enterococcus* prevalence (P > 0.05). However, there was a significant interaction between BiW class and experimental *E. faecium* treatment, with NBW Treatment piglets having significantly higher prevalence of *Enterococcus* than LBW and NBW Control piglets (**figure 5.7b**). The relative abundance of *Enterococcus* was similar to that previously observed for age matched piglets in Chapters 2 and 4 (**figure 5.7c, d**).

a) b)

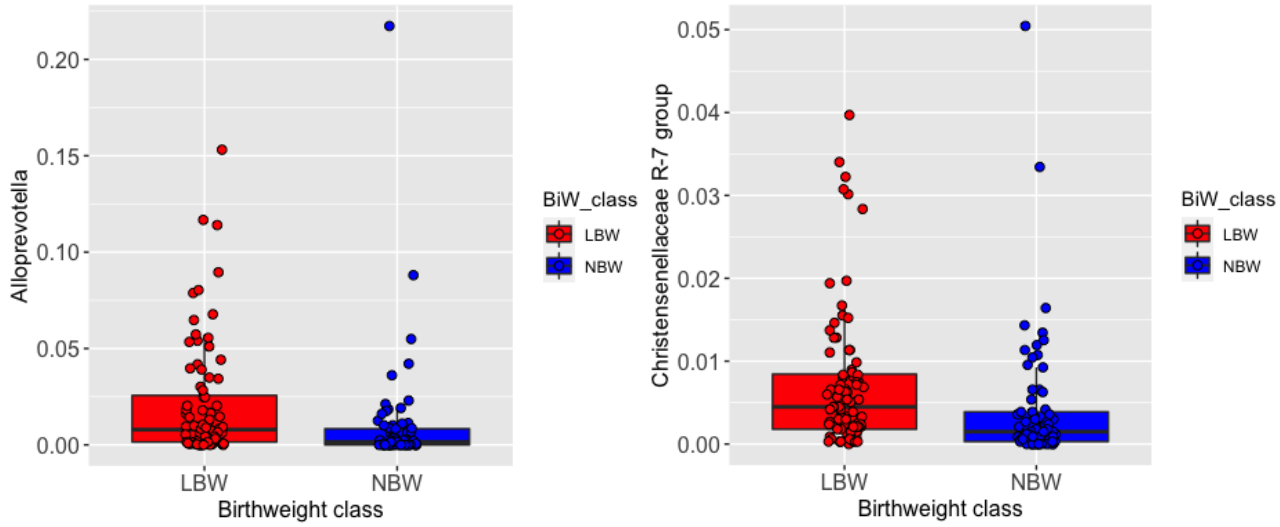
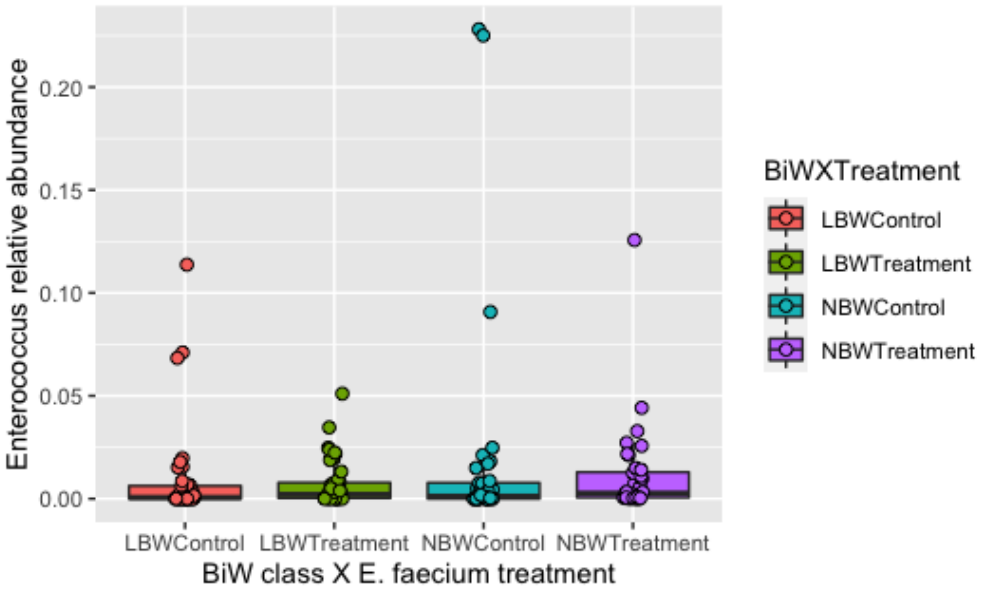
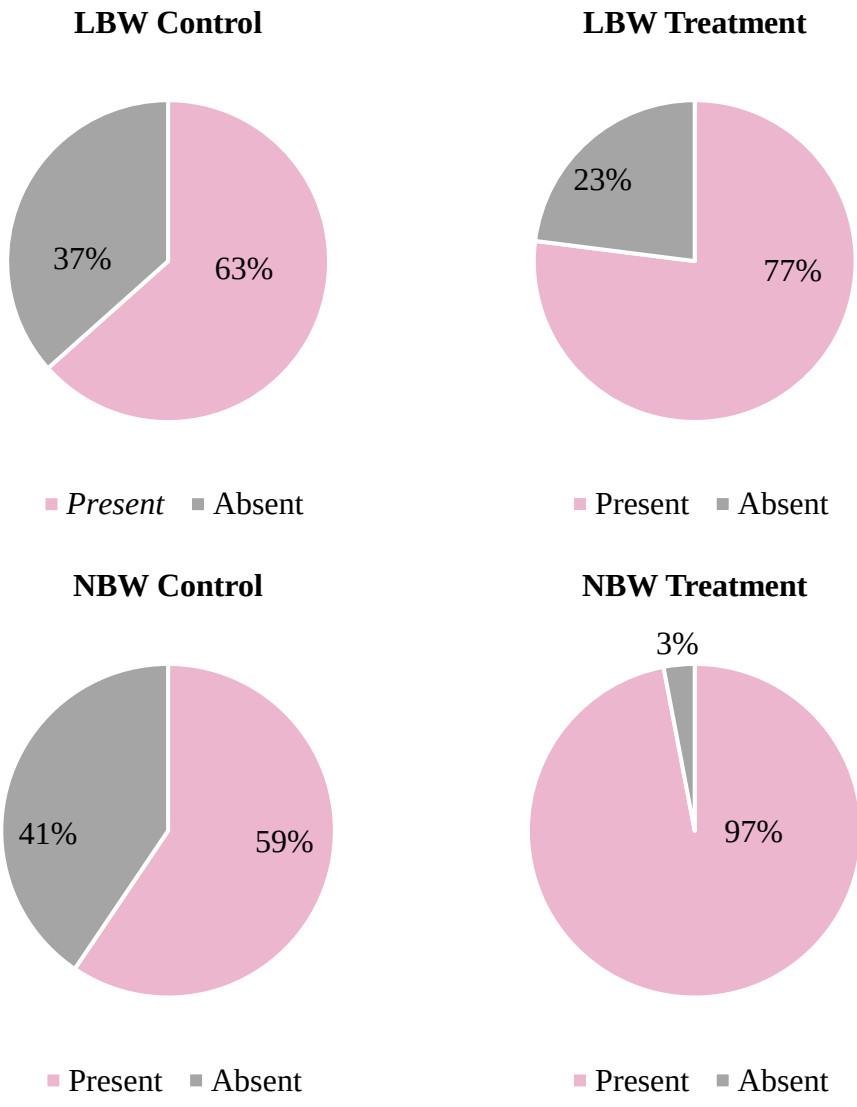


Figure 5.6. The relative abundance of *Alloprevotella* (a) and *Christensenellaceae R-7 group* (b) for each birthweight (BiW) class (LBW = low BiW, NBW = normal BiW). The raw relative abundance values are plotted opposed to the arcsine square root transformed values used in the analysis.

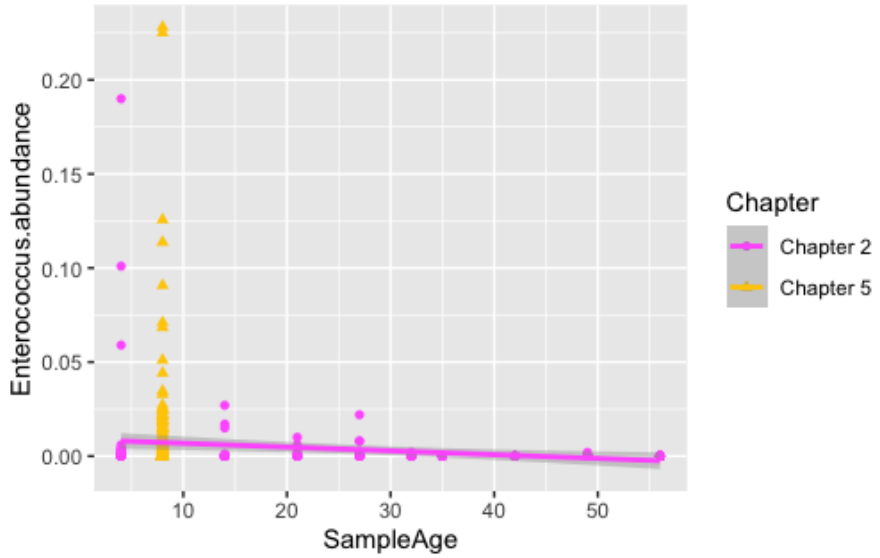
a)


Enterococcus relative abundance
0.20
0.15
0.10
0.05
0.00
LBWControl
LBWTreatment
NBWControl
NBWTreatment
BiW class X E. faecium treatment
BiWXTreatment
LBWControl
LBWTreatment
NBWControl
NBWTreatment

b)


LBW Control
37%
63%
Present
Absent
LBW Treatment
23%
77%
Present
Absent
NBW Control
41%
59%
Present
Absent
NBW Treatment
3%
97%
Present
Absent

c)



d)

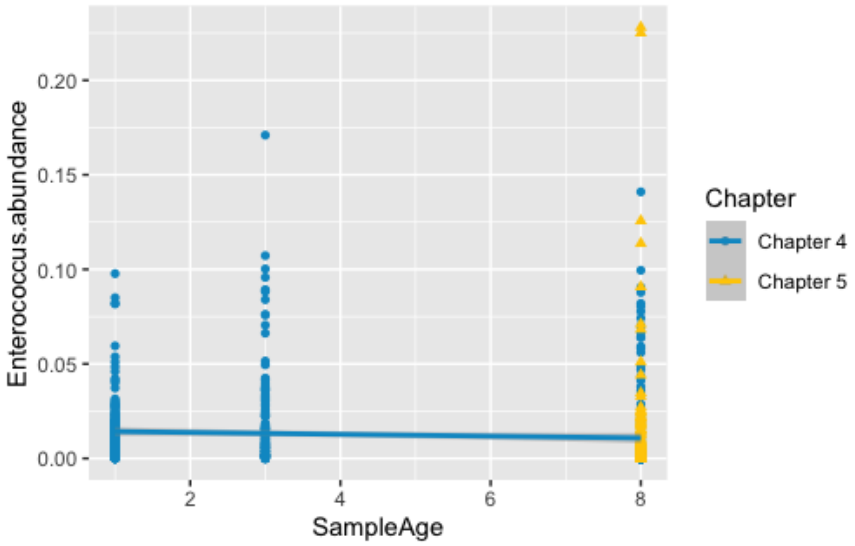


Figure 5.7. The relative abundance (a) and prevalence (b) of *Enterococcus* in experimental piglets according to piglet birthweight class (low birthweight (LBW) or normal birthweight (NBW)) and experimental *E. faecium* treatment (Treatment or Control). The proportion of piglets within each birthweight class and experimental *E. faecium* treatment where the presence of *Enterococcus* was detected are coloured in pink (b). The relative abundance of *Enterococcus* observed in the present study in relation to Chapters 2 and 4 (c, d).

5.4 Discussion

The study hypothesised that experimental *E. faecium* treatment over the first 7 days of life would alter the microbiota profile at 8 days of age and improve pre- and post-weaning performance in a BiW dependant manner. The hypothesis was rejected as there was no

significant difference in the microbiota or performance of *E. faecium* Treatment and Control piglets, nor an interaction with BiW class. However, *E. faecium* treatment was able to reduce diarrhoea occurrence. The study also hypothesised that there would be an interaction between birthweight and experimental *E. faecium* treatment on the microbiota profile of piglets. This was rejected as there was no differences in the microbiota profile of LBW and NBW piglets, nor an interaction with experimental *E. faecium* treatment. However, NBW pigs had superior performance throughout the experiment.

5.4.1 The effect of E. faecium on the microbiota profile and piglet performance

The experimental *E. faecium* treatment displayed no significant effect on the microbiota profile of piglets in relation to alpha diversity, beta diversity or genera relative abundance at 8 days of age. Therefore, the results of this study demonstrated the *E. faecium* treatment was unable to modulate the microbiota outside of the experimental *E. faecium* treatment period which ceased > 24 hours prior to faecal sampling of piglets. Day 8 was selected for analysis as part of the study aimed to determine whether early life DFM could modulate the microbiota beyond the immediate period of experimental *E. faecium* treatment. The treatment period was selected to overlap with the period of microbiota developmental plasticity, thus the period with greatest potential to alter the developmental pattern of the microbiota and subsequently piglet health and performance. In the present study, no effects on the microbiota were observed. However, future studies should also analyse the microbiota profile during the DFM treatment period to determine whether modulation of the microbiota occurred whilst piglets were actively receiving the DFM treatment.

Peng *et al.* (2019) provided piglets with *E. faecium* NCIMB 10415 (3×10^9 CFU/day) treatment between days 1-7 of life and reported no significant effect on alpha or beta diversity at 10 days of age, similarly to the findings of the present study, although a reduction in the genus *Bilophilia* was observed. Wang *et al.* (2016b) dosed piglets with an autogenous *E. faecium* strain EF1 (6×10^8 CFU/ml) in 2ml 10% sterile skimmed milk on days 1, 3 and 5 of life, which is a similar experimental model to the present study where $4.19 – 4.68 \times 10^8$ CFU/ml was given on days 1 - 7 of life. Wang *et al.* (2016b) conducted a small study, determining the caecal microbiota profile on days 25 (weaning) and 33 of age with 3 piglets per treatment, reporting that alpha diversity was not be affected by *E. faecium* treatment pre-weaning, but increased alpha diversity post-weaning. They further reported that beta diversity was be significantly affected pre- and post-weaning by *E. faecium* treatment. Moreover, the study reported an increase in the abundance of *Lactobacillus, Ruminococcus* and *Collineslla*

and a reduction on *Bacteroides* and *Fusobacterium* pre-weaning, but did not report changes to *Enterococcus* abundance. However post-weaning *Enterococcus* abundance was notably increased along with the abundance of *Bacteroides*, *Paraprevotella*, *Ruminococcus* and *Faecalibacterium*, whilst the abundance of *Blautia* was reduced (Wang *et al.*, 2016b). Therefore, although *E. faecium* treatment ceased on day 5, a difference in the relative abundance of *Enterococcus* was not observed until post-weaning, suggesting that early life DFM intervention has more apparent effects on the microbiota profile in response to periods of stress and possible microbiota dysbiosis. When *E. faecium* NCIMB 11181 was given to piglets post-weaning for 2 weeks there were significant changes to the microbiota community composition, with increased alpha diversity and an increase in the relative abundance of *Lactobacillus* (*L.johnsonii* and *L.fermentum*), *Clostridium* (*C.butyricum*), *Enterococcus, Megasphera, Oscillibacter* and *Succinivibrio,* whilst the relative abundance of *Eschericchia* (*E.coli*) and *Dialister* were reduced (Pajarillo *et al.*, 2015). Whilst no effects on the microbiota, or *Enterococcus* abundance were noted in the present study pre-weaning, similarly to Peng *et al.* (2019) and Wang *et al.* (2016b), the effects on the microbiota could be more long term (Wang *et al.*, 2016b). Consequently, future work should also analyse the post-weaning microbiota in response to early life DFM treatment, but also GIT segments, as the DFM treatment may affect the microbiota of each GIT section differently. Faecal microbiota samples were collected pre- and post-weaning on each weigh day, however due to the global COVID-19 pandemic it was not possible to analyse all of these samples.

Despite no significant effect of experimental *E. faecium* treatment on the microbiota profile in the present study, precautions were taken to maximise the potential for the experimental *E. faecium* treatment to colonise the neonatal GIT. As previously shown in Chapter 4 the microbiota community composition changes within the first week of life, and so the bacteria cultured from older piglets (such as day 8 samples used in laboratory pilot studies) may not be suitable to colonise the GIT of piglets on day 1 of life compared with early life samples. For this reason, *E. faecium* was cultured from day 3 samples. Furthermore, *E. faecium* was selected for experimental work as it is a facultative anaerobe, thus able to colonise the GIT which is not strictly anaerobic until a few days of age in piglets. *Enterococcus* abundance also appears to decline with age in piglets as demonstrated in Figure 8, therefore justifying the use of a day 3 faecal sample in order to successfully isolate *E. faecium*.

Part of the thesis aim was to promote growth of piglets through manipulation of the microbiota, particularly in LBW piglets. Thus, *E. faecium* was cultured from a faecal sample from a LBW piglet which had superior pre-weaning ADG in the experiment described in

Chapter 4. This was done in order to utilise a bacterium which should be able to colonise the GIT of LBW piglets, as differences in the GIT microbiota exist between LBW and NBW piglets (Chapter 2, Gaukroger *et al.*, 2020; Li *et al.*, 2018, 2019, Zhang *et al.*, 2019a).

In studies which have isolated and used other autogenous LAB strains for the probiotic treatment of neonates (Liu *et al.*, 2014; Yang *et al.*, 2020, Zhang *et al.*, 2019a), the effects of freezing without a cryopreservative on the defrosted viable counts of a DFM have not been ascertained. Freezing an inoculum, rather than preparing an inoculum fresh in a sterile environment, offers the most practical solution for on-farm application, and thus needs to be explored. In the present study we reported that the action of freezing induces varied effects on defrosted *E. faecium* viability, with on average 59% $\pm$ 29.5% SD loss in viability, however we showed that viable counts after defrosting were stable with up to 5 months of freezing to -80ºC. Based on the loss of viable counts and decision not to use a cryopreservative agent (including glycerol or sterile skimmed milk, due to the effects this may have on diarrhoea and microbiota composition, respectively), we increased the original concentration of the experimental *E. faecium* treatment prior to freezing, so that viable counts after freezing were close to the those which have been used in studies which reported beneficial effects on piglet performance following oral delivery (Zeyner and Boldt, 2006; Wang *et al.*, 2016b), or in pre- and post-weaning feed (Scharek *et al.*, 2007; Pajarillo *et al.*, 2015).

Care was taken to ensure experimental piglets received the full *E. faecium* dose. Firstly, the experimental *E. faecium* treatment was delivered slowly to the back of the tongue with the piglets oriented slightly backwards to facilitate swallowing and to minimise dribbling loss of the inoculum. Piglets were only placed back in the pen once they were observed to swallow the inoculum, with any dribbling piglets recorded each day. The 1 ml dosage for the first 7 days of life was used to facilitate swallowing, as larger volumes would increase the risk of dribbling loss, especially in LBW neonates. Thus, a high *E. faecium* inoculum dose once per day was deemed to be the most effective mode of delivery compared to the 2ml every other day as in Wang *et al.* (2016b), particularly as the *E. faecium* was suspended in PBS rather than the more appetising skimmed milk.

However, in spite of our best efforts, the experimental *E. faecium* treatment did not significantly affect the microbiota of experimental piglets, with *Enterococcus* relative abundance in line with age matched samples from Chapter 2 and 4 (Figure 8). The absence of detectable changes in the microbiota on day 8 suggests the *E. faecium* was not able to colonise the GIT at higher levels after the termination of experimental *E. faecium* treatment. This may be due to the faster GIT transit time observed in suckling piglets compared with weaned piglets (Snoeck *et al.*, 2004; Everaert *et al.*, 2017), such that additional *Enterococcus*

abundance resulting from administration only to day 7 would not be detected in faecal samples on day 8. However, in the present study, within the *E. faecium* treated piglets there was a significantly higher prevalence of *Enterococcus* on day 8, indicating that, whilst the *E. faecium* treatment did not increase overall relative abundance of *Enterococcus*, it did increase representation of the genus amongst piglets (more piglets colonised with Enterococcus). In an earlier study by Vahjen *et al.* (2007), sows were given *E. faecium* NCIMB 10415 during gestation and lactation. Piglets from *E. faecium* fed sows had lower total *Enterococcus* and *E. faecalis* in the colon at 14 days of age, demonstrating *E. faecium* to regulate the *Enterococcus* genera abundance. Furthermore, Starke *et al.* (2015) reported that, *in vitro*, *E. faecium* NCIMB 10415 had direct growth effects on porcine GIT species/strains. *E. faecium* NCIMB 10415, in co-culture, did not affect the growth of 3 out of 4 *E. faecium* strains but did reduced the growth of 3 out of 4 *E. faecalis* strains. Moreover, the *E. faecium* NCIMB 10415 increased the growth of *L. johnsonni DSM 10333* and *L. reuteri DSM 20016*, but had no effect on 2 strains of *Salmonella Typhimurium* and *E. coli*. Both Vahjen *et al.* (2007) and Starke *et al.* (2015) showed *Enterococcus* species abundance changed in response to *E. faecium* treatment. Therefore, it could be suggested that the absence of change in *Enterococcus* relative abundance, which was low on day 8, may be due to regulation of other *Enterococcus* species by *E. faecium* resulting in no change to the overall relative abundance of the genus. However, in this experiment we could not determine differences in the microbiota of *E. faecium* Treatment piglets down to species level; using metagenomic sequencing may have been able to resolve this but use of this more costly sequencing method was not possible within the budget of the current project. Furthermore, qPCR may have been useful to detect specific changes in species abundance in response to experimental *E. faecium* treatment, or to identify treatment responders and non-responders within the experimental *E. faecium* treatment, as done by Starke *et al.* (2013), enabling further grouping of piglets to conduct a 2 x 2 x 2 factorial design on the microbiota and performance data analysis. Although qPCR and metagenomic sequencing were outside of the scope of this study, they should be considered for future research along with longitudinal microbiota analyses.

Experimental *E. faecium* treatment did not significantly affect pre- or post-weaning performance, feed intake or FCR. The results of this experiment are consistent with some other reports in the literature (Taras *et al.*, 2006; Broom *et al.*, 2006; Martin *et al.*, 2012; Peng *et al.*, 2019). This was unsurprising as no modulation to the microbiota profile by experimental *E. faecium* treatment occurred. However, in other studies, the lack of improvement to performance may be attributed to the strain of *E. faecium* used, dose, length

of treatment and form (orally or in-feed). Direct oral delivery of *E. faecium* NCIMB 10415 until weaning (Zeyner and Boldt, 2006), rather than only providing *E. faecium* in creep feed (Taras *et al.*, 2006; Martin *et al.*, 2012), was better able to significantly improve piglet performance as creep feed intake is very low pre-weaning (Pajor *et al.*, 1991), with intakes only really increasing from day 19 of age (Huting *et al.*, 2017). Therefore, selecting to deliver *E. faecium* orally should have been the most beneficial mode of delivery to promote piglet performance. A review by Barba-Vidal *et al.* (2018), discussing the effectiveness of probiotics in post-weaning pigs, described inconsistencies in results to be related to the high variability in trial designs, with probiotics used for different time periods, days of age, different probiotic concentrations and different modes of probiotic delivery (orally, top dressed or in feed). Furthermore, Barba-Vidal *et al.* (2018) noted that the results of probiotic trials depend on host genetics and health status of the environment tested, in accordance with Broom *et al.* (2006), as the microbiota of pigs differs between genetics (Kubasova *et al.*, 2018; Xiao *et al.*, 2016; Xiao *et al.*, 2017; Bergamaschi *et al.*, 2020a) and with sanitary status (Mulder *et al.*, 2009; Te Pas *et al.*, 2020). The review concluded that strains should be selected for specific purposes (therapeutic or prophylactic treatment and for specific purposes within each treatment), rather than to improve overall performance and health of pigs.

There was a low incidence of pre-weaning and post-weaning diarrhoea in the present study which may explain why no improvements in performance were noted between experimental E. faecium treatment groups. Broom *et al.* (2006) suggested that a lack of beneficial effect of *E. faecium* on piglet performance may be related to farm health status, whereby any beneficial effects of *E. faecium* are masked under adequate - high health conditions. However, despite the low rates of pre- and post-weaning diarrhoea, *E. faecium* Treatment significantly reduced diarrhoea occurrence across the 39 day experimental trial, and specifically during the initial post-weaning period. In the literature, the main effect of *E. faecium* probiotics, namely *E. faecium* NCIMB 10415, seems to be related to reducing diarrhoea incidence and duration (Taras *et al.*, 2006; Zeyner and Boldt, 2006) and modulation of the immune system. *E. faecium* reportedly reduces *E. coli* adherence to the intestinal mucosa (Bednorz *et al.*, 2013b), ß-haemolytic and 0141 serovars of *E. coli* in the intestinal contents of piglets (Scharek *et al.*, 2007) and faecal *E. coli*; whilst Taras *et al.* (2006) also noted that *E. faecium* reduced the virulence genes of *E. coli*. *E. faecium* is also able to exhibit antiviral properties, reducing rotavirus shedding (Kreuzer *et al.* 2012). The antiviral properties are proposed to be via virus entrapment (Wang *et al.*, 2013c), increasing nitric oxide release from epithelium cells and immunoregulation (Wang *et al.*, 2013c; Kreuzer *et al.*, 2012). Wang *et al.* (2016b) reported a

significant reduction in the incidence of pre- and post-weaning diarrhoea in piglets treated with *E. faecium* orally during early life. Consequently, increased prevalence of *Enterococcus* in early life may have conferred protective effects against diarrhoea post-weaning. Future studies should monitor *Enterococcus* prevalence, in relation to diarrhoea, across multiple sampling time points pre- and post-weaning. Moreover, future studies should also collect blood and intestinal tissues to determine the effects of direct fed autogenous strains of bacteria on the immune function in the absence of pathogen challenge, and whether these effects are BiW class dependant, as differences in the immune function exist between LBW and NBW piglets (Lessard *et al.*, 2018; Lo Verso *et al.*, 2020).

Kiros *et al.* (2019) demonstrated that either 5 x 10^9 or 2.5 x 10^{10} CFU/day *Saccharomyces cerevisiae* on alternative days between days 1 – 28 of age was able to increase piglet performance and alter the microbiota community composition and genera abundances of caecal contents at 28 days of age. However, the study did not establish whether the effects on the microbiota profile were retained after removal of *S. cerevisiae*. Similarly, Yang *et al.* (2020) demonstrated 5 x 10^7 CFU/ml of *Lactobacillus reuteri* KT260178 oral inoculum for the first 7 days of life, followed by 1.0 x 10^7 CFU/g creep feed and starter diet until 28 days of age (weaning day 21) was able to increase the *Lactobacillus* and *Bifidobacterium* abundance in the caecum and reduce the abundance of *E. coli* and *Staphylococcus.* Meanwhile, *L. reuteri* colonisation of the distal jejunum and ileum was increased. However, the study did not monitor any modulation to the microbiota after termination of *L. reuteri* KT260178 treatment. Thus, based on the piglet literature, it appears that a variety of different probiotic species can modulate the microbiota when administered during early life. However, evidence for microbiota modulation after cessation of probiotics treatment is yet to be fully ascertained and must be addressed in future research.

5.4.2 The effect of BiW class on piglet performance and microbiota profile

Low birthweight piglets had significantly lower LW at all time points pre- and post-weaning, consistent with the findings in Chapter 2 (Gaukroger *et al.*, 2020) and in the literature (Douglas *et al.*, 2014a,c; Huting *et al.*, 2017, 2018, 2019; Li *et al.*, 2018; Zhang *et al.*, 2019a). Furthermore, LBW piglets had significantly lower ADG between time points 5-8, 8-13, 19-27 and 32-39 days and for the whole 39-day experimental period. Similarly, poorer ADG in LBW piglets has been reported in the literature (Douglas *et al.*, 2014a,c; Huting *et al.*, 2017, 2018, 2019; Li *et al.*, 2018; Zhang *et al.*, 2019a). However, no significant difference between BiW classes were noted between days 13-19, similarly to Gaukroger *et al.* (2020; Chapter 2). This may be related to LBW piglets not displaying severe intra-uterine growth restriction in

this study, with the associated subsequent impairments (Rutherford *et al.*, 2013). Therefore, as a teat order is established and peak lactation is reached, NBW piglets might become more restricted by intake until solid feed consumption becomes significant. The ADG was not significantly different between LBW and NBW piglets between days 27-35, coinciding with a significant reduction in ADG in an otherwise sigmoidal growth curve, with ADG during this period similar to that between days 3-5 of age, as observed in Chapter 2 (Gaukroger *et al.*, 2020). The decline in ADG immediately post-weaning was related to weaning stressors, many of which affect both NBW and LBW piglets in a similar manner, including removal from the sow, loss of passive immunity, handling stress, new pen environment and change in diet type with the removal of sow milk (Campbell *et al.*, 2013). Piglets were grouped at weaning according to rearing cohort and experimental *E. faecium* treatment, thus mixing stress and fighting post-weaning were minimised. However, NBW piglets eat more creep feed than LBW pre-weaning, with a larger proportion of the litter classed as 'eaters' at weaning (Huting *et al.*, 2017, 2019), and are thus expected to show a significantly higher feed intake post-weaning. However, whilst NBW piglets had a higher feed intake post-weaning, this did not result in a significant improvement in FCR, as previously reported (Huting *et al.*, 2019).
The LBW piglet microbiota had a significantly higher number of OTUs and Shannon diversity index on day 8. Conversely, Zhang *et al.* (2019a) reported LBW pigs to have a lower alpha diversity in the jejunum at 7 days of age. The microbiota community composition was significantly different between LBW and NBW piglets at 8 days of age, based on weighted and unweighted UniFrac distances. Similarly, Li *et al.* (2018) reported significant differences in Bray Curtis distances between BiW classes on day 8, and Zhang *et al.* (2019a) reported significant differences in the jejunum and ileum microbiota community composition between BiW classes on days 7, 21 and 28, based on unweighted UniFrac distances. Conversely, Li *et al.* (2019) reported no significant difference in the microbiota community composition between BiW classes along any GIT segment using unweighted UniFrac distances. Differences in microbiota community composition, particularly weighted UniFrac distance, could potentially be related to significant increases in the abundance of *Alloprevotella* and *Christensenellaceae R-7 group* in LBW piglets. *Christensenellaceae R-7 group* had previously been reported to have higher abundance in LBW piglet faeces at 21 days of age (Li *et al.*, 2018). Increased Christensenellaceae family abundance has also been associated with lower LW in pigs at 103 days of age (Oh *et al.*, 2020). Conversely, Quan *et al.* (2018) reported *Christensenellaceae R-7 group* to be significantly more abundant in the ileum of finisher pigs with a lower FCR, this may be related to the SCFA production by carbohydrate fermentation (Morotomi *et al.*, 2011), thus improving gut health. Higher abundance of the

Christensenellaceae family has also been reported finisher pigs with a low residual intake (higher feed efficiency) (McCormack *et al.*, 2017). *Alloprevotella* was also significantly increased in the faecal microbiota of LBW piglets on D8; similarly, Li *et al.* (2018) reported that the abundance of *Alloprevotella* to be increased in LBW piglet faeces at 14 days of age. *Alloprevotella* has previously been shown to be negatively correlated with the *Escherichia* genus, which was identified as the key node in a correlation network analysis associated with diarrhoea in piglets (Sun *et al.*, 2019), and found in higher abundance in healthy vs diarrhoetic piglets (Han *et al.*, 2019). Both *Alloprevotella* and *Christensenellaceae R-7 group* belong to bacteria families which produce SCFA, suggesting LBW piglets may have altered microbiota fermentation patterns compared to NBW piglets in early life.

To conclude, the autogenous *E. faecium* strain given to piglets for the first 7 days of life at a concentration of 4.19 - 4.68 x 10^8 CFU/ml PBS had no significant effect on performance or microbiota profile in NBW and LBW pigs. Meanwhile, *E faecium* treatment was able to significantly reduce incidences of diarrhoea, particularly post-weaning diarrhoea. It is suggested that the lack of significant change to the microbiota profile may explain the absence of improved performance following early life experimental *E. faecium* treatment. However, prevalence of *Enterococcus* (the number of piglets colonised with *Enterococcus*) was higher *in E. faecium* Treatment piglets, suggesting that although *E. faecium* treatment increased *Enterococcus* prevalence, it did not increase relative abundance. Thus, *E. faecium* was either out-competed by the commensal early life microbiota, explaining the absence of changes in the relative abundance of other genera, and/or *E. faecium* may have modulated the *Enterococcus* relative abundance at the species level, as opposed to additively increasing *Enterococcus* relative abundance. Meanwhile, LBW pigs had a different microbiota profile on day 8, with LBW piglets displaying increased microbiota richness and diversity and a significantly higher abundance of *Alloprevotella* and *Christensenellaceae R7 group*, suggesting differences in microbiota fermentation patterns.

Chapter 6. General discussion

The aim of the thesis was to understand how the piglet microbiota establishes and develops during early life, and whether this is affected by piglet BiW class, and to investigate whether microbiota markers for superior early life performance could be identified and utilised. As part of this aim, it was necessary to understand the influence of maternal and management factors in relation to altering the microbiota and performance of piglets, including birth sow parity and cross-fostering. Furthermore, the potential of an autogenous *E. faecium* treatment to modulate the early life microbiota development and performance of piglets was assessed. The findings of the thesis in relation to these aims, and the future research opportunities which these suggest, are discussed in this chapter.

6.1 Microbiota seeding of the GIT by the sow and the importance of sow

The sow presents one source of bacteria for seeding of the GIT of the neonatal piglet. The sow faecal microbiota undergoes significant change during the periparturient period, with sows exhibiting a significantly different microbiota community composition on day 3 of lactation (Chapter 3 and 5; Cheng *et al.*, 2018), compared with the end of gestation and day 8 of lactation. Moreover, the experiment reported in Chapter 3 demonstrated that multiparous sows had significantly higher alpha diversity during the periparturient period, while in Chapter 4 it is reported that progeny from multiparous sows had significantly higher Shannon diversity index during the neonatal period than progeny from primiparous sows. Therefore, neonatal piglet microbiota evenness seems inherently affected by that of their birth mother in a parity dependant manner. The microbiota community composition was also significantly different between progeny from multiparous and primiparous sows during the neonatal period (Chapter 4), coinciding with the period of sow microbiota dysbiosis (Chapter 3). Future research should identify microbiota taxonomic and metabolic functional differences between progeny from primiparous and multiparous sows to determine whether differences in the microbiota contribute to the mechanisms by which progeny from primiparous sows exhibit poorer performance (Carney-Hinkle *et al.*, 2013; Craig *et al.*, 2017)

Both maternal and environmental microbiota sources have a fundamental role in shaping the neonatal piglet microbiota profile. Previous research has reported the farrowing pen floor, vagina and milk to be the first sources of microbiota to transiently colonise the neonatal GIT, whereas the sow nipple and faeces act as longer-term microbiota sources, important for the development of the piglet microbiota during lactation (Chen *et al.*, 2018; Liu *et al.*, 2019a). In accordance with this, Chapter 4 reported that the sow udder microbiota, as opposed to sow

faeces, was the most important maternal microbiota source for colonizing the neonatal GIT on day 1, demonstrated by significantly lower weighted UniFrac distances between sow udder – piglet faeces compared to sow faeces – piglet faeces, and based on the results of the SourceTracker analysis. The sow udder microbiota at farrowing had a consistent influence on the microbiota development throughout the neonatal period, whilst the sow faeces became increasingly more important for piglet microbiota colonisation from 3 days of age, supporting the findings by Chen *et al.* (2018) and Liu *et al.* (2019a). In Chapter 4 over 95% of the microbiota was explained by unknown sources during the neonatal period, as only the sow udder and faecal samples were used as possible microbiota sources in the Source Tracker analysis. However, both Chen *et al.* (2018) and Liu *et al.* (2019a) also reported unknown sources to contribute to the microbiota of piglets, despite utilising a greater variety of maternal and environmental sources in their Source Tracker analysis (64.7% - 98.7% between days 3 – 21 (Chen *et al.*, 2018) and 65 – 75% between days 7 – 35 days (Liu *et al.*, 2019a)). Unknown sources acting as a prominent microbiota source for pre-weaned piglets could be related to the increased GIT maturity of piglets, providing a selective pressure for colonisers, as well as increased exploratory behaviour and creep feed intake. Future research should not only monitor time matched sow faeces but also time matched environment samples, to decipher where the origin of the unknown component of the piglet faecal microbiota.

The day 3 post-partum sow microbiota displayed a significant increase in the relative abundance of *Escherichia/Shigella, Fusobacterium* and *Bacteroides* (Chapter 3, Cheng *et al.*, 2018) and an increase in the relative abundance of *Christensenellaceae R7 group* and *Ruminococcaceae UCG-002* (Chapter 3) during lactation. Similarly, the neonatal microbiota is characterised by a higher relative abundance of *Escherichia/Shigella, Fusobacterium* and *Bacteroides* (Chapter 2; Mach *et al.*, 2015; Bian *et al.*, 2016; Chen *et al.*, 2018; Li *et al.*, 2018; Choudhury *et al.*, 2020) and *Christensenellaceae R7 group* and *Ruminococcaceae UCG-002* (Chapter 2). As the sow faecal microbiota becomes increasingly important for seeding of the neonatal GIT from day 3, this suggests that sow faeces are the source of these early life genera in piglet faeces.

Chapter 4 reported a significant litter effect on the microbiota community composition on day 1 and 3, with significantly lower weighted UniFrac distances between siblings than between non-siblings reared in the same litter. However, the observed litter specificity was lost by day 8. The results indicate the birth sow to have an important role in shaping the neonatal microbiota profile, with primiparous sows negatively affecting microbiota diversity of progeny. Similarly, Larivière-Gauthier *et al.* (2019) demonstrated litter specific and parity

dependant microbiota community composition to exist at 20 days of age, supporting the findings of Chapter 4. Their study reported the relative abundance of *Bacteroides*, *Butyricimonas* and unclassified Ruminococcaceae to be dependent on birth sow ID. Furthermore, progeny of sows of lower parity were characterised by the relative abundance of *Clostridium XIVa*, *Anaerococcus*, *Butyricimonas* and unclassified Clostridiales, Ruminococcaceae and Coriobacteriaceae; whilst progeny of higher parity sows were characterised by the relative abundance of *Alloprevotella*, unclassified Fusobacteriaceae, *Ordibacter* and *Mitsukella*. Similarly, Han *et al.* (2018) reported a sow effect on the microbiota profile of piglets up to 63 days of age. A more recent study by Larivière-Gauthier *et al.* (2020) reported birth sow ID to significantly affect the microbiota community composition of finisher pigs (13 weeks of age), highlighting the long-term impact of the sow on the development of the pig microbiota profile. The studies by Larivière-Gauthier *et al.* (2019, 2020) do not report any cross-fostering of experimental piglets, thus the significant effect of birth sow ID can also be considered as rearing sow ID effects. In Chapter 4, birth sow effects diminished between days 3 – 8 of age, which were superseded by a rearing litter effect, which may persist long term as reported in the studies by Larivière-Gauthier *et al.* (2019, 2020). Future studies should determine whether, following cross-fostering, rearing sow ID effects persist over time. Larivière-Gauthier *et al.* (2020) also reported nursery and fattening farm to significantly influence the microbiota development of pigs at finishing, thus the specific rearing farm environment also has a significant effect on the microbiota development of pigs.

6.1.1 Manipulating the piglet microbiota by targeted modulation of the sow microbiota with pre- and probiotics

The studies by Larivière-Gauthier *et al.* (2019, 2020) proposed altering the sow microbiota in late gestation and lactation as a potential means of long-term modulation of the piglet microbiota. However, the use of probiotics in sow diets has exhibited different effects in relation to modulation of the sow and piglet microbiota (Baker *et al.*, 2013; Starke *et al.*, 2013), although some studies have reported an increase in the prevalence and abundance of probiotic species in piglets following supplementation of probiotics to sow diets (Buddington *et al.*, 2010; Menegat *et al.*, 2019).

With regards to prebiotic interventions in sows, Hasan *et al.* (2018) reported the supplementation of 2g/kg yeast derivative to increase colostrum yield and consequently the relative abundance of milk oligosaccharide degrading bacteria in piglets, including *Oscillibacter, Clostridium IV* and *Blautia*. Therefore, modulation occurred in the piglet

microbiota in response to dietary substrate availability, as opposed to modulation of the seeding population. Shang *et al.* (2019) reported fibre source in the sow diet to have a prebiotic effect on the microbiota of piglets at 21 days of age. From 85 days of gestation, sows received either a control corn-soybean meal diet, a wheat bran diet which contained 30% wheat bran in gestation and 15% in lactation, or a sugar beet pulp (SBP) diet supplemented with 20% SBP in gestation and 10% in lactation. Sows fed the SBP produced piglets with a microbiota profile significantly higher in the relative abundance of Christensenellaceae and had a higher butyrate concentration in the colon at 21 days of age, whilst sows fed the wheat bran diet produced piglets with a significantly higher relative abundance of Lactobacilliaceae. Shang *et al.* (2019) reported that feeding sows the SBP diet increased milk quality (IL-10 and IgA concentration), improved growth rates and intestinal barrier function, and reduced intestinal inflammation of piglets at 21 days of age. Future research should look at the use of probiotics and prebiotics in late gestation and lactation sow diets with the aim of reducing microbiota dysbiosis associated with the periparturient period and to increase microbiota diversity in primiparous sows. Future research studies should also monitor the effects of prebiotic and probiotic sow dietary interventions on piglet performance, microbiota profile and metabolic function, to establish if/how interventions to the sow diet can modulate long term microbiota and performance effects on offspring.

6.2 Longitudinal development of the early life faecal microbiota

The GIT microbiota community composition displays significant changes with age (Chapter 2 and 4) related to increased GIT maturation and the introduction of solid feed associated with weaning, resulting in increased microbiota stability and lower inter-pig variability post-weaning (Frese *et al.*, 2015; Mach *et al.*, 2015; Bian *et al.*, 2016; Chen *et al.*, 2017; 2018; Li *et al.*, 2018; Wang *et al.*, 2019a; Ke *et al.*, 2019). The change in microbiota community composition is modulated, in part, by increased microbiota richness and evenness with age (Frese *et al.*, 2015; Chen *et al.*, 2015; Li *et al.*, 2018; Wang *et al.*, 2019a; Ke *et al.*, 2019). Chapter 2 reported a significant reduction in the number of observed OTUs and Shannon diversity index on day 32 compared with day 27 (day before weaning) before increasing and then plateauing. Wang *et al.* (2019a) also reported lower Shannon diversity index immediately post-weaning, followed by an increase and plateau during the post-weaning period. The reduction in alpha diversity measures immediately post-weaning may be explained by weaning-induced microbiota dysbiosis, detected in Chapter 2 and Wang *et al.* (2019a) as a result of more frequent sampling than other longitudinal studies.

Chapter 2 and 5 reported that the predominant phyla in piglet faeces were Firmicutes and Bacteroidetes, irrespective of age, with a reduction in Proteobacteria noted pre-weaning, as reported in the literature (Mach *et al.*, 2015; Bian *et al.*, 2016; Chen *et al.*, 2017, 2018; Li *et al.*, 2018; Wang *et al.*, 2019a; Guevarra *et al.*, 2019). At the genus level, the pre-weaning microbiota (Chapter 2) was characterised by *Lactobacillus, Bacteroides, Escherchia/Shigella, Clostridium sensu stricto 1, Ruminococcaceae UCG-002* and *Christensenellaceae R-7 group*, in agreement with the literature (Mach *et al.*, 2015; Bian *et al.*, 2016; Chen *et al.*, 2018; Li *et al.*, 2018). However, a shift in genera relative abundance occurred after weaning, characterised by higher relative abundance of *Prevotella, Rikensenellaceae RC9 gut group, Ruminococcaceae UCG-002* and *UCG-014, Subdoligranulum* and *[Eubacterium] Coprostanoligenes group*. Similarly, in the literature, weaning and the introduction of solid feed intake results in the microbiota profile consisting of predominantly *Prevotella, Rikenellaceae RC9 gut group, Ruminococcus, Subdoligranulum, Blautia* and *Roseburia* (Mach *et al.*, 2015, Bian *et al.*, 2016; Chen *et al.*, 2017, 2018; Li *et al.*, 2018; Choudhury *et al.*, 2020). The shift in genera relative abundance is associated with change in dietary substrate at weaning, from a predominantly highly digestible milk diet to a less digestible plant-based diet containing complex polysaccharides, as well as maturation of the GIT environment (Mach *et al.*, 2015; Frese *et al.*, 2015; Bian *et al.*, 2016).

6.2.1 Maturation of the piglet microbiota by solid feed intake

Creep feed is provided during lactation to ease the transition to solid feed (associated with the abrupt nature of weaning), increasing familiarisation of the piglets to diet form, whilst encouraging GIT maturation prior to weaning in order to digest solid feed. Increased creep feed intake pre-weaning reduces latency to solid feed intake post-weaning (Bruininx *et al.*, 2002) and increases post-weaning performance (Bruininx *et al.*, 2002, 2004; Sulabo *et al.*, 2010; Collins *et al.*, 2013; Huting *et al.*, 2017, 2019). Creep feed intake has recently been shown to modulate the faecal microbiota development of piglets (Choudhury *et al.*, 2020). Compared to piglets who received no creep feed, the provision of creep feed (26% non-starch polysaccharide) between 2 – 28 days of age increased microbiota diversity on days 15, 21 and 28 of age. The increase in microbiota diversity was similar to the diversity seen in post-weaned piglets. Increased creep feed intake from 15 days of age was not only correlated with increased alpha diversity, but also with the earlier loss of pre-weaning genera including *Bacteroides, Fusobacterium and Esherichia/Shigella*, and earlier increase in the relative abundance of post-weaning associated genera including *Prevotella 9, Roseburia, Faecalibacterium* and *Subdoligranulum*. In piglets who did not receive creep feed, there was a

clear segregation between pre- and post-weaning microbiota community composition. However, in creep-fed piglets, the day 21 and 28 microbiota community compositions were intermediate between the pre- and post-weaning community composition, demonstrating a smooth and gradual maturation of the GIT microbiota. Similar results were reported in Chapter 2. Future studies monitoring the microbiota development with age should classify piglets as 'eaters' or 'non-eaters' using dyed feed and the colorimeter faecal analysis methods described by Huting *et al.* (2017) and factor this values into the microbiota analysis.

Increasing the level of dietary fibre in creep feed with the addition of 1% pure cellulose or 1.3% alfalfa has demonstrated positive effects on the microbiota composition, including increased butyrate concentration in the large intestine (Mu *et al.*, 2017). Butyrate acts as the preferred energy source for colonocytes, assisting with maintenance of GIT integrity. Van Hees *et al.* (2019) also demonstrated that 5% purified cellulose in milk replacer between days 2 -14 and creep feed from days 15 – 26 was able to reduce ileal pH, increase volatile fatty acid concentration in the caecum and mid-colon, whilst reducing *E. coli* and increasing *Ruminococcus* relative abundance. Thus, the use of additional fibre sources in creep feed can exert beneficial effects on the microbiota community composition of suckling piglets and should be explored further, particularly in relation to its capacity to reduce post-weaning diarrhoea by diminishing relative abundance of *E. coli.*

Furthermore, it is important to increase creep feed intake in suckling piglets to promote maturation of both the GIT and the GIT microbiota in preparation of weaning and a plant-based diet. Whilst utilising a play feeder (normal creep feeder with canvas cloth, cotton ropes and a plastic tube attached) to deliver creep feed pre-weaning did not increase pre-weaning performance or weaning weight, it significantly increased ADFI and ADG in the first two weeks post-weaning (Middelkoop *et al.*, 2019a). A subsequent study by Middelkoop *et al.* (2019b) demonstrated that pre-weaning intake by piglets could be increased by the provision of a diverse feed (creep feed, honey loops, celery and peanuts vs creep feed alone), which resulted in an increase in pre-weaning ADG and post-weaning ADG and ADFI for the first two weeks. However, when plain creep feed was provided, the provision of a rooting substrate (sand) was able to increase intake, supporting the notion that encouragement/stimulation of foraging behaviour is important to increase solid feed intake in young piglets. Thus, to increase microbiota maturation in suckling piglets and post-weaning performance, increasing creep feed intake by encouraging foraging behaviour in combination with the adaption to feeder design and creep feed form requires further research.

However, LBW piglets eat very little creep feed compared with NBW piglets (Huting *et al.*, 2017, 2019), thus presenting a challenge in how to promote the GIT and microbiota maturation. LBW piglets are normally raised in litters of similarly sized piglets following cross-fostering shortly after birth. Douglas *et al.* (2014a) demonstrated LBW in homogenous litters to consume significantly more supplementary milk than those in heterogenous litters. Therefore, manipulating the supplementary milk composition to promote maturation of the GIT and microbiota may be a more effective intervention than creep feed modifications, for LBW piglets in particular. Amdi *et al.* (2020) demonstrated that gradually increasing wheat flour dry matter percentage of milk formula, from 10% on day 11 to 40% by day 26, was able to significantly increase maltase and sucrase activity in the proximal small intestine compared with piglets receiving supplementary milk containing only bovine milk and whey. Thus, gradually increasing wheat flour content to supplementary milk increased the digestive capacity of piglets to a plant-based diet. However, the effect of this treatment on the post-weaning performance, feed intake and diarrhoea occurrence need to be established with further research. In addition, research needs to determine whether these alterations to supplementary milk composition increase the maturation of the GIT microbiota in a similar manner to creep feed, especially in LBW piglets.

6.3 Associations between piglet birthweight and microbiota development

The early life microbiota of LBW (0.80 – 1.25kg) and NBW (1.50 – 2.00kg) piglets was monitored in Chapters 2, 4 and 5, to determine whether faecal microbiota profiles differed between BiW classes and whether differences were consistent across studies. Whilst LBW pigs had significantly poorer performance and lower LWs across all three Chapters, the effect of BiW class on the microbiota was inconsistent. Neither Chapter 2 nor 4 reported differences in alpha diversity measures during the first week of life between BiW classes, although Chapter 5 reported that LBW piglets had a higher number of observed OTUs and Shannon diversity index on day 8, higher diversity is generally considered a characteristic of a healthy animal. Chapter 2 reported the number of observed OTUs of LBW pigs to be significantly lower at 21 days of age, but higher on days 27, 32 and 56 of age. In accordance with results in Chapters 2 and 4, Li *et al.* (2018, 2019) reported no difference in alpha diversity of ileal or colonic microbiota on days 7, 21 and 28 of life, or of faecal microbiota between days 3 – 35 of life. Meanwhile, Huang *et al.* (2019a) reported no difference in alpha diversity on day 1 between BiW classes, as observed in Chapter 4. However, Zhang *et al.* (2019a) monitored the

SI microbiota of LBW and NBW piglets and reported that the microbiota in the jejunum of LBW pigs had lower richness at 7 and 21 days of age.

The microbiota community composition was not significantly different between LBW and NBW piglets in Chapter 2; instead changes were driven by age, in agreement with Li *et al.* (2018, 2019). However, in Chapters 4 there was a significant difference between BiW classes for unweighted UniFrac distances on days 3 and 8, meanwhile in Chapter 5 significant differences between BiW classes on day 8 were reported for both weighted and unweighted UniFrac distances. Huang *et al.* (2019a) also reported a significant difference in the Bray Curtis distances between BiW classes at 12 hours of age. Moreover, Zhang *et al.* (2019a) reported differences in jejunal and ileal community composition on days 7, 21 and 28. Thus, clear contradictions exist between different studies in the literature and thesis chapters.

Chapter 2 reported differences in genera abundance between BiW classes, with LBW pigs having significantly lower relative abundance of *Ruminococcaceae UCG-005* on day 21 and higher abundances of *Ruminococcaceae UCG-014* on day 32. The lower faecal relative abundance of *Ruminococcaceae UCG-005* was in agreement with Zhang *et al.* (2019a), who reported lower abundance of this genus on day 21 in the jejunum of LBW piglets. In contrast, Li *et al.* (2018) reported the relative abundance of *Ruminococcaceae UCG-005* to be significantly higher in the faeces of LBW piglets on days 7 and 21. Chapter 5 reported a significantly higher relative abundance of *Alloprevotella* and *Christensenellaceae R7 group* in LBW piglet faeces on day 8, whereas Li *et al.* (2018) reported a significantly higher relative abundance of *Christensenellaceae R7 group* in LBW piglet faeces on day 21. With the exception of the aforementioned similarities in genera abundance, no other genera markers for LBW have been reported consistently between studies (Li *et al.*, 2018, 2019; Huang *et al.*, 2019a; Zhang *et al.*, 2019a) or between thesis chapters. However, between studies there has been more consistency between the reported microbiota metabolic function of LBW piglets. LBW pigs were reported to display reduced metabolic functional pathways related to carbohydrate, lipid and amino acid metabolism (Li *et al.*, 2018, 2019; Zhang *et al.*, 2019a).

The differences in results of different studies may have arisen from a range of factors related to trial design. Firstly, the microbiota composition is affected by GIT segment analysed (Zhao *et al.*, 2015; Li *et al.*, 2019). Whilst faecal samples were utilised in Chapters 2, 4 and 5, and the papers of Li *et al.* (2018) and Huang *et al.* (2019a), ileum and colon samples were analysed by Li *et al.* (2019) and jejunum and ileum samples by Zhang *et al.* (2019a). Faecal samples were utilised in the studies reported here to facilitate longitudinal analyses of the

piglet microbiota to be conducted, with the repeated measures enabling the analysis to be conducted taking into consideration individual piglet variability within the microbiota. Differences in weaning age may have contributed to differences in the results between thesis chapters and the literature, with weaning occurring at 28 days in thesis chapters and 21 days in the studies by Li *et al.* (2018, 2019) and Zhang *et al.* (2019a). Therefore, whilst on day 27 piglets would have been consuming predominantly milk and some creep feed in Chapter 2, and the day 32 samples taken shortly after weaning during a time of microbiota dysbiosis, the faecal microbiota on days 28 and 35 for Li *et al.* (2018, 2019) and Zhang *et al.* (2019a) would have been assessed after the microbiota had adapted to a solid feed diet post-weaning. Moreover, creep feed was only available from 10 days of age in Chapter 2, whilst creep was provided from 3 – 5 days of age by Li *et al.* (2018, 2019) and Zhang *et al.* (2019a). This earlier availability of creep feed could potentially have altered the microbiota of piglets, particularly in NBW pigs who consume more creep feed than LBW piglets (Huting *et al.*, 2017, 2019). Finally, birthweight of LBW piglets was lower in studies by Huang *et al.* (2019a) (0.92kg $\pm$ 0.04), Li *et al.* (2018) (0.75 – 0.95kg) and Li *et al.* (2019) (0.878kg $\pm$ 0.044). The piglets in Chapters 2, 4 and 5 had a BiW between 0.80 – 1.25kg, in accordance with the LBW criteria stated by Douglas *et al.* (2014a). Piglets with lower BiW often express increased IUGR, thus those reported in the literature may have had altered GIT morphology and immune function compared to the experimental LBW piglets used in thesis chapters. The final difference between literature studies and thesis chapters was the maternal environment to which piglets were exposed. A lack of consistency in conclusions about microbiota differences between feed efficiency studies in the literature, and even within studies between batches or experimental farms where care has been taken to standardise genetics, feed and management practices, has been attributed to the maternal influence and early life environmental effects on the microbiota (McCormack *et al.*, 2019a; Vigors *et al.*, 2020a). Larivière-Gauthier *et al.* (2019) reported differences in the microbiota profile at 20 days of age existed between pigs reared in different farrowing rooms on the same farm, which may further explain the inconsistencies in the microbiota profile between LBW and NBW piglets reported in Chapters 2, 4 and 5. Studies which report differences in the microbiota between BiW classes have been small with regard to replication, with 6 – 8 piglets per BiW group and with different pigs sampled at each time point, thus potentially not adequately accounting for large inter-piglet variability, especially during early life when the microbiota composition is less stable.

Despite the studies conducted throughout the thesis spanning several batches and containing a much higher sample replicate, thus statistical power, than previous research, future studies should include different cohorts spanning multiple batches, at preferably different sites, in order to establish more reliable markers of BiW class. Future studies should also include measurement of piglet head shape to determine IUGR severity (Hales *et al.*, 2013; Amdi *et al.*, 2013), irrespective of BiW class and include these measures as fixed effects in microbiota analysis. To date, studies have not characterised the microbiota of IUGR LBW piglets compared to those of LBW which display normal allometry. Furthermore, increasing the number of BiW categories in larger trials to, for example, < 1kg, 1 – 1.25kg, 1.25 – 1.5kg, 1.5 - 1.75kg, 1.75 – 2.00kg, and combining BiW with IUGR head shape score, may help to establish a cut off weight at which BiW affects the microbiota and indicate whether IUGR is more detrimental to the microbiota development, as IUGR can also affect NBW piglets (Matheson *et al.*, 2018).

6.3.1 Morphometric measures to help identify microbiota markers associated with compensatory growth in LBW piglets

Some LBW pigs are able to exhibit compensatory growth, whilst others remain stunted. The ability to exhibit compensatory growth has been associated with an increased BMI and abdominal circumference at birth and birthweight: cranial circumference (disproportionally larger head size to birthweight is indicative of IUGR) (Douglas *et al.*, 2016; Huting *et al.*, 2018). However, an explanatory mechanism for the positive association between birth morphology (BMI and abdominal circumference in particular) and piglet performance remains to be eluded. In humans, the Christensenellaceae family has been reportedly associated with a lower BMI (Goodrich *et al.*, 2014). LBW piglets have significantly lower BMI at birth (Douglas *et al.*, 2016) which provides a potential explanation for the higher abundance of *Christensenellaceae R7 group* abundance in LBW piglets on day 8 in Chapter 5. Future studies should correlate birthweight, IUGR head shape score and morphometric measures at birth with the microbiota profile, in order to help decipher characteristics in the microbiota profile which might be related to increased compensatory growth in piglets. Furthermore, analysis of the microbiota functional capacity between LBW piglets who are able to exhibit compensatory growth and those who are not may help to provide an explanation for the morphometric measures phenomenon reported by Douglas *et al.* (2016) and Huting *et al.* (2018). Understanding differences in the microbiota metabolic function may help to inform management interventions, aimed at the microbiota, in order to shift the microbiota metabolic profile towards that of piglets with superior performance.

6.4 Associations between piglet performance and microbiota development

One of the experimental aims of Chapter 2 was to identify microbiota markers associated with LBW pigs which could exhibit compensatory growth. Whilst there were no microbiota markers segregating LBW "good" from "poor" ADG piglets, the study found three genera markers associated with superior performance between birth – 56 days of age, irrespective of BiW class. These markers included a significant increase in *Lactobacillus* relative abundance on day 4 and a numerical increase on day 8, a significant increase in the relative abundance of unclassified Prevotellaceae on day 8 and *Ruminococcaceae UCG-005* on day 14. To date, very little research has been conducted to determine potential early life microbiota markers associated with performance (Mach *et al.*, 2015; Morissette *et al.*, 2018; Ding *et al.*, 2019). *Ruminococcaceae UCG-005* relative abundance on day 14 was positively associated with ADG between birth – day 56. Mach *et al.* (2015) reported the faecal microbiota of piglets with superior pre-weaning performance compared with poorer performers, to differentiate into two enterotypes. The microbiota enterotype of superior pre-weaning performance was characterised by higher abundance of Ruminococcace. Similarly, Morissette *et al.* (2018) characterised superior growth rates in early lactation to be associated with increased Ruminococcaceae abundance. Their study concluded that superior performance and modulation of the microbiota composition arises in response to milk intake. Chapter 2 also reported increased relative abundance on day 8 of unclassified Prevotellaceae to be associated with increased piglet ADG between birth – day 56. Bian *et al.* (2016) correlated unclassified Prevotellaceae relative abundance to milk lactose content. Higher milk intake and subsequently lactose availability within the GIT could induce a rise in the relative abundance of unclassified Prevotellaceae and may explain the higher relative abundance of this taxa in piglets with a higher ADG. This supports the proposal by Morissette *et al.* (2018) that increased colostrum and milk intake of piglets with superior performance induces significant changes to genera relative abundance in their faeces.

The relative abundance of *Lactobacillus* was significantly increased in neonates with superior ADG between birth – 56 days of age. Ding *et al.* (2019) also reported *Lactobacillus* relative abundance to be positively correlated with pre-weaning performance in the caecum and colon of piglets at 21 days of age, whilst Hasan *et al.* (2018) reported increased relative abundance of *Lactobacillus* in the first week of life to be associated with neonatal ADG. *Lactobacillus* is a core member of the pig GIT microbiota (Holman *et al.*, 2015). Early life research has demonstrated the use of different *Lactobacillus* species/strains to have beneficial effects on the microbiota development, growth and immunomodulation of pre-weaned piglets, thus

supporting the findings of Chapter 2. Wang *et al.* (2020) reported an autogenous *L. reuteri* D8 strain to promote intestinal mucosa development and barrier function in neonatal piglets when provided as 10^9 CFU/ml in 2ml PBS between days 3 – 8 of age. Wang *et al.* (2019c) dosed neonatal piglets on days 1, 3 and 5 with 5 x 10^8 CFU/ml of *L. rhamnosus* GG ATCC53103 in 2ml skimmed milk and reported improvements to intestinal barrier function, increased pre-weaning ADG and weaning weight, and a reduction in diarrhoea incidence. Moreover, Liu *et al.* (2014) reported daily dosing of 6 x 10^9 CFU/ml of *L. fermentum* I5007 to formula-fed piglets between days 4 – 14 of age improved ADG, intestinal morphology and colonic butyrate concentration. Finally, dosing piglets with 1ml of 5 x 10^7 CFU/ml of an autogenous *L. reuteri* KT260178 strain for the first 7 days of age, followed by 1 x 10^7 CFU/g in creep feed reduced *E. coli* and *Staphylococcus* counts in the caecum at 28 days of age, and increased antioxidant status and performance of piglets, as well as increasing counts of *Lactobacillus* and *Bifidobacterium* (Yang *et al.*, 2020). Therefore, increasing *Lactobacillus* relative abundance in the GIT of neonates increases barrier function and subsequently reduces mucosal inflammation and the energy required to mount an immune response to bacteria translocation or pathogen challenge, retaining more energy for piglet growth.

Due to the differences in microbiota markers for feed efficiency and growth between studies, as well as differences between farms or batches of pigs within the same study (McCormack *et al.*, 2019a; Vigors *et al.*, 2020a), Gardiner *et al.* (2020) proposed identifying personalised microbiota markers or microbiota metabolic functions associated with performance, specific to the farm studied. Gardiner *et al.* (2020) highlighted that the species identified as markers for performance need to be carefully considered, before potential use as an autogenous probiotic treatment, due to the variability in effects associated with difference species or strains within a particular genus or species, respectively. Meanwhile, Liao and Nyachoti (2017) stressed the importance of targeted selection of a potential probiotic based on specific mode of action rather than simply using a probiotic and expecting it to improve overall health and performance. This was demonstrated in Chapter 5, whereby early life administration of an autogenous *E. faecium* inoculum was able to reduce the occurrence of diarrhoea, but failed to improve overall piglet performance. *E. faecium* probiotic strains are best regarded for their anti-diarrhoeal properties, as opposed to growth promotion (Taras *et al.*, 2006; Zeyner and Boldt, 2006; Wang *et al.*, 2016b). Of the three genera markers reported to be positively associated with performance in Chapter 2, *Lactobacillus* holds the most potential as an autogenous probiotic strain since, as discussed previously, this species is widely reported to have beneficial effects on performance and health of piglets. However, 16S rRNA gene sequencing does not enable robust species level identification. Therefore, subsequent research

should be subjected to qPCR using a range of *Lactobacillus* specific primers to identify species. Alternatively, *Lactobacillus* species could also be cultured from the faeces of high performing piglets and the whole genome sequenced to establish species and strain, as well as attributes related to gene functions. A series of early life trials should then be conducted on a large scale to test each and/or a combination of the *Lactobacillus* isolates and quantify their effect on performance, thus establishing which species/strain has to the potential for an autogenous probiotic use at a given farm. Of note, is the need to repeat dose piglets with an autogenous probiotic, especially with *Lactobacillus* which is an anaerobe, as the neonatal GIT is not anaerobic until a few days of age. In support of this, Hou *et al.* (2015) demonstrated increased efficacy of *L. reuteri* I5007 treatment on piglet performance and immunomodulation by dosing piglets every 4 days between days 1 – 17, as opposed to the first 4 days of life. However, frequent repeat dosing of individual piglets is impractical commercially due to the labour intensive and time-consuming nature of this practice. Alternative probiotic dosing either via the sow (faeces) or as part of a soluble solution in an electrolyte bowl, for example, present more commercially applicable solutions, but further research would be required to establish the stability and efficacy of a *Lactobacillus* probiotic strain in aerobic conditions.

Studies on the inclusion of microbiota data to predict grower/finisher performance have suggested that microbiota markers can be utilised to predict ADG and backfat depth, although the microbiota markers for performance recorded at weaning were less predictive than those recorded at 15- and 22-weeks post-weaning (Lu *et al.*, 2018; Maltecca *et al.*, 2019). Indeed, Bergamaschi *et al.* (2020b) reported a 245 OTUs to be associated with ADG (101 positively) in 1028 pigs at weaner, grower and finisher phases; 5% of the OTUs belonged to *Prevotella*, 4.5% to *Lactobacillus* and 3.9% to *Ruminoccocus*, similar to the genera identified in Chapter 2 as being early life markers for performance. Whilst heritability estimates of OTUs associated with performance were generally low, Bergamaschi *et al.* (2020b) further concluded that some OTUs were moderately heritable, including one *Prevotella* OTU which was positively correlated with ADG at weaning and weeks 14 and 22 post-weaning. Thus, microbiota markers, particularly in grower/finisher pigs could be used in selection programmes in the future. Conversely, Weishaar *et al.* (2020) reported that it was only possible to breed for heritability in the microbiota profile associated with feed efficiency ((residual feed intake) enhancing the profile of the microbiota for this trait specifically), and not FCR and ADG which may limit commercial application. Weishaar *et al.* (2020) noted that

looking at breeding for microbiota profiles associated with 'total merit' of the animal may be more appropriate rather than individual performance parameters.

6.4.1 Future directions of performance and microbiota research

Consequently, there are several future avenues for microbiota research (in relation to ADG with respect to this thesis). The first is increased research into early life microbiota markers for performance, with a focus on a farm specific microbiota profile as well as metabolic functional profile, utilising shotgun metagenomic sequencing, proteomics and metabolomics to identify species specific functional pathways enriched in high performing pigs. These data can then be utilised to develop an autogenous early life probiotic for use over successive time points in order to target a specific problem or microbiota function (e.g reducing diarrhoea or increasing gut integrity, thus, potentially performance). However, the current high costs associated with metagenomic shotgun sequencing may prohibit commercial application for some time. An alternative approach could be to utilise 16S rRNA sequencing to establish the farm specific microbiota community profile and identify differences in the microbiota profile related to performance, as reported in Chapter 2. Based on the microbiota profile, in the absence of being able to generate an autogenous probiotic, the most relevant, commercially available, probiotic could be selected to promote piglet performance by mitigating either lower relative abundance in poorly performing piglets of genera associated with high performance, or to supress pathogenic strains (such as ETEC if pre- and post-weaning diarrhoea is a problem on farm). Alternatively, in creep feed and post-weaning diets, the use of prebiotics and feed additives could be utilised to indirectly shift the microbiota in a direction more closely aligned to the identified microbiota profile associated with high performing pigs, as discussed in Chapter 1. However, in grower/finisher pigs, it is harder to discern cause and effect in the microbiota profile of high and low performing pigs. Despite inconsistencies in taxonomic markers for FCR and ADG between studies (Mach *et al.*, 2015; Han *et al.*, 2017; Torres-Pitarch *et al.*, 2020; Quan *et al.*, 2018; McCormack *et al.*, 2017; 2019a, Vigors *et al.*, 2020a), there is increased consistency in relation to metabolic function of the microbiota profile associated with superior performing pigs (Tan *et al.*, 2017; Yang *et al.*, 2017; McCormack *et al.*, 2017; Metzler-Zebeli *et al.*, 2018). A microbiota enriched in metabolic functions for energy sequestration, lipid metabolism and immunomodulatory effects (anti-inflammatory) is positively associated with performance (Gardiner *et al.*, 2020). Thus, Gardiner *et al.* (2020) suggest future research should identify microbiota functionality, as opposed to taxa composition, associated with superior performance. This information can then be used as a selection criterion for performance, or to select appropriate feed

additives/manipulations to diet formulations in order to achieve the desired microbiota functionality. Consequently, research is predominately moving in the direction of shotgun metagenomic sequencing, budget permitting, in order to profile the metabolic capacity of the whole microbiome, rather than the taxonomic assemblage of the microbiota and its predicted functionality (using PICRUSt bioinformatics software) which can be achieved with 16S rRNA gene sequencing.

6.5 Conclusions and practical implications

The thesis reports the sow, in particular the areolar skin, to act as an important source of neonatal microbiota seeding of the GIT in the immediate post-natal period, whilst the sow faeces acts as a continuous GIT microbiota seeding source as piglets age. Birth sow had a significant impact on the microbiota of neonates, with a litter specific microbiota community composition existing for the first 3 days of age, irrespective of cross-fostering practices. In addition, birth sow parity significantly affected microbiota diversity and community composition of neonates. Sows exhibit microbiota dysbiosis in response to farrowing, with a significant increase in potential pathogens including *Escherichia/Shigella* and *Fusobacterium*, whilst primiparous sows display reduced microbiota diversity compared with multiparous sows. Due to the importance of maternal microbiota sources in relation to the microbiota development of the piglets, future studies should focus on modulating the sow microbiota in order to alter the microbiota and performance of piglets, particularly in primiparous sows. Piglet microbiota development was mainly driven by age, although the thesis also identified that differences in the faecal microbiota existed between LBW and NBW piglets, demonstrating that birthweight is an important factor influencing the microbiota development of piglets. Although, a reliable intervention for LBW piglets in order to promote compensatory growth through manipulation of the microbiota remains to be elaborated. The thesis also identified genera markers in early life that were associated with superior performance to 56 days of age, including *Lactobacillus*, unclassified Prevotellaceae and *Ruminococcaceae UCG-005*. Future research should explore the genome properties of the genera associated with superior performance, in order to try and identify potential probiotic species/strains which could be administered in early life to promote performance via manipulation of the GIT microbiota. Furthermore, the thesis demonstrated that treatment with an autogenous *E. faecium* inoculum for the first 7 days of life was able to significantly reduce diarrhoea occurrence up to 39 days of age, although the *E. faecium* inoculum did not significantly affect the microbiota profile or performance of piglets.

Cumulatively, the thesis provides evidence for the importance of the sow and early life microbiota profile in relation to piglet health and performance, irrespective of differences in the microbiota that exist between LBW and NBW piglets.

References

AHDB. (2020a) *Indoor breeding herd Key Performance Indicators (KPIs)*. Available at: https://porktools.ahdb.org.uk/prices-stats/costings-herd-performance/indoor-breeding-herd/ (Accessed 15 November 2020).

AHDB. (2020b) Small piglet management. Available at: https://ahdb.org.uk/knowledge-library/small-piglet-management (Accessed: 14 November 2020).

Algers, B., Jensen, P. & Steinwall, L. (1990) Behaviour and weight changes at weaning and regrouping of pigs in relation to teat quality. *Applied Animal Behaviour Science*. 26 (1–2), pp. 143–155.

Algers, B. & Jensen, P. (1991) Teat stimulation and milk production during early lactation in sows: Effects of continuous noise. *Canadian Journal of Animal Science*. 71 (1), pp. 51–60.

Alvarenga, A.L.N., Chiarini-Garcia, H., Cardeal, P.C., Moreira, L.P., Foxcroft, G.R., Fontes, D.O. & Almeida, F.R.C.L. (2013) Intra-uterine growth retardation affects birthweight and postnatal development in pigs, impairing muscle accretion, duodenal mucosa morphology and carcass traits. *Reproduction, Fertility and Development*. 25 (2), pp. 387–395.

Amdi, C., Krogh, U., Flummer, C., Oksbjerg, N., Hansen, C.F. & Thei, P.K. (2013) Intrauterine growth restricted piglets defined by their head shape ingest insufficient amounts of colostrum. *Journal of Animal Science*. 91 (12), pp. 5605–5613.

Amdi, C., Klarlund, M. V., Hales, J., Thymann, T. & Hansen, C.F. (2016) Intrauterine growth-restricted piglets have similar gastric emptying rates but lower rectal temperatures and altered blood values when compared with normal-weight piglets at birth. *Journal of Animal Science*. 94 (11), pp. 4583–4590.

Amdi, C., Pedersen, M. L. M., Klaaborg, J., Myhill, L. J., Engelsmann, M. N., Williams, A. R. & Thymann, T. (2020) Pre-weaning adaptation responses in piglets fed milk replacer with gradually increasing amounts of wheat. *British Journal of Nutrition*. 126 (3), pp. 375-382.

Ames, N.J., Ranucci, A., Moriyama, B. & Wallen, G.R. (2017) The human microbiome and understanding the 16S rRNA gene in translational nursing science. *Nursing Research.* 66 (2), pp.184–197.

Anderson, M.J. (2001) A new method for non-parametric multivariate analysis of variance. *Austral Ecology*. 26 (1), pp. 32–46.

Anderson, M.J. & Walsh, D.C.I. (2013) PERMANOVA, ANOSIM, and the Mantel test in the face of heterogeneous dispersions: What null hypothesis are you testing? *Ecological Monographs*. 83 (4), pp. 557–574.

Appleby, M.C., Pajor, E.A. & Fraser, D. (1992) Individual variation in feeding and growth of piglets: Effects of increased access to creep food. *Animal Production*. 55 (1), pp. 147–152.

Aviles-Rosa, E.O., Rakhshandeh, A. & McGlone, J.J. (2019) Preliminary study: Depriving piglets of maternal feces for the first seven days post-partum changes piglet physiology and performance before and after weaning. *Animals*. 9 (5), 268.

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

Azad, M.B., Bridgman, S.L., Becker, A.B. & Kozyrskyj, A.L. (2014) Infant antibiotic exposure and the development of childhood overweight and central adiposity. *International Journal of Obesity*. 38 (10), pp. 1290–1298.

Bach Knudsen, K.E. (1997) Carbohydrate and lignin contents of plant materials used in animal feeding. *Animal Feed Science and Technology*. 67 (4), pp. 319–338.

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

Baldwin, D.M. & Stabenfeldt, G.H. (1975) Endocrine changes in the pig during late pregnancy, parturition and lactation. *Biology of Reproduction*. 12 (4), pp. 508–515.

Balzani, A., Cordell, H.J., Sutcliffe, E. & Edwards, S.A. (2016) Sources of variation in udder morphology of sows. *Journal of Animal Science*. 94 (1), pp. 394–400.

Barba-Vidal, E., Martín-Orué, S.M. & Castillejos, L. (2018) Review: Are we using probiotics correctly in post-weaning piglets? *Animal*. 12 (12), pp. 2489–2498.

Barnett, K.L., Kornegay, E.T., Risley, C.R., Lindemann, M.D. & Schurig, G.G. (1989) Characterization of creep feed consumption and its subsequent effects on immune response, scouring index and performance of weanling pigs. *Journal of Animal Science*. 67 (10), pp. 2698–2708.

Bauer, E., Metzler-Zebeli, B.U., Verstegen, M.W.A. & Mosenthin, R. (2011) Intestinal gene expression in pigs: Effects of reduced feed intake during weaning and potential impact of dietary components. *Nutrition Research Reviews.* 24 (2) pp.155–175.

Bauer, R., Walter, B., Hoppe, A., Gaser, E., Lampe, V., Kauf, E. & Zwiener, U. (1998a) Body weight distribution and organ size in newborn swine (sus scrofa domestica) - A study describing an animal model for asymmetrical intrauterine growth retardation. *Experimental and Toxicologic Pathology*. 50 (1), pp. 59–65.

Bauer, R., Walter, B., Gaser, E., Rösel, T., Kluge, H. & Zwiener, U. (1998b) Cardiovascular function and brain metabolites in normal weight and intrauterine growth restricted newborn piglets - Effect of mild hypoxia. *Experimental and Toxicologic Pathology*. 50 (4–6), pp. 294–300.

Baxter, E.M., Jarvis, S., D'Eath, R.B., Ross, D.W., Robson, S.K., Farish, M., Nevison, I.M., Lawrence, A.B. & Edwards, S.A. (2008) Investigating the behavioural and physiological indicators of neonatal survival in pigs. *Theriogenology*. 69 (6), pp.773–783.

Baxter, E.M., Rutherford, K.M.D., D'Eath, R.B., Arnott, G., Turner, S.P., Sandøe, P., Moustsen, V.A., Thorup, F., Edwards, S.A. & Lawrence, A.B. (2013) The welfare implications of large litter size in the domestic pig II: Management factors. *Animal Welfare*. 22 (2), pp. 219–238.

Beaulieu, A.D., Aalhus, J.L., Williams, N.H. & Patience, J.F. (2010) Impact of piglet birth weight, birth order, and litter size on subsequent growth performance, carcass quality, muscle composition, and eating quality of pork. *Journal of Animal Science*. 88 (8), pp. 2767–2778.

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

Bednorz, C., Guenther, S., Oelgeschläger, K., Kinnemann, B., Pieper, R., Hartmann, S., Tedin, K., Semmler, T., Neumann, K., Schierack, P., Bethe, A. & Wieler, L.H. (2013b) Feeding the probiotic Enterococcus faecium strain NCIMB 10415 to piglets specifically reduces the number of Escherichia coli pathotypes that adhere to the gut mucosa. *Applied and Environmental Microbiology*. 79 (24), pp. 7896–7904.

Benjamini, Y. & Hochberg, Y. (1995) Controlling the False Discovery Rate: A Practical and Powerful Approach to Multiple Testing. *Journal of the Royal Statistical Society: Series B (Methodological)*. 57 (1), pp. 289–300.

Bérard, J., Kreuzer, M. & Bee, G. (2008) Effect of litter size and birth weight on growth, carcass and pork quality, and their relationship to postmortem proteolysis. *Journal of Animal Science*. 86 (9), pp. 2357–2368.

Berg, G., Rybakova, D., Fischer, D., Cernava, T., Vergès, M.C.C., Charles, T., Chen, X., Cocolin, L., Eversole, K., Corral, G.H., Kazou, M., Kinkel, L., Lange, L., Lima, N., Loy, A., Macklin, J.A., Maguin, E., Mauchline, T., McClure, R., et al. (2020) Microbiome definition re-visited: old concepts and new challenges. *Microbiome*. 8 (1), pp. 103-122.

Bergamaschi, M., Tiezzi, F., Howard, J., Huang, Y.J., Gray, K.A., Schillebeeckx, C., McNulty, N.P. & Maltecca, C. (2020a) Gut microbiome composition differences among breeds impact feed efficiency in swine. *Microbiome*. 8 (1), 110.

Bergamaschi, M., Maltecca, C., Schillebeeckx, C., McNulty, N.P., Schwab, C., Shull, C., Fix, J. & Tiezzi, F. (2020b) Heritability and genome-wide association of swine gut microbiome features with growth and fatness parameters. *Scientific Reports*. 10 (1), 10134.

Beyer, M., Jentsch, W., Kuhla, S., Wittenburg, H., Kreienbring, F., Scholze, H., Rudolph, P.E. & Metges, C.C. (2007) Effects of dietary energy intake during gestation and

lactation on milk yield and composition of first, second and fourth parity sows. *Archives of Animal Nutrition*. 61 (6), pp. 452–468.

Bharti, R. & Grimm, D.G. (2019) Current challenges and best-practice protocols for microbiome analysis. *Briefings in Bioinformatics*. 22 (1), pp. 178-193.

Bhattarai, Y. (2018) Microbiota-gut-brain axis: Interaction of gut microbes and their metabolites with host epithelial barriers. *Neurogastroenterology and Motility,* 30 (6), E13366.

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

Biddle, A., Stewart, L., Blanchard, J. & Leschine, S. (2013) Untangling the genetic basis of fibrolytic specialization by lachnospiraceae and ruminococcaceae in diverse gut communities. *Diversity*. 5 (3), pp. 627–640.

Bjerre, D., Mark, T., Sørensen, P., Proschowsky, H.F., Vernerse, A., Jørgensen, C.B. & Fredholm, M. (2010) Investigation of candidate regions influencing litter size in Danish Landrace sows. *Journal of Animal Science*. 88 (5), pp. 1603–1609.

Bøe, K. (1991) The process of weaning in pigs: when the sow decides. *Applied Animal Behaviour Science*. 30 (1–2), pp. 47–59.

Boehm, G., Bierbach, U., Senger, H., Jakobsson, I., Minoli, I., Moro, G. & Räihä, N. (1991) Activities of lipase and trypsin in duodenal juice of infants small for gestational age. *Journal of Pediatric Gastroenterology and Nutrition*. 12 (3), pp. 324–327.

Bolyen, E., Rideout, J.R., Dillon, M.R., Bokulich, N.A., Abnet, C.C., Al-Ghalith, G.A., Alexander, H., Alm, E.J., Arumugam, M., Asnicar, F., Bai, Y., Bisanz, J.E., Bittinger, K., Brejnrod, A., Brislawn, C.J., Brown, C.T., Callahan, B.J., Caraballo-Rodríguez, A.M., Chase, J., et al. (2019) Reproducible, interactive, scalable and extensible microbiome data science using QIIME 2. *Nature Biotechnology.* 37 (8) pp.852–857.

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

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

Bruininx, E.M.A.M., Binnendijk, G.P., Van Der Peet-Schwering, C.M.C., Schrama, J.W., Den Hartog, L.A., Everts, H. & Beynen, A.C. (2002) Effect of creep feed consumption on individual feed intake characteristics and performance of group-housed weanling pigs. *Journal of Animal Science*. 80 (6), pp. 1413–1418.

Bruininx, E.M.A.M., Schellingerhout, A.B., Binnendijk, G.P., Van Der Peet-Schwering, C.M.C., Schrama, J.W., Den Hartog, L.A., Everts, H. & Beynen, A.C. (2004) Individually assessed creep food consumption by suckled piglets : Influence on post-weaning food intake characteristics and indicators of gut structure and hind-gut fermentation. *Animal Science*. 78 (1), pp. 67–75.

Buddington, R.K., Williams, C.H., Kostek, B.M., Buddington, K.K. & Kullen, M.J. (2010) Maternal-to-infant transmission of probiotics: Concept validation in mice, rats, and pigs. *Neonatology*. 97 (3), pp. 250–256.

Cabrera, R.A., Boyd, R.D., Jungst, S.B., Wilson, E.R., Johnston, M.E., Vignes, J.L. & Odle, J. (2010) Impact of lactation length and piglet weaning weight on long-term growth and viability of progeny. *Journal of Animal Science*. 88 (7), pp. 2265–2276.

Cahenzli, J., Köller, Y., Wyss, M., Geuking, M.B. & McCoy, K.D. (2013) Intestinal microbial diversity during early-life colonization shapes long-term IgE levels. *Cell Host and Microbe*. 14 (5), pp. 559–570.

Calderón Díaz, J.A., Boyle, L.A., Diana, A., Leonard, F.C., Moriarty, J.P., McElroy, M.C., McGettrick, S., Kelliher, D. & García Manzanilla, E. (2017a) Early life indicators predict mortality, illness, reduced welfare and carcass characteristics in finisher pigs. *Preventive Veterinary Medicine*. 146, pp.94–102.

Calderón Díaz, J.A.., Diana, A., Boyle, L.A., Leonard, F.C., McElroy, M., McGettrick, S., Moriarty, J. & Manzanilla, E.G. (2017b) Delaying pigs from the normal production flow is associated with health problems and poorer performance. *Porcine Health Management*. 3 (1), 13.

Camargo, E.G., Rego, J.C.C., Dias, L.T. & Teixeira, R. de A. (2013) Effects of cross-fostering on performance of piglets. *Revista Brasileira de Saude e Producao Animal*. 14 (1), pp. 142–148.

Campbell, J.M., Crenshaw, J.D. & Polo, J. (2013) The biological stress of early weaned piglets. *Journal of Animal Science and Biotechnology*. 4 (1), pp. 124-127.

Campos, P.H.R.F., Silva, B.A.N., Donzele, J.L., Oliveira, R.F.M. & Knol, E.F. (2012) Effects of sow nutrition during gestation on within-litter birth weight variation: A review. *Animal*. 6 (5), pp.797–806.

Carney-Hinkle, E.E., Tran, H., Bundy, J.W., Moreno, R., Miller, P.S. & Burkey, T.E. (2013) Effect of dam parity on litter performance, transfer of passive immunity, and progeny microbial ecology. *Journal of Animal Science*. 91 (6), pp. 2885–2893.

Cavaco, L.M., Hasman, H., Aarestrup, F.M., Wagenaa, J.A., Graveland, H., Veldman, K., Mevius, D., Fetsch, A., Tenhagen, B.A., Porrero, M.C., Dominguez, L., Granie, S.A., Jouy, E., Butaye, P., Kaszanyitzky, E., Dán, A., Zmudzki, J., Battisti, A. & Franco, A. (2011) Zinc resistance of Staphylococcus aureus of animal origin is strongly associated with methicillin resistance. *Veterinary Microbiology*. 150 (3–4), pp. 344–348.

Cecchinato, A., Bonfatti, V., Gallo, L. & Carnier, P. (2008) Survival analysis of preweaning piglet survival in a dry-cured ham-producing crossbred line. *Journal of Animal Science*. 86 (10), pp. 2486–2496.

Chevaux, E., Sacy, A., Le Treut, Y. & Martineau, G.P. (2010) Intra-Uterine Growth Retardation (IUGR): morphological and behavioural description. In: 21st IPVS congress. Vancouver, Canada, p 209.

Chen, F., Wang, T., Feng, C., Lin, G., Zhu, Y., Wu, G., Johnson, G. & Wang, J. (2015) Proteome differences in placenta and endometrium between normal and intrauterine growth restricted pig fetuses. *PLoS ONE*. 10 (11), E0142396.

Chen, H., Mao, X., He, J., Yu, B., Huang, Z., Yu, J., Zheng, P. & Chen, D. (2013) Dietary fibre affects intestinal mucosal barrier function and regulates intestinal bacteria in weaning piglets. *British Journal of Nutrition*. 110 (10), pp. 1837–1848.

Chen, L., Xu, Y., Chen, X., Fang, C., Zhao, L. & Chen, F. (2017) The maturing development of gut microbiota in commercial piglets during the weaning transition. *Frontiers in Microbiology*. 8, 1688.

Chen, X., Xu, J., Ren, E., Su, Y. & Zhu, W. (2018) Co-occurrence of early gut colonization in neonatal piglets with microbiota in the maternal and surrounding delivery environments. *Anaerobe*. 49, pp.30–40.

Cheng, C.S., Wei, H.K., Wang, P., Yu, H.C., Zhang, X.M., Jiang, S.W. & Peng, J. (2019) Early intervention with faecal microbiota transplantation: An effective means to improve growth performance and the intestinal development of suckling piglets. *Animal*. 13 (3), pp. 533–541.

Cheng, C., Wei, H., Yu, H., Xu, C., Jiang, S. & Peng, J. (2018) Metabolic syndrome during perinatal period in sows and the link with gut microbiota and metabolites. *Frontiers in Microbiology*. 9, 1989.

Chevaux, E., Sacy, A., Le Treut, Y. and Martineau, G. (2010) 'Intrauterine growth retardation (IUGR): Morphological and behavioural description'. International Pig Veterinary Society – Biennial International Congress. Vancouver, Canada, 21/07/2010, p. 209.

Choct, M. (1997) Feed non-starch polysaccharides: Chemical structures and nutritional significance. *Feed Milling International*. pp.13–26.

Choudhury, R., Middelkoop, A., Bolhuis, J.E., Kleerebezem, M. (2019) Legitimate and reliable determination of the age-related intestinal microbiome in young piglets; rectal swabs and fecal samples provide comparable insights. *Frontiers in Microbiology*. 10, 1886.

Choudhury, R., Middelkoop, A., Gerrits, W.J.J., Kemp, B., Bolhuis, J.E. & Kleerebezem, M. (2021) Early-life feeding accelerates gut microbiome maturation in piglets. Scientific Reports. 11, 4213.

Ciesinski, L., Guenther, S., Pieper, R., Kalisch, M., Bednorz, C. & Wieler, L.H. (2018) High dietary zinc feeding promotes persistence of multi-resistant E. coli in the swine gut. *PLoS ONE*. 13 (1), E0191660.

Clarridge, J.E. (2004) Impact of 16S rRNA gene sequence analysis for identification of bacteria on clinical microbiology and infectious diseases. *Clinical Microbiology Reviews*. 17 (4) pp.840–862.

Clowes, E.J., Williams, I.H., Baracos, V.E., Pluske, J.R., Cegielski, A.C., Zak, L.J. & Aherne, F.X. (1998) Feeding Lactating Primiparous Sows to Establish Three Divergent Metabolic States: II. Effect on Nitrogen Partitioning and Skeletal Muscle Composition. *Journal of Animal Science*. 76 (4), pp. 1154–1164.

Collins, C.L., Morrison, R.S., Smits, R.J., Henman, D.J., Dunshea, F.R. & Pluske, J.R. (2013) Interactions between piglet weaning age and dietary creep feed composition on lifetime growth performance1. *Animal Production Science*. 53 (10), pp. 1025–1032.

Collins, C.L., Pluske, J.R., Morrison, R.S., McDonald, T.N., Smits, R.J., Henman, D.J., Stensland, I. & Dunshea, F.R. (2017) Post-weaning and whole-of-life performance of pigs is determined by live weight at weaning and the complexity of the diet fed after weaning. *Animal Nutrition*. 3 (4), pp. 372–379.

Conterno, L., Fava, F., Viola, R. & Tuohy, K.M. (2011) Obesity and the gut microbiota: Does up-regulating colonic fermentation protect against obesity and metabolic disease? *Genes and Nutrition*. 6 (3) pp.241–260.

Costello, E.K., Carlisle, E.M., Bik, E.M., Morowitz, M.J. & Relman, D.A. (2013) Microbiome assembly across multiple body sites in low-birthweight infants. *mBio*. 4 (6), E00782-00713.

Cottrell, J.J., Craig, J., Wijesiriwardana, U.A., Fothergill, L., Ringuet, M.T., O'Hallorhan, K., Turpin, D.L., Munoz, L.M., Collins, C.L., Furness, J.B., Dunshea, F. R. & Pluske, J. (2017) The gastrointestinal tract of piglets from first parity sows develops more slowly and is more permeable than piglets from later parity sows. *FASEB Journal*. 31, p.792.

Craig, J.R., Collins, C.L., Bunter, K.L., Cottrell, J.J., Dunshea, F.R. & Pluske, J.R. (2017) Poorer lifetime growth performance of gilt progeny compared with sow progeny is largely due to weight differences at birth and reduced growth in the preweaning period,

and is not improved by progeny segregation after weaning. *Journal of Animal Science*. 95 (11), pp. 4904–4916.

Craig, J.R., Dunshea, F.R., Cottrell, J.J., Wijesiriwardana, U.A. & Pluske, J.R. (2019) Primiparous and multiparous sows have largely similar colostrum and milk composition profiles throughout lactation. *Animals*. 9 (2), 35.

Cryan, J.F. & Dinan, T.G. (2012) Mind-altering microorganisms: The impact of the gut microbiota on brain and behaviour. *Nature Reviews Neuroscience*. 13 (10) pp.701–712.

Curtis, T.P. & Sloan, W.T. (2004) Prokaryotic diversity and its limits: microbial community structure in nature and implications for microbial ecology. *Current Opinion in Microbiology*. 7 (3), pp. 221–226.

D'Eath, R.B. (2005) Socialising piglets before weaning improves social hierarchy formation when pigs are mixed post-weaning. *Applied Animal Behaviour Science*. 93 (3–4), pp. 199–211.

D'Inca, R., Che, L., Thymann, T., Sangild, P.T. & Le Huërou-Luron, I. (2010) Intrauterine growth restriction reduces intestinal structure and modifies the response to colostrum in preterm and term piglets. *Livestock Science*. 133 (1–3), pp. 20–22.

D'Inca, R., Gras-Le Guen, C., Che, L., Sangild, P.T. & Le Huërou-Luron, I. (2011) Intrauterine growth restriction delays feeding-induced gut adaptation in term newborn pigs. *Neonatology*. 99 (3), pp. 208–216.

Daft, J.G., Ptacek, T., Kumar, R., Morrow, C. & Lorenz, R.G. (2015) Cross-fostering immediately after birth induces a permanent microbiota shift that is shaped by the nursing mother. *Microbiome*. 3 (1), 17.

Daudelin, J.F., Lessard, M., Beaudoin, F., Nadeau, É., Bissonnette, N., Boutin, Y., Brousseau, J.P., Lauzon, K. & Fairbrother, J. (2011) Administration of probiotics influences F4 (K88)-positive enterotoxigenic Escherichia coli attachment and intestinal cytokine expression in weaned pigs. *Veterinary Research*. 42 (1), 69.

De Goffau, M.C., Fuentes, S., Van Den Bogert, B., Honkanen, H., De Vos, W.M., Welling, G.W., Hyöty, H. & Harmsen, H.J.M. (2014) Aberrant gut microbiota composition at the onset of type 1 diabetes in young children. *Diabetologia*. 57 (8), pp. 1569–1577.

De Rodas, B., Youmans, B.P., Danzeisen, J.L., Tran, H. & Johnson, T.J. (2018) Microbiome profiling of commercial pigs from farrow to finish. *Journal of Animal Science*. 96 (5), pp. 1778–1794.

Deen, M.G.H. and Bilkei, G. (2004) Cross fostering of low-birthweight piglets. *Livestock Production Science*. 90 (2–3), pp. 279–284.

Delzenne, N.M., Neyrinck, A.M. & Cani, P.D. (2011) Modulation of the gut microbiota by nutrients with prebiotic properties: Consequences for host health in the context of obesity and metabolic syndrome. *Microbial Cell Factories*. 10 (supplement 1), S10.

Desbonnet, L., Clarke, G., Shanahan, F., Dinan, T.G. & Cryan, J.F. (2014) Microbiota is essential for social development in the mouse. *Molecular Psychiatry*. 19 (2), pp.146–148.

Devillers, N., Farmer, C., Le Dividich, J. & Prunier, A. (2007) Variability of colostrum yield and colostrum intake in pigs. *Animal*. 1 (7), pp.1033–1041.

Dibner, J.J. & Richards, J.D. (2005) Antibiotic growth promoters in agriculture: History and mode of action. *Poultry Science*. 84 (4), pp. 634–643.

Ding, X., Lan, W., Liu, G., Ni, H. & Gu, J.D. (2019) Exploring possible associations of the intestine bacterial microbiome with the pre-weaned weight gaining performance of piglets in intensive pig production. *Scientific Reports*. 9 (1), 15534.

Dong, G.Z. & Pluske, J.R. (2007) The low feed intake in newly-weaned pigs: Problems and possible solutions. *Asian-Australasian Journal of Animal Sciences*. 20 (3), pp. 440–452.

Dong, L., Zhong, X., Ahmad, H., Li, W., Wang, Y., Zhang, L. & Wang, T. (2014) Intrauterine growth restriction impairs small intestinal mucosal immunity in neonatal piglets. *Journal of Histochemistry and Cytochemistry*. 62 (7), pp. 510–518.

Dou, S., Gadonna-Widehem, P., Rome, V., Hamoudi, D., Rhazi, L., Lakhal, L., Larcher, T., Bahi-Jaber, N., Pinon-Quintana, A., Guyonvarch, A., Huërou-Luron, I.L.E. & Abdennebi-Najar, L. (2017) Characterisation of early-life fecal microbiota in susceptible and healthy pigs to post-weaning diarrhoea. *PLoS ONE*. 12 (1), E0169851.

Douglas, S.L., Edwards, S.A., Sutcliffe, E., Knap, P.W. & Kyriazakis, I. (2013) Identification of risk factors associated with poor lifetime growth performance in pigs. *Journal of Animal Science*. 91 (9), pp. 4123–4132.

Douglas, S.L., Edwards, S.A. & Kyriazakis, I. (2014a) Management strategies to improve the performance of low birth weight pigs to weaning and their long-term consequences. *Journal of Animal Science*. 92 (5), pp. 2280–2288.

Douglas, S.L., Edwards, S.A. & Kyriazakis, I. (2014b) Too late to catch up: A high nutrient specification diet in the grower phase does not improve the performance of low birth weight pigs. *Journal of Animal Science*. 92 (10), pp. 4577–4584.

Douglas, S.L., Wellock, I., Edwards, S.A. & Kyriazakis, I. (2014c) High specification starter diets improve the performance of low birth weight pigs to 10 weeks of age. *Journal of Animal Science*. 92 (10), pp. 4741–4750.

Douglas, S.L., Edwards, S.A. & Kyriazakis, I. (2016) Are all piglets born lightweight alike? Morphological measurements as predictors of postnatal performance. *Journal of Animal Science*. 94 (8), pp. 3510–3518.

Dourmad, J., Quiniou, N., Heugebaert, S., Paboeuf, F. & Ngo, T. 2012. Effect of parity and number of suckling piglets on milk production of sows. In: Proceedings of the 63rd Annual Meeting of the European Federation of Animal Science, Bratislava, Slovakia, p. 44.

Duarte, M.E., Zhou, F.X., Dutra, W.M. & Kim, S.W. (2019) Dietary supplementation of xylanase and protease on growth performance, digesta viscosity, nutrient digestibility, immune and oxidative stress status, and gut health of newly weaned pigs. *Animal Nutrition*. 5 (4), pp.351–358.

Dubreuil, J.D. (2017) Enterotoxigenic Escherichia coli and probiotics in swine: What the bleep do we know? *Bioscience of Microbiota, Food and Health*. 36 (3) pp.75–90.

Edgar, R.C. (2010) Search and clustering orders of magnitude faster than BLAST. *Bioinformatics.* 26 (19), pp. 2460-2461.

Edgar, R. C., Haas, B. J., Clemente, J. C., Quince, C. and Knight, R. (2011) UCHIME improves sensitivity and speed of chimera detection. *Bioinformatics.* 27 (16), pp. 2194–2200.

El-Aidy, S., Hooiveld, G., Tremaroli, V., Bäckhed, F. & Kleerebezem, M. (2013) The gut microbiota and mucosal homeostasis: Colonized at birth or at adulthood, does it matter? *Gut Microbes*. 4 (2), pp. 118–124.

English, J.G.H. & Bilkei, G. (2004) The effect of litter size and littermate weight on pre-weaning performance of low-birth-weight piglets that have been cross-fostered. *Animal Science*. 79 (3), pp. 439–443.

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

Fança-Berthon, P., Hoebler, C., Mouzet, E., David, A. & Michel, C. (2010) Intrauterine growth restriction not only modifies the cecocolonic microbiota in neonatal rats but also affects its activity in young adult rats. *Journal of Pediatric Gastroenterology and Nutrition*. 51 (4), pp. 402–413.

Farmer, C. (2018) Nutritional impact on mammary development in pigs: A review. *Journal of Animal Science*. 96 (9), pp. 3748–3756.

FAO/WHO (2001) Evaluation of Health and Nutritional Properties of Probiotics in Food Including Powder Milk with Live Acid Bacteria. Report of a Joint FAO/WHO Expert Consultation, Córdoba, Argentina. Available at: http://www.fao.org/tempref/docrep/fao/meeting/009/y6398e.pdf (Accessed: 3rd December 2020).

Feldpausch, J.A., Jourquin, J., Bergstrom, J.R., Bargen, J.L., Bokenkroger, C.D., Davis, D.L., Gonzalez, J.M., Nelssen, J.L., Puls, C.L., Trout, W.E. & Ritter, M.J. (2019) Birth weight threshold for identifying piglets at risk for preweaning mortality. *Translational Animal Science*. 3 (2), pp. 633–640.

Ferenc, K., Pilzys, T., Skrzypek, T., Garbicz, D., Marcinkowski, M., Dylewska, M., Gładysz, P., Skorobogatov, O., Gajewski, Z., Grzesiuk, E. & Zabielski, R. (2017) Structure and

function of enterocyte in intrauterine growth retarded pig neonates. *Disease Markers*. 2017, 5238134.

Ferrari, C. V., Sbardella, P.E., Bernardi, M.L., Coutinho, M.L., Vaz, I.S., Wentz, I. & Bortolozzo, F.P. (2014) Effect of birth weight and colostrum intake on mortality and performance of piglets after cross-fostering in sows of different parities. *Preventive Veterinary Medicine*. 114 (3–4), pp. 259–266.

Flint, H.J. & Bayer, E.A. (2008) Plant cell wall breakdown by anaerobic microorganisms from the mammalian digestive tract. *Annals of the New York Academy of Sciences*. 1125 (1), pp. 280–288.

Flint, H.J., Bayer, E.A., Rincon, M.T., Lamed, R. & White, B.A. (2008) Polysaccharide utilization by gut bacteria: Potential for new insights from genomic analysis. *Nature Reviews Microbiology*. 6 (2) pp.121–131.

Foxcroft, G.R., Dixon, W.T., Dyck, M.K., Novak, S., Harding, J.C. & Almeida, F.C. (2009) Prenatal programming of postnatal development in the pig. *Society of Reproduction and Fertility*. supplement 66, p.213–231.

Foxcroft, G.R., Dixon, W.T., Novak, S., Putman, C.T., Town, S.C. & Vinsky, M.D. (2006) The biological basis for prenatal programming of postnatal performance in pigs. *Journal of Animal Science*, 84 (13, supplement 1). pp. 105-112.

Frese, S.A., Parker, K., Calvert, C.C. & Mills, D.A. (2015) Diet shapes the gut microbiome of pigs during nursing and weaning. *Microbiome*. 3 (1), 28.

Gardiner, G.E., Metzler-Zebeli, B.U.& Lawlor, P.G. (2020) Impact of intestinal microbiota on growth and feed efficiency in pigs: A Review. *Microorganisms*, 8 (12), 1886.

Gaukroger, C.H., Stewart, C.J., Edwards, S.A., Walshaw, J., Adams, I.P. & Kyriazakis, I. (2020) Changes in Faecal Microbiota Profiles Associated with Performance and Birthweight of Piglets. *Frontiers in Microbiology.* 11, 917.

Gaukroger, C.H., S.A. Edwards., J. Walshaw., A. Nelson., I. P Adams., C.J. Stewart and I. Kyriazakis. (2021). Shifting sows: longitudinal changes in the periparturient faecal microbiota of primiparous and multiparous sows. *Animal*. 15(3), 100135.

Gensollen, T., Iyer, S.S., Kasper, D.L. & Blumberg, R.S. (2016) How colonization by microbiota in early life shapes the immune system. *Science*, 352 (6285) p.539–544.

George, P.B. (1975) *Glucose metabolism by gravid sows.* PhD thesis. Oregan State University. Available at: https://scholar.google.com/scholar_lookup?title=Glucose%20metabolism%20in%20gravid%20sows&publication_year=1975&author=P.B.%20George (Accessed: 13/06/2020).

Gérard, P. (2016) Gut microbiota and obesity. *Cellular and Molecular Life Sciences*, 73, pp. 147-162.

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

Gollwitzer, E.S., Saglani, S., Trompette, A., Yadava, K., Sherburn, R., McCoy, K.D., Nicod, L.P., Lloyd, C.M. & Marsland, B.J. (2014) Lung microbiota promotes tolerance to allergens in neonates via PD-L1. *Nature Medicine*. 20 (6), pp. 642–647.

Gondret, F., Lefaucheur, L., Louveau, I., Lebret, B., Pichodo, X. & Le Cozler, Y. (2005) Influence of piglet birth weight on postnatal growth performance, tissue lipogenic capacity and muscle histological traits at market weight. *Livestock Production Science*. 93 (2), pp. 137–146.

Gondret, F., Lefaucheur, L., Juin, H., Louveau, I. & Lebret, B. (2006) Low birth weight is associated with enlarged muscle fiber area and impaired meat tenderness of the longissimus muscle in pigs. *Journal of Animal Science*. 84 (1), pp. 93–103.

Goodrich, J.K., Waters, J.L., Poole, A.C., Sutter, J.L., Koren, O., Blekhman, R., Beaumont, M., Van Treuren, W., Knight, R., Bell, J.T., Spector, T.D., Clark, A.G. & Ley, R.E. (2014) Human genetics shape the gut microbiome. *Cell*. 159 (4), pp. 789–799.

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

Grier, A., Qiu, X., Bandyopadhyay, S., Holden-Wiltse, J., Kessler, H.A., Gill, A.L., Hamilton, B., Huyck, H., Misra, S., Mariani, T.J., Ryan, R.M., Scholer, L., Scheible, K.M., Lee, Y.H., Caserta, M.T., Pryhuber, G.S. & Gill, S.R. (2017) Impact of prematurity and nutrition on the developing gut microbiome and preterm infant growth. *Microbiome*. 5 (1), 158.

Grześkowiak, Ł., Martínez-Vallespín, B., Dadi, T.H., Radloff, J., Amasheh, S., Heinsen, F.A., Franke, A., Reinert, K., Vahjen, W., Zentek, J. & Pieper, R. (2018) Formula feeding predisposes neonatal piglets to clostridium difficile gut infection. *Journal of Infectious Diseases*. 217 (9), pp.1442–1452.

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

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

Guinane, C.M. & Cotter, P.D. (2013) Role of the gut microbiota in health and chronic gastrointestinal disease: Understanding a hidden metabolic organ. *Therapeutic Advances in Gastroenterology*. 6 (4), pp. 295–308.

Hales, J., Moustsen, V.A., Nielsen, M.B.F. & Hansen, C.F. (2013) Individual physical characteristics of neonatal piglets affect preweaning survival of piglets born in a noncrated system. *Journal of Animal Science.* 91 (10), pp. 4991–5003.

Han, G.G., Lee, J.Y., Jin, G.D., Park, J., Choi, Y.H., Chae, B.J., Kim, E.B. & Choi, Y.J. (2017) Evaluating the association between body weight and the intestinal microbiota of weaned piglets via 16S rRNA sequencing. *Applied Microbiology and Biotechnology*. 101 (14), pp. 5903–5911.

Han, G.G., Lee, J.Y., Jin, G.D., Park, J., Choi, Y.H., Kang, S.K., Chae, B.J., Kim, E.B. & Choi, Y.J. (2018) Tracing of the fecal microbiota of commercial pigs at five growth stages from birth to shipment. *Scientific Reports*. 8 (1), 6012.

Han, C., Dai, Y., Liu, B., Wang, L., Wang, J. & Zhang, J. (2019) Diversity analysis of intestinal microflora between healthy and diarrheal neonatal piglets from the same litter in different regions. *Anaerobe*.55, pp.136–141.

Hansen, A. V., Strathe, A.B., Kebreab, E., France, J. & Theil, P.K. (2012) Predicting milk yield and composition in lactating sows: A Bayesian approach. *Journal of Animal Science*. 90 (7), pp. 2285–2298.

Hansen, C.F., Hales, J., Amdi, C. & Moustsen, V.A. (2019) Intrauterine growth-restricted piglets defined by their head shape have impaired survival and growth during the suckling period. *Animal Production Science*. 59 (6), pp. 1056–1062.

Härtel, C., Pagel, J., Spiegler, J., Buma, J., Henneke, P., Zemlin, M., Viemann, D., Gille, C., Gehring, S., Frommhold, D., Rupp, J., Herting, E. & Göpel, W. (2017) Lactobacillus acidophilus/Bifidobacterium infantis probiotics are associated with increased growth of VLBWI among those exposed to antibiotics. *Scientific Reports*. 7 (1), 5633.

Hasan, S., Junnikkala, S., Peltoniemi, O., Paulin, L., Lyyski, A., Vuorenmaa, J. & Oliviero, C. (2018) Dietary supplementation with yeast hydrolysate in pregnancy influences colostrum yield and gut microbiota of sows and piglets after birth. *PLoS ONE*. 13 (5), E0197586.

Hawe, S.J., Scollan, N., Gordon, A. & Magowan, E. (2020) What is the current significance of low birthweight pigs on commercial farms in Northern Ireland in terms of impaired growth and mortality? *Translational Animal Science*, 4 (3), txaa147.

Hayakawa, T., Masuda, T., Kurosawa, D. & Tsukahara, T. (2016) Dietary administration of probiotics to sows and/or their neonates improves the reproductive performance, incidence of post-weaning diarrhea and histopathological parameters in the intestine of weaned piglets. *Animal Science Journal*. 87 (12), pp. 1501–1510.

He, Y., Deen, J., Shurson, G.C., Wang, L., Chen, C., Keisler, D.H. & Li, Y.Z. (2016) Identifying factors contributing to slow growth in pigs. *Journal of Animal Science*. 94 (5), pp. 2103–2116.

Heijtz, R.D., Wang, S., Anuar, F., Qian, Y., Bjorkholm, B. & Samuelsson, A. (2011) Normal gut microbiota modulates brain development and behavior. *Proceeding of the National Academy of Sciences*. 108 (7), pp. 3047-3052.

Heo, J.M., Kim, J.C., Hansen, C.F., Mullan, B.P., Hampson, D.J. & Pluske, J.R. (2008) Effects of feeding low protein diets to piglets on plasma urea nitrogen, faecal ammonia nitrogen, the incidence of diarrhoea and performance after weaning. *Archives of Animal Nutrition*. 62 (5), pp. 343–358.

Heo, J.M., Kim, J.C., Hansen, C.F., Mullan, B.P., Hampson, D.J. & Pluske, J.R. (2009) Feeding a diet with decreased protein content reduces indices of protein fermentation and the incidence of postweaning diarrhea in weaned pigs challenged with an enterotoxigenic strain of Escherichia coli. *Journal of Animal Science*. 87 (9), pp. 2833–2843.

Heo, J.M., Kim, J.C., Hansen, C.F., Mullan, B.P., Hampson, D.J. & Pluske, J.R. (2010) Feeding a diet with a decreased protein content reduces both nitrogen content in the gastrointestinal tract and post-weaning diarrhoea, but does not affect apparent nitrogen digestibility in weaner pigs challenged with an enterotoxigenic strain of Escherichia coli. *Animal Feed Science and Technology*. 160 (3–4), pp. 148–159.

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

Herpin, P., Damon, M. & Le Dividich, J. (2002) Development of thermoregulation and neonatal survival in pigs. *Livestock Production Science*. 78 (1), pp. 25–45.

Hill, G.M., Mahan, D.C., Carter, S.D., Cromwell, G.L., Ewan, R.C., Harrold, R.L., Lewis, A.J., Miller, P.S., Shurson, G.C., Veum, T.L., Cline, T.R., Crenshaw, T.D., Hollis, G.R., Libal, G.W., Nelssen, J.L., Yen, J.T. & Layman, D.K. (2001) Effect of pharmacological concentrations of zinc oxide with or without the inclusion of an antibacterial agent on nursery pig performance. *Journal of Animal Science*. 79 (4), pp. 934–941.

Højberg, O., Canibe, N., Poulsen, H.D., Hedemann, M.S. & Jensen, B.B. (2005) Influence of dietary zinc oxide and copper sulfate on the gastrointestinal ecosystem in newly weaned piglets. *Applied and Environmental Microbiology*. 71 (5), pp. 2267–2277.

Holman, D.B., Brunelle, B.W., Trachsel, J. & Allen, H.K. (2017) Meta-analysis to define a core microbiota in the swine gut. *mSystems*. 2 (3), E00004-17.

Horrell, I. & Hodgson, J. (1992a) The bases of sow-piglet identification. 1. The identification by sows of their own piglets and the presence of intruders. *Applied Animal Behaviour Science*. 33 (4), pp. 319–327.

Horrell, I. & Hodgson, J. (1992b) The bases of sow-piglet identification. 2. Cues used by piglets to identify their dam and home pen. *Applied Animal Behaviour Science*. 33 (4), pp. 329–343.

Hou, C., Liu, H., Zhang, J., Zhang, S., Yang, F., Zeng, X., Thacke, P.A., Zhang, G. & Qiao, S. (2015) Intestinal microbiota succession and immunomodulatory consequences after introduction of Lactobacillus reuteri I5007 in neonatal piglets. *PLoS ONE*. 10 (3), E0119505.

Hu, C., Song, J., Li, Y., Luan, Z. & Zhu, K. (2013) Diosmectite-zinc oxide composite improves intestinal barrier function, modulates expression of pro-inflammatory cytokines and tight junction protein in early weaned pigs. *British Journal of Nutrition*. 110 (4), pp. 681–688.

Hu, C.H., Song, Z.H., Xiao, K., Song, J., Jiao, L.F. & Ke, Y.L. (2014) Zinc oxide influences intestinal integrity, the expressions of genes associated with inflammation and TLR4-myeloid differentiation factor 88 signaling pathways in weanling pigs. *Innate Immunity*. 20 (5), pp. 478–486.

Hu, J., Nie, Y., Chen, J., Zhang, Y., Wang, Z., Fan, Q. & Yan, X. (2016a) Gradual changes of gut microbiota in weaned miniature piglets. *Frontiers in Microbiology*. 7, 1727.

Hu, L., Peng, X., Chen, H., Yan, C., Liu, Y., Xu, Q., Fang, Z., Lin, Y., Xu, S., Feng, B., Li, J., Wu, D. & Che, L. (2016b) Effects of intrauterine growth retardation and Bacillus subtilis PB6 supplementation on growth performance, intestinal development and immune function of piglets during the suckling period. *European Journal of Nutrition*. 56 (4), pp. 1753-1765.

Hu, L., Geng, S., Li, Y., Cheng, S., Fu, X., Yue, X. & Han, X. (2018) Exogenous fecal microbiota transplantation from local adult pigs to crossbred newborn piglets. *Frontiers in Microbiology*. 8, 2663.

Huang, C., Song, P., Fan, P., Hou, C., Thacker, P. & Ma, X. (2015) Dietary sodium butyrate decreases postweaning diarrhea by modulating intestinal permeability and changing the bacterial communities in weaned piglets. *Journal of Nutrition*. 145 (12), pp. 2774–2780.

Huang, M.Z., Wang, S.Y., Wang, H., Cui, D.A., Yang, Y.J., Liu, X.W., Kong, X.J. & Li, J.Y. (2018) Differences in the intestinal microbiota between uninfected piglets and piglets infected with porcine epidemic diarrhea virus. *PLoS ONE*. 13 (2), E0192992.

Huang, S., Li, N., Liu, C., Li, T., Wang, W., Jiang, L., Li, Z., Han, D., Tao, S. & Wang, J. (2019a) Characteristics of the gut microbiota colonization, inflammatory profile, and plasma metabolome in intrauterine growth restricted piglets during the first 12 hours after birth. *Journal of Microbiology*. 57 (9), pp. 748–758.

Huang, X., Gao, J., Zhao, Y., He, M., Ke, S., Wu, J., Zhou, Y., Fu, H., Yang, H., Chen, C. & Huang, L. (2019b) Dramatic remodeling of the gut microbiome around parturition and its relationship with host serum metabolic changes in sows. *Frontiers in Microbiology*. 10, 2123.

Huang, Y., Li, Y. li, Huang, Q., Cui, Z. wen, Yu, D. you, Rajput, I.R., Hu, C. hong & Li, W. fen (2012) Effect of orally administered Enterococcus faecium EF1 on Intestinal cytokines and chemokines production of suckling piglets. *Pakistan Veterinary Journal*. 32 (1), pp. 81–84.

Huting, A.M.S., Almond, K., Wellock, I. & Kyriazakis, I. (2017) What is good for small piglets might not be good for big piglets: The consequences of cross-fostering and creep feed provision on performance to slaughter. *Journal of Animal Science*. 95 (11), pp. 4926–4944.

Huting, A.M.S., Sakkas, P., Wellock, I., Almond, K. & Kyriazakis, I. (2018) Once small always small? To what extent morphometric characteristics and postweaning starter regime affect pig lifetime growth performance. *Porcine Health Management*. 4 (1), 21.

Huting, A.M.S., Sakkas, P. & Kyriazakis, I. (2019) Sows in mid parity are best foster mothers for the pre- A nd post-weaning performance of both light and heavy piglets. *Journal of Animal Science*. 97 (4), pp. 1656–1670.

Huygelen, V., De Vos, M., Prims, S., Vergauwen, H., Fransen, E., Casteleyn, C., Van Cruchten, S. & Van Ginneken, C. (2015) Birth weight has no influence on the

morphology, digestive capacity and motility of the small intestine in suckling pigs. *Livestock Science*. 182, pp. 129–136.

Inman, C.F., Haverson, K., Konstantinov, S.R., Jones, P.H., Harris, C., Smidt, H., Miller, B., Bailey, M. & Stokes, C. (2010) Rearing environment affects development of the immune system in neonates. *Clinical and Experimental Immunology*. 160 (3), pp. 431–439.

Inoue, R., Tsukahara, T., Nakanishi, N. & Ushida, K. (2005) Development of the intestinal microbiota in the piglet. *The Journal of General and Applied Microbiology*. 51 (4), pp. 257–265.

Jarvis, S., Van der Vegt, B.J., Lawrence, A.B., McLean, K.A., Deans, L.A., Chirnside, J. & Calvert, S.K. (2001) The effect of parity and environmental restriction on behavioural and physiological responses of pre-parturient pigs. *Applied Animal Behaviour Science*. 71 (3), pp. 203–216.

Jarvis, S., Calvert, S.K., Stevenson, J., van Leeuwen, N. & Lawrence, A.B. (2002) Pituitary-adrenal activation in pre-parturient pigs (sus scrofa) is associated with behavioural restriction due to lack of space rather than nesting substrate. *Animal Welfare*. 11 (4), pp. 371–384.

Jensen-Waern, M., Melin, L., Lindberg, R., Johannisson, A., Petersson, L. & Wallgren, P. (1998) Dietary zinc oxide in weaned pigs - Effects on performance, tissue concentrations, morphology, neutrophil functions and faecal microflora. *Research in Veterinary Science*. 64 (3), pp. 225–231.

Jensen, P. (1994) Fighting between unacquainted pigs - effects of age and of individual reaction pattern. *Applied Animal Behaviour Science*. 41 (1–2), pp. 37–52.

Jensen, P. & Recén, B. (1989) When to wean - Observations from free-ranging domestic pigs. *Applied Animal Behaviour Science*. 23 (1–2), pp. 49–60.

Jiang, Z., Wei, S., Wang, Z., Zhu, C., Hu, S., Zheng, C., Chen, Z., Hu, Y., Wang, L., Ma, X. & Yang, X. (2015) Effects of different forms of yeast Saccharomyces cerevisiae on growth performance, intestinal development, and systemic immunity in early-weaned piglets. *Journal of Animal Science and Biotechnology*. 6, 47.

Jolley, K.A. & Maiden, M.C.J. (2010) BIGSdb: Scalable analysis of bacterial genome variation at the population level. *BMC Bioinformatics*. 11 (1), 595.

Joint Genome Institute. (2020) *BBDuk Guide*. Available at: https://jgi.doe.gov/data-and-tools/bbtools/bb-tools-user-guide/bbduk-guide/ (Accessed: 6th December 2020).

Kabat, A.M., Srinivasan, N. & Maloy, K.J. (2014) Modulation of immune development and function by intestinal microbiota. *Trends in Immunology*. 35 (11) p.507–517.

Kamada, N. & Núñez, G. (2014) Regulation of the immune system by the resident intestinal bacteria. *Gastroenterology*. 146 (6), pp. 1477–88.

Katouli, M., Lund, A., Wallgren, P., Kühn, I., Söderlind, O. & Möllby, R. (1997) Metabolic fingerprinting and fermentative capacity of the intestinal flora of pigs during pre- and post-weaning periods. *Journal of Applied Microbiology*. 83 (2), pp. 147–154.

Kats, L.J., Tokach, M.D., Goodband, R.D. & Nelssen, J.L. (1992) Influence of weaning weight and growth during the first week postweaning on subsequent pig performance. *Kansas Agricultural Experiment Station Research Reports*. (10), pp. 15–17.

Ke, S., Fang, S., He, M., Huang, X., Yang, H., Yang, B., Chen, C. & Huang, L. (2019) Age-based dynamic changes of phylogenetic composition and interaction networks of health pig gut microbiome feeding in a uniformed condition. *BMC Veterinary Research*. 15 (1), 172.

Khot, P.D. & Fisher, M.A. (2013) Novel approach for differentiating shigella species and Escherichia coli by matrix-assisted laser desorption ionization-time of flight mass spectrometry. *Journal of Clinical Microbiology*. 51 (11), pp. 3711–3716.

Kim, E.Y., Kim, Y.H., Rhee, M.H., Song, J.C., Lee, K.W., Kim, K.S., Lee, S.P., Lee, I.S. & Park, S.C. (2007) Selection of lactobacillus sp. PSC101 that produces active dietary enzymes such as amylase, lipase, phytase and protease in pigs. *Journal of General and Applied Microbiology*. 53 (2), pp. 111–117.

Kim, H.B., Borewicz, K., White, B.A., Singer, R.S., Sreevatsan, S., Tu, Z.J. & Isaacson, R.E. (2011) Longitudinal investigation of the age-related bacterial diversity in the feces of commercial pigs. *Veterinary Microbiology*. 153 (1–2), pp. 124–133.

Kim, J.C., Hansen, C.F., Mullan, B.P. & Pluske, J.R. (2012) Nutrition and pathology of weaner pigs: Nutritional strategies to support barrier function in the gastrointestinal tract. *Animal Feed Science and Technology*. 173 (1–2), pp. 3–16.

Kim, J., Nguyen, S.G., Guevarra, R.B., Lee, I. & Unno, T. (2015) Analysis of swine fecal microbiota at various growth stages. *Archives of Microbiology*. 197 (6), pp. 753–759.

King, R.H., Mullan, B.P., Dunshea, F.R. & Dove, H. (1997) The influence of piglet body weight on milk production of sows. *Livestock Production Science*. 47 (2), pp. 169–174.

King, R.L., Matheson, S.M., Baxter, E.M. & Edwards, S.A. (2020) Sow behaviour and piglet weight gain after late cross-fostering in farrowing crates and pens. *Animal*. 14 (9), pp. 1923-1933.

Kiros, T.G., Luise, D., Derakhshani, H., Petri, R., Trevisi, P., D'Inca, R., Auclair, E. & van Kessel, A.G. (2019) Effect of live yeast Saccharomyces cerevisiae supplementation on the performance and cecum microbial profile of suckling piglets. *PLoS ONE*. 14 (7), E0219557.

Kittana, H., Gomes-Neto, J.C., Heck, K., Geis, A.L., Segura Muñoz, R.R., Cody, L.A., Schmaltz, R.J., Bindels, L.B., Sinha, R., Hostetter, J.M., Benson, A.K. & Ramer-Tait, A.E. (2018) Commensal Escherichia coli strains can promote intestinal inflammation via differential interleukin-6 production. *Frontiers in Immunology*. 9, 2318.

Klaaborg, J. & Amdi, C. (2020) Administration of glucose at litter equalization as a strategy to increase energy in intrauterine growth restricted piglets. *Animals*. 10 (7), pp. 1–10.

Klingspor, S., Martens, H., Çaushi, D., Twardziok, S., Aschenbach, J.R. & Lodemann, U. (2013) Characterization of the effects of Enterococcus faecium on intestinal epithelial transport properties in piglets. *Journal of Animal Science*. 91 (4), pp.1707–1718.

Klingspor, S., Bondzio, A., Martens, H., Aschenbach, J.R., Bratz, K., Tedin, K., Einspanier, R. & Lodemann, U. (2015) Enterococcus faecium NCIMB 10415 modulates epithelial integrity, heat shock protein, and proinflammatory cytokine response in intestinal cells. *Mediators of Inflammation*. 2015, 304149.

Knights, D., Kuczynski, J., Charlson, E.S., Zaneveld, J., Mozer, M.C., Collman, R.G., Bushman, F.D., Knight, R. & Kelley, S.T. (2011) Bayesian community-wide culture-independent microbial source tracking. *Nature Methods*. 8 (9), pp.761–765.

Koh, H.W., Kim, M.S., Lee, J.S., Kim, H. & Park, S.J. (2015) Changes in the swine gut microbiota in response to porcine epidemic diarrhoea infection. *Microbes and Environments*. 30 (3), pp. 284–287.

Koketsu, Y., Tani, S. & Iida, R. (2017) Factors for improving reproductive performance of sows and herd productivity in commercial breeding herds. *Porcine Health Management.* 3 (1), 1.

Kolacek, S., Puntis, J.W.L., Lloyd, D.R., Brown, G.A. & Booth, I.W. (1990) Ontogeny of pancreatic exocrine function. *Archives of Disease in Childhood*. 65 (2), pp. 178–181.

Koren, O., Goodrich, J.K., Cullender, T.C., Spor, A., Laitinen, K., Kling Bäckhed, H., Gonzalez, A., Werner, J.J., Angenent, L.T., Knight, R., Bäckhed, F., Isolauri, E., Salminen, S. & Ley, R.E. (2012) Host remodeling of the gut microbiome and metabolic changes during pregnancy. *Cell*. 150 (3), pp. 470–480.

Kozich, J.J., Westcott, S.L., Baxter, N.T., Highlander, S.K. & Schloss, P.D. (2013) Development of a dual-index sequencing strategy and curation pipeline for analyzing amplicon sequence data on the miseq illumina sequencing platform. *Applied and Environmental Microbiology*. 79 (17), pp. 5112–5120.

Kreuzer-Redmer, S., Bekurtz, J.C., Arends, D., Bortfeldt, R., Kutz-Lohroff, B., Sharbati, S., Einspanier, R. & Brockmann, G.A. (2016) Feeding of Enterococcus faecium NCIMB 10415 leads to intestinal miRNA-423-5p-induced regulation of immunerelevant genes. *Applied and Environmental Microbiology*. 82 (8), pp. 2263–2269.

Kreuzer, S., MacHnowska, P., Amus, J., Sieber, M., Pieper, R., Schmidt, M.F., Brockmann, G.A., Scharek-Tedin, L. & Johne, R. (2012) Feeding of the probiotic bacterium Enterococcus faecium NCIMB 10415 differentially affects shedding of enteric viruses in pigs. *Veterinary Research*. 43 (1), 58.

Krueger, R., Derno, M., Goers, S., Metzler-Zebeli, B.U., Nuernberg, G., Martens, K., Pfuhl, R., Nebendahl, C., Zeyner, A., Hammon, H.M. & Metges, C.C. (2014) Higher body fatness in intrauterine growth retarded juvenile pigs is associated with lower fat and

higher carbohydrate oxidation during ad libitum and restricted feeding. *European Journal of Nutrition*. 53 (2), pp. 583–597.

Kubasova, T., Davidova-Gerzova, L., Babak, V., Cejkova, D., Montagne, L., Le-Floc'h, N. & Rychlik, I. (2018) Effects of host genetics and environmental conditions on fecal microbiota composition of pigs. *PLoS ONE*. 13 (8), E0201901.

Kubasova, T., Davidova-Gerzova, L., Merlot, E., Medvecky, M., Polansky, O., Gardan-Salmon, D., Quesnel, H. & Rychlik, I. (2017) Housing systems influence gut microbiota composition of sows but not of their piglets. *PLoS ONE*. 12 (1), E0170051.

Kuller, W.I., Soede, N.M., Van Beers-Schreurs, H.M.G., Langendijk, P., Taverne, M.A.M., Kemp, B. & Verheijden, J.H.M. (2007a) Effects of intermittent suckling and creep feed intake on pig performance from birth to slaughter. *Journal of Animal Science*. 85 (5), pp. 1295–1301.

Kuller, W.I., van Beers-Schreurs, H.M.G., Soede, N.M., Langendijk, P., Taverne, M.A.M., Kemp, B. & Verheijden, J.H.M. (2007b) Creep feed intake during lactation enhances net absorption in the small intestine after weaning. *Livestock Science*. 108 (1–3), pp. 99–101.

Lallès, J.P., Boudry, G., Favier, C., Le Floc'h, N., Luron, I., Montagne, L., Oswald, I.P., Pié, S., Piel, C. & Sève, B. (2004) Gut function and dysfunction in young pigs: Physiology. *Animal Research.* 53 (4), pp. 301–316.

Lallès, J.-P., Bosi, P., Smidt, H. & Stokes, C.R. (2007) Weaning — A challenge to gut physiologists. *Livestock Science*. 108 (1–3), pp. 82–93.

Lallès, J.P., Bizon, A., Taekema, A., Arnal, M.E., Stokes, C.R., Bailey, M., Smidt, H., Jansman, A. & Koopmans, S.J. (2014) Microbiota diversity during neonatal colonization impacts gut physiology in a pig model. *Proceedings of the Nutrition Society*. 73.

Lanferdini, E., Andretta, I., Fonseca, L.S., Moreira, R.H.R., Cantarelli, V.S., Ferreira, R.A., Saraiva, A. & Abreu, M.L.T. (2018) Piglet birth weight, subsequent performance, carcass traits and pork quality: A meta-analytical study. *Livestock Science*. 214, pp. 175–179.

Larivière-Gauthier, G., Thibodeau, A., Letellier, A., Yergeau & Fravalo, P. (2019) Salmonella shedding status of the sow affects the microbiota of their piglets at weaning. *Journal of Applied Microbiology*. 126 (2), pp. 411–423.

Larivière-Gauthier, G., Thibodeau, A., Letellier, A., Yergeau & Fravalo, P. (2020) Sows affect their piglets' faecal microbiota until fattening but not their Salmonella enterica shedding status. *Letters in Applied Microbiology.* 72 (2), pp. 113-120.

Le Chatelier, E., Nielsen, T., Qin, J., Prifti, E., Hildebrand, F., Falony, G., Almeida, M., Arumugam, M., Batto, J. M., Kennedy, S., Leonard, P., Li, J., Burgdorf, K., Grarup, N., Jorgensen, T., Branslund, I., Nielsen, H.B., Juncker, A. S., Bertalan, M. *et al.* (2013) Richness of human gut microbiome correlates with metabolic markers. *Nature*. 500, pp. 541–546.

Le Dividich, J. & Herpin, P. (1994) Effects of climatic conditions on the performance, metabolism and health status of weaned piglets: a review. *Livestock Production Science*. 38 (2), pp. 79–90.

Le Sciellour, M., Labussière, E., Zemb, O. & Renaudeau, D. (2018) Effect of dietary fiber content on nutrient digestibility and fecal microbiota composition in growing-finishing pigs. *PLoS ONE*. 13 (10), E0206159.

Lei, X.J. & Kim, I.H. (2018) Low dose of coated zinc oxide is as effective as pharmacological zinc oxide in promoting growth performance, reducing fecal scores, and improving nutrient digestibility and intestinal morphology in weaned pigs. *Animal Feed Science and Technology*. 245, pp. 117–125.

Lessard, M., Blais, M., Beaudoin, F., Deschene, K., Verso, L. Lo, Bissonnette, N., Lauzon, K. & Guay, F. (2018) Piglet weight gain during the first two weeks of lactation influences the immune system development. *Veterinary Immunology and Immunopathology*. 206, pp. 25–34.

Lewis, M.C., Inman, C.F., Patel, D., Schmidt, B., Mulder, I., Miller, B., Gill, B.P., Pluske, J., Kelly, D., Stokes, C.R. & Bailey, M. (2012) Direct experimental evidence that early-life farm environment influences regulation of immune responses. *Pediatric Allergy and Immunology*. 23 (3), pp. 265–269.

Ley, R.E., Turnbaugh, P.J., Klein, S. & Gordon, J.I. (2006) Microbial ecology: Human gut microbes associated with obesity. *Nature*. 444 (7122), pp.1022–1023.

Li, M., Bauer, L.L., Chen, X., Wang, M., Kuhlenschmidt, T.B., Kuhlenschmidt, M.S., Fahey, G.C. & Donovan, S.M. (2012) Microbial composition and in vitro fermentation patterns of human milk oligosaccharides and prebiotics differ between formula-fed and sow-reared piglets. *Journal of Nutrition*. 142 (4), pp. 681–689.

Li, N., Huang, S., Jiang, L., Wang, W., Li, T., Zuo, B., Li, Z. & Wang, J. (2018) Differences in the gut microbiota establishment and metabolome characteristics between low- and normal-birth-weight piglets during early-life. *Frontiers in Microbiology*. 9, 1789.

Li, N., Huang, S., Jiang, L., Dai, Z., Li, T., Han, D. & Wang, J. (2019) Characterization of the early life microbiota development and predominant Lactobacillus species at distinct gut segments of low- And normal-birth-weight piglets. *Frontiers in Microbiology*. 10, 797.

Liao, S.F. & Nyachoti, M. (2017) Using probiotics to improve swine gut health and nutrient utilization. *Animal Nutrition.* 3 (4) pp.331–343.

Lin, H. V., Frassetto, A., Kowalik, E.J., Nawrocki, A.R., Lu, M.M., Kosinski, J.R., Hubert, J.A., Szeto, D., Yao, X., Forrest, G. & Marsh, D.J. (2012) Butyrate and propionate protect against diet-induced obesity and regulate gut hormones via free fatty acid receptor 3-independent mechanisms. *PLoS ONE*. 7 (4), E35240.

Lindemann, M.D., Cornelius, S.G., el Kandelgy, S.M., Moser, R.L. & Pettigrew, J.E. (1986) Effect of age, weaning and diet on digestive enzyme levels in the piglet. *Journal of animal science*. 62 (5), pp.1298–1307.

Liu, H., Zhang, J., Zhang, S., Yang, F., Thacker, P.A., Zhang, G., Qiao, S. & Ma, X. (2014) Oral administration of lactobacillus fermentum I5007 favors intestinal development and alters the intestinal microbiota in formula-fed piglets. *Journal of Agricultural and Food Chemistry*. 62 (4), pp. 860–866.

Liu, C., Zhu, Q., Chang, J., Yin, Q., Song, A., Li, Z., Wang, E. & Lu, F. (2017) Effects of Lactobacillus casei and Enterococcus faecalis on growth performance, immune function and gut microbiota of suckling piglets. *Archives of Animal Nutrition*. 71 (2), pp. 120–133.

Liu, H., Zeng, X., Zhang, G., Hou, C., Li, N., Yu, H., Shang, L., Zhang, X., Trevisi, P., Yang, F., Liu, Z. & Qiao, S. (2019a) Maternal milk and fecal microbes guide the spatiotemporal development of mucosa-associated microbiota and barrier function in the porcine neonatal gut. *BMC Biology*. 17 (1), 106.

Liu, H., Hou, C., Li, N., Zhang, X., Zhang, G., Yang, F., Zeng, X., Liu, Z. & Qiao, S. (2019b) Microbial and metabolic alterations in gut microbiota of sows during pregnancy and lactation. *FASEB Journal*. 33 (3), pp. 4490–4501.

Liu, S., Zhao, L., Zhai, Z., Zhao, W., Ding, J., Dai, R., Sun, T. & Meng, H. (2015) Porcine Epidemic Diarrhea Virus fnfection induced the unbalance of gut microbiota in piglets. *Current Microbiology*. 71 (6), pp. 643–649.

Liu, T.W., Park, Y.M., Holscher, H.D., Padilla, J., Scroggins, R.J., Welly, R., Britton, S.L., Koch, L.G., Vieira-Potter, V.J. & Swanson, K.S. (2015) Physical activity differentially affects the cecal microbiota of ovariectomized female rats selectively bred for high and low aerobic capacity. *PLoS ONE*. 10 (8), E0136150.

Liu, Y., Espinosa, C.D., Abelilla, J.J., Casas, G.A., Lagos, L.V., Lee, S.A., Kwon, W.B., Mathai, J.K., Navarro, D.M.D.L., Jaworski, N.W. & Stein, H.H. (2018) Non-antibiotic feed additives in diets for pigs: A review. *Animal Nutrition*. 4 (2) pp.113–125.

Lodemann, U., Strahlendorf, J., Schierack, P., Klingspor, S., Aschenbach, J.R. & Martens, H. (2015) Effects of the probiotic Enterococcus faecium and pathogenic Escherichia coli strains in a pig and human epithelial intestinal cell model. *Scientifica*. 2015, E235184.

López-Vergé, S., Gasa, J., Coma, J., Bonet, J. & Solà-Oriol, D. (2019) Effect of lactation length caused by the management production system on piglet performance until slaughter. *Livestock Science*. 224, pp. 26–30.

López-Vergé, S., Gasa, J., Farré, M., Coma, J., Bonet, J. & Solà-Oriol, D. (2018) Potential risk factors related to pig body weight variability from birth to slaughter in commercial conditions. *Translational Animal Science*. 2 (4), pp. 383–395.

Lo Verso, L., Matte, J.J., Lapointe. J., Talbot, G., Bissonnette, N., Blais, M., Guay, F. and Lessard, M. (2020) Impact of birth weight and neonatal nutritional interventions with micronutrients and bovine colostrum on the development of piglet immune response

during the peri-weaning period. *Veterinary Immunology and Immunopathology*. 226, 110072.

Lozupone, C.A., Hamady, M., Kelley, S.T. & Knight, R. (2007) Quantitative and qualitative β diversity measures lead to different insights into factors that structure microbial communities. *Applied and Environmental Microbiology.* 73 (5) pp.1576–1585.

Lozupone, C. & Knight, R. (2005) UniFrac: A new phylogenetic method for comparing microbial communities. *Applied and Environmental Microbiology*. 71 (12), pp. 8228–8235.

Lu, D., Tiezzi, F., Schillebeeckx, C., McNulty, N.P., Schwab, C., Shull, C. & Maltecca, C. (2018) Host contributes to longitudinal diversity of fecal microbiota in swine selected for lean growth. *Microbiome*. 6 (1), 4.

Lynegaard, J.C., Hales, J., Nielsen, M.N., Hansen, C.F. & Amdi, C. (2020) The stomach capacity is reduced in intrauterine growth restricted piglets compared to normal piglets. *Animals*. 10 (8), pp. 1–7.

Mach, N., Berri, M., Estellé, J., Levenez, F., Lemonnier, G., Denis, C., Leplat, J.J., Chevaleyre, C., Billon, Y., Doré, J., Rogel-Gaillard, C. & Lepage, P. (2015) Early-life establishment of the swine gut microbiome and impact on host phenotypes. *Environmental Microbiology Reports*. 7 (3), pp. 554–569.

Mahan, D.C. & Lepine, A.J. (1991) Effect of pig weaning weight and associated nursery feeding programs on subsequent performance to 105 kilograms body weight. *Journal of Animal Science*. 69 (4), pp. 1370–1378.

Maltecca, C., Lu, D., Schillebeeckx, C., McNulty, N.P., Schwab, C., Shull, C. & Tiezzi, F. (2019) Predicting growth and carcass traits in swine using microbiome data and machine learning algorithms. *Scientific Reports*. 9 (1), 6574.

Maradiaga, N., Aldridge, B., Zeineldin, M. & Lowe, J. (2018) Gastrointestinal microbiota and mucosal immune gene expression in neonatal pigs reared in a cross-fostering model. *Microbial Pathogenesis*. 121, pp. 27–39.

Martin, L., Pieper, R., Kröger, S., Goodarzi Boroojeni, F., Vahjen, W., Neumann, K., Van Kessel, A.G. & Zentek, J. (2012) Influence of age and Enterococcus faecium NCIMB

10415 on development of small intestinal digestive physiology in piglets. *Animal Feed Science and Technology*. 175 (1–2), pp. 65–75.

Martineau G. P., Badouard B. (2009) Managing highly prolific sows/ In: Tools of the Trade. London Swine Conference, London, Ontario. pp 13 -19.

Matheson, S.M., Walling, G.A. & Edwards, S.A. (2018) Genetic selection against intrauterine growth retardation in piglets: A problem at the piglet level with a solution at the sow level. *Genetics Selection Evolution*. 50 (1).

McCormack, U.M., Curião, T., Buzoianu, S.G., Prieto, M.L., Ryan, T., Varley, P., Crispie, F., Magowan, E., Metzler-Zebeli, B.U., Berry, D., O'Sullivan, O., Cotter, P.D., Gardiner, G.E. & Lawlor, P.G. (2017) Exploring a possible link between the intestinal microbiota and feed efficiency in pigs. *Applied and Environmental Microbiology*. 83 (15), E00380-17.

McCormack, U.M., Curião, T., Wilkinson, T., Metzler-Zebeli, B.U., Reyer, H., Ryan, T., Calderon-Diaz, J.A., Crispie, F., Cotter, P.D., Creevey, C.J., Gardiner, G.E. & Lawlor, P.G. (2018) Fecal microbiota transplantation in gestating sows and neonatal offspring alters lifetime intestinal microbiota and growth in offspring. *mSystems*. 3 (3), E00134-17.

McCormack, U.M., Curião, T., Metzler-Zebeli, B.U., Magowan, E., Berry, D.P., Reyer, H., Prieto, M.L., Buzoianu, S.G., Harrison, M., Rebeiz, N., Crispie, F., Cotter, P.D., O'Sullivan, O., Gardiner, G.E. & Lawlor, P.G. (2019a) Porcine feed efficiency-associated intestinal microbiota and physiological traits: finding consistent cross-locational biomarkers for residual feed intake. *mSystems*. 4 (4), E00324-18.

McCormack, U.M., Curião, T., Metzler-Zebeli, B.U., Wilkinson, T., Reyer, H., Crispie, F., Cotter, P.D., Creevey, C.J., Gardiner, G.E. & Lawlor, P.G. (2019b) Improvement of feed efficiency in pigs through microbial modulation via fecal microbiota transplantation in sows and dietary supplementation of inulin in offspring. *Applied and Environmental Microbiology*. 85 (22), E01255-19.

McCracken, B.A., Spurlock, M.E., Roos, M.A., Zuckermann, F.A. & Gaskins, H.R. (1999) Weaning anorexia may contribute to local inflammation in the piglet small intestine. *Journal of Nutrition*. 129 (3), pp. 613–619.

McMillen, I.C., Adams, M.B., Ross, J.T., Coulter, C.L., Simonetta, G., Owens, J.A., Robinson, J.S. & Edwards, L.J. (2001) Fetal growth restriction: Adaptations and consequences. *Reproduction*. 122 (2) pp.195–204.

Mende, D.R., Letunic, I., Maistrenko, O.M., Schmidt, T.S.B., Milanese, A., Paoli, L., Hernández-Plaza, A., Orakov, A.N., Forslund, S.K., Sunagawa, S., Zeller, G., Huerta-Cepas, J., Coelho, L.P. & Bork, P. (2020) ProGenomes2: An improved database for accurate and consistent habitat, taxonomic and functional annotations of prokaryotic genomes. *Nucleic Acids Research*. 48 (1), pp. 621–625.

Menegat, M.B., DeRouchey, J.M., Woodworth, J.C., Dritz, S.S., Tokach, M.D. & Goodband, R.D. (2019) Effects of Bacillus subtilis C-3102 on sow and progeny performance, fecal consistency, and fecal microbes during gestation, lactation, and nursery periods. *Journal of Animal Science*. 97 (9), pp. 3920–3937.

Merrifield, C.A., Lewis, M.C., Berger, B., Cloarec, O., Heinzmann, S.S., Charton, F., Krause, L., Levin, N.S., Duncker, S., Mercenier, A., Holmes, E., Bailey, M. & Nicholson, J.K. (2016) Neonatal environment exerts a sustained influence on the development of the intestinal microbiota and metabolic phenotype. *ISME Journal*. 10 (1), pp. 145–157.

Metzler-Zebeli, B.U., Lawlor, P.G., Magowan, E. & Zebeli, Q. (2018) Interactions between metabolically active bacteria and host gene expression at the cecal mucosa in pigs of diverging feed efficiency. *Journal of Animal Science*. 96 (6), pp. 2249–2264.

Michiels, J., De Vos, M., Missotten, J., Ovyn, A., De Smet, S. & Van Ginneken, C. (2013) Maturation of digestive function is retarded and plasma antioxidant capacity lowered in fully weaned low birth weight piglets. *British Journal of Nutrition*. 109 (1), pp. 65–75.

Middelkoop, A., Costermans, N., Kemp, B. & Bolhuis, J.E. (2019a) Feed intake of the sow and playful creep feeding of piglets influence piglet behaviour and performance before and after weaning. *Scientific Reports*. 9 (1), 16140.

Middelkoop, A., van Marwijk, M.A., Kemp, B. & Bolhuis, J.E. (2019b) Pigs like it varied; feeding behavior and pre- and post-weaning performance of piglets exposed to dietary diversity and feed hidden in substrate during lactation. *Frontiers in Veterinary Science*. 6, 408.

Miller, Y.J., Collins, A.M., Smits, R.J., Thomson, P.C. & Holyoake, P.K. (2012) Providing supplemental milk to piglets preweaning improves the growth but not survival of gilt progeny compared with sow progeny. *Journal of Animal Science*. 90 (13), pp. 5078–5085.

Milligan, B.N., Fraser, D. & Kramer, D.L. (2002) Within-litter birth weight variation in the domestic pig and its relation to pre-weaning survival, weight gain, and variation in weaning weights. *Livestock Production Science*. 76 (1–2), pp. 181–191.

Milligan, B.N., Fraser, D. & Kramer, D.L. (2001) The effect of littermate weight on survival, weight gain, and suckling behavior of low-birth-weight piglets in cross-fostered litters. *Journal of Swine Health and Production*. 9 (4), pp. 161–166.

Molist, F., Gómez de Segura, A., Pérez, J.F., Bhandari, S.K., Krause, D.O. & Nyachoti, C.M. (2010) Effect of wheat bran on the health and performance of weaned pigs challenged with Escherichia coli K88+. *Livestock Science*. 133 (1–3), pp. 214–217.

Molist, F., Hermes, R.G., De Segura, A.G., Martí-N-Orúe, S.M., Gasa, J., Manzanilla, E.G. & Pérez, J.F. (2011) Effect and interaction between wheat bran and zinc oxide on productive performance and intestinal health in post-weaning piglets. *British Journal of Nutrition*. 105 (11), pp. 1592–1600.

Molist, F., Manzanilla, E.G., Pérez, J.F. & Nyachoti, C.M. (2012) Coarse, but not finely ground, dietary fibre increases intestinal Firmicutes:Bacteroidetes ratio and reduces diarrhoea induced by experimental infection in piglets. *British Journal of Nutrition*. 108 (1), pp. 9–15.

Montoro, J.C., Manzanilla, E.G., Solà-Oriol, D., Muns, R., Gasa, J., Clear, O. & Díaz, J.A.C. (2020) Predicting productive performance in grow-finisher pigs using birth and weaning body weight. *Animals*. 10 (6), pp. 1–14.

Mor, G. & Cardenas, I. (2010) The Immune System in Pregnancy: A Unique Complexity. *American Journal of Reproductive Immunology*. 63 (6), pp.425–433.

Morissette, B., Talbot, G., Beaulieu, C. & Lessard, M. (2018) Growth performance of piglets during the first two weeks of lactation affects the development of the intestinal microbiota. *Journal of Animal Physiology and Animal Nutrition*. 102 (2), pp. 525–532.

Morotomi, M., Nagai, F. & Watanabe, Y. (2011) Description of Christensenella minuta gen. nov., sp. nov., isolated from human faeces, which forms a distinct branch in the order Clostridiales, and proposal of Christensenellaceae fam. nov. *International Journal of Systematic and Evolutionary Microbiology*. 62 (Pt 1), pp. 144–149.

Mu, C., Zhang, L., He, X., Smidt, H. & Zhu, W. (2017) Dietary fibres modulate the composition and activity of butyrate-producing bacteria in the large intestine of suckling piglets. *Antonie van Leeuwenhoek, International Journal of General and Molecular Microbiology*. 110 (5), pp. 687–696.

Mu, C., Bian, G., Su, Y. & Zhu, W. (2019) Differential effects of breed and nursing on early-life colonic microbiota and immune status as revealed in a cross-fostering piglet model. *Applied and Environmental Microbiology*. 85 (9), E02510-18.

Mulder, I.E., Schmidt, B., Stokes, C.R., Lewis, M., Bailey, M., Aminov, R.I., Prosser, J.I., Gill, B.P., Pluske, J.R., Mayer, C.D., Musk, C.C. & Kelly, D. (2009) Environmentally-acquired bacteria influence microbial diversity and natural innate immune responses at gut surfaces. *BMC Biology*. 7 (1), 79.

Mulligan, C.M. & Friedman, J.E. (2017) Maternal modifiers of the infant gut microbiota: Metabolic consequences. *Journal of Endocrinology.* 235 (1) pp. 1–12.

Muns, R., Silva, C., Manteca, X. & Gasa, J. (2014) Effect of cross-fostering and oral supplementation with colostrums on performance of newborn piglets. *Journal of Animal Science*. 92 (3), pp. 1193–1199.

Nair, M. S., Eucker, T., Martinson, B., Neubauer, A., Victoria, J., Nicholson, B. And Peeters, M. (2019) Influence of the pig gut microbiota on Mycoplasma hyopneumoniae susceptibly. *Veterinary Research*. 50, 86.

Naqid, I.A., Owen, J.P., Maddison, B.C., Gardner, D.S., Foster, N., Tchórzewska, M.A., La Ragione, R.M. & Gough, K.C. (2015) Prebiotic and probiotic agents enhance antibody-based immune responses to Salmonella Typhimurium infection in pigs. *Animal Feed Science and Technology*. 201, pp. 57–65.

Neu, J., Douglas-Escobar, M. & Lopez, M. (2007) Microbes and the developing gastrointestinal tract. *Nutrition in Clinical Practice.* 22 (2), pp.174–182.

Newberry, R.C. & Wood-Gush, D.G.M. (1985) The Suckling Behaviour of Domestic Pigs in A Semi-Natural Environment. *Behaviour*. 95 (1–2), pp. 11–25.

Niederwerder, M.C. (2017) Role of the microbiome in swine respiratory disease. *Veterinary Microbiology.* 209, pp. 97–106.

Niederwerder, M.C., Jaing, C.J., Thissen, J.B., Cino-Ozuna, A.G., McLoughlin, K.S. & Rowland, R.R.R. (2016) Microbiome associations in pigs with the best and worst clinical outcomes following co-infection with porcine reproductive and respiratory syndrome virus (PRRSV) and porcine circovirus type 2 (PCV2). *Veterinary Microbiology*. 188, pp. 1–11.

Nissen, P.M. & Oksbjerg, N. (2011) Birth weight and postnatal dietary protein level affect performance, muscle metabolism and meat quality in pigs. *Animal*. 5 (9), pp. 1382–1389.

Niu, Q., Li, P., Hao, S., Zhang, Y., Kim, S.W., Li, H., Ma, X., Gao, S., He, L., Wu, W., Huang, X., Hua, J., Zhou, B. & Huang, R. (2015) Dynamic distribution of the gut microbiota and the relationship with apparent crude fiber digestibility and growth stages in pigs. *Scientific Reports*. 5 (10), 9938.

Nowland, T.L., Plush, K.J., Barton, M. & Kirkwood, R.N. (2019) Development and function of the intestinal microbiome and potential implications for pig production. *Animals*. 9 (3), 76.

Nowland, T.L. & Kirkwood, R.N. (2020) Faecal microbiota transplantation: Is it the future for pig production? *Microbiology Australia*. 41 (2), pp.91–94.

Nowland, T.L., Torok, V.A., Low, W.Y., Plush, K.J., Barton, M.D. & Kirkwood, R.N. (2020) A single faecal microbiota transplantation altered the microbiota of weaned pigs. *Life*. 10 (9), pp. 1–17.

Nurk, S., Bankevich, A., Antipov, D., Gurevich, A., Korobeynikov, A., Lapidus, A., Prjibelsky, A., Pyshkin, A., Sirotkin, A., Sirotkin, Y., Stepanauskas, R., McLean, J., Lasken, R., Clingenpeel, S.R., Woyke, T., Tesler, G., Alekseyev, M.A. & Pevzner, P.A. (2013) Assembling genomes and mini-metagenomes from highly chimeric reads, in *Lecture Notes in Computer Science (including subseries Lecture Notes in Artificial Intelligence and Lecture Notes in Bioinformatics)*. pp. 158–170.

O'Callaghan, T.F., Ross, R.P., Stanton, C. & Clarke, G. (2016) The gut microbiome as a virtual endocrine organ with implications for farm and domestic animal endocrinology. *Domestic Animal Endocrinology*. 56 (Supp1) pp. 44-55.

Ocepek, M., Andersen-Ranberg, I., Edwards, S.A. & Andersen, I.L. (2016) Udder characteristics of importance for teat use in purebred and crossbred pigs. *Journal of Animal Science*. 94 (2), pp. 780–788.

Oh, J. K., Jong, P. C., Pajarillo, E. A. B., Kim, S. H., Kwak, M., Eun, J., Chee, S. W., Whang, K., Kim, S. and Kang, D. (2020) "Association between the Body Weight of Growing Pigs and the Functional Capacity of Their Gut Microbiota. *Animal Science Journal.* 91(1), E13418

Oikonomou, G., Teixeira, A.G.V., Foditsch, C., Bicalho, M.L., Machado, V.S. & Bicalho, R.C. (2013) Fecal Microbial Diversity in Pre-Weaned Dairy Calves as Described by Pyrosequencing of Metagenomic 16S rDNA. Associations of Faecalibacterium Species with Health and Growth. *PLoS ONE*. 8 (4) 63157.

Olszak, T., An, D., Zeissig, S., Vera, M.P., Richter, J., Franke, A., Glickman, J.N., Siebert, R., Baron, R.M., Kasper, D.L. & Blumberg, R.S. (2012) Microbial exposure during early life has persistent effects on natural killer T cell function. *Science*. 336 (6080), pp. 489–493.

Oostindjer, M., Kemp, B., van den Brand, H. & Bolhuis, J.E. (2014) Facilitating 'learning from mom how to eat like a pig' to improve welfare of piglets around weaning. *Applied Animal Behaviour Science*. 160 (1), pp. 19–30.

Opapeju, F.O., Krause, D.O., Payne, R.L., Rademacher, M. & Nyachoti, C.M. (2009) Effect of dietary protein level on growth performance, indicators of enteric health, and gastrointestinal microbial ecology of weaned pigs induced with postweaning colibacillosis. *Journal of Animal Science*. 87 (8), pp. 2635–2643.

Osman, M.A., Neoh, H.M., Mutalib, N.S.A., Chin, S.F. & Jamal, R. (2018) 16S rRNA gene sequencing for deciphering the colorectal cancer gut microbiome: current protocols and workflows. *Frontiers in Microbiology*. 9, 767.

Ou, D., Li, D., Cao, Y., Li, X., Yin, J., Qiao, S. & Wu, G. (2007) Dietary supplementation with zinc oxide decreases expression of the stem cell factor in the small intestine of weanling pigs. *Journal of Nutritional Biochemistry*. 18 (12), pp. 820–826.

Pajarillo, E.A.B., Chae, J.P., Balolong, M.P., Kim, H.B., Park, C.S. & Kang, D.K. (2015) Effects of probiotic Enterococcus faecium NCIMB 11181 administration on swine fecal microbiota diversity and composition using barcoded pyrosequencing. *Animal Feed Science and Technology*. 201, pp. 80–88.

Pajor, E.A., Fraser, D. & Kramer, D.L. (1991) Consumption of solid food by suckling pigs: individual variation and relation to weight gain. *Applied Animal Behaviour Science*. 32 (2–3), pp.139–155.

Paredes, S.P., Jansman, A.J.M., Verstegen, M.W.A., Awati, A., Buist, W., den Hartog, L.A., van Hees, H.M.J., Quiniou, N., Hendriks, W.H. & Gerrits, W.J.J. (2012) Analysis of factors to predict piglet body weight at the end of the nursery phase. *Journal of Animal Science*. 90 (9), pp. 3243–3251.

Patil, A., Kumar, S., Verma, A. & Baghel, R. (2015) Probiotics as Feed Additives in Weaned Pigs: A Review. *Livestock Research International*. 3 (2), pp. 31–39.

Pedersen, M. L., Moustsen, V.A., Nielsen, M. B. F. and Kristensen, A. R. (2011). Improved udder access prolongs duration of milk letdown and increases piglet weight gain. *Livestock Science*. 140, pp. 253–261.

Peng, X., Wang, R., Hu, L., Zhou, Q., Liu, Y., Yang, M., Fang, Z., Lin, Y., Xu, S., Feng, B., Li, J., Jiang, X., Zhuo, Y., Li, H., Wu, D. & Che, L. (2019) Enterococcus faecium NCIMB 10415 administration improves the intestinal health and immunity in neonatal piglets infected by enterotoxigenic Escherichia coli K88. *Journal of Animal Science and Biotechnology*. 10 (1), pp. 132-146.

Père, M.C., Etienne, M. & Dourmad, J.Y. (2000) Adaptations of glucose metabolism in multiparous sows: Effects of pregnancy and feeding level. *Journal of Animal Science*. 78 (11), pp. 2933–2941.

Père, M.C. & Etienne, M. (2007) Insulin sensitivity during pregnancy, lactation, and postweaning in primiparous gilts. *Journal of Animal Science*. 85 (1), pp. 101–110.

Père, M.C. & Etienne, M. (2019) Influence of litter size on insulin sensitivity in multiparous sows. *Journal of Animal Science*. 97 (2), pp. 874–884.

Petri, D., Hill, J.E. & Kessel, A.G. (2010) Microbial succession in the Gastrointestinal Tract (GIT) of the preweaned pig. *Livestock Science*. 133 (1), pp. 107-109.

Pié, S., Lallès, J.P., Blazy, F., Laffitte, J., Sève, B. & Oswald, I.P. (2004) Weaning is associated with an upregulation of expression of inflamatory cytokines in the intestine of piglets. *Journal of Nutrition*. 134 (3), pp. 641–647.

Pieper, R., Dadi, T.H., Pieper, L., Vahjen, W., Franke, A., Reinert, K. & Zentek, J. (2020) Concentration and chemical form of dietary zinc shape the porcine colon microbiome, its functional capacity and antibiotic resistance gene repertoire. *ISME Journal*. 14 (11), 2783–2793.

Pierzynowski, S.G., Weström, B.R., Svendsen, J. & Karlsson, B.W. (1990) Development of exocrine pancreas function in chronically cannulated pigs during 1-13 weeks of postnatal life. *Journal of Pediatric Gastroenterology and Nutrition*. 10 (2), pp. 206–212.

Pierzynowski, S.G., Westrom, B.R., Erlanson-Albertsson, C., Ahre'N, B., Svendsen, J. & Karlsson, B.W. (1993) Induction of exocrine pancreas maturation at weaning in young developing pigs. *Journal of Pediatric Gastroenterology and Nutrition*. 16 (3), pp. 287–293.

Pierzynowski, S.G., Weström, B.R., Svendsen, J., Svendsen, L. & Karlsson, B.W. (1995) Development and regulation of porcine pancreatic function. *International Journal of Pancreatology*. 18 (2), pp. 81–94.

Piñeiro, C., Manso, A., Manzanilla, E.G. & Morales, J. (2019) Influence of sows' parity on performance and humoral immune response of the offspring. *Porcine Health Management*. 5 (1), 1.

Pluske, J.R., Williams, I.H. & Aherne, F.X. (1996a) Maintenance of villous height and crypt depth in piglets by providing continuous nutrition after weaning. *Animal Science*. 62 (1), pp. 131–144.

Pluske, J.R., Williams, I.H. & Aherne, F.X. (1996b) Villous height and crypt depth in piglets in response to increases in the intake of cows' milk after weaning. *Animal Science*. 62 (1), pp. 145–158.

Pluske, J.R., Hampson, D.J. & Williams, I.H. (1997) Factors influencing the structure and function of the small intestine in the weaned pig: A review. *Livestock Production Science.* 51 (1–3), pp. 215–236.

Pluske, J.R., Williams, I.H., Zak, L.J., Clowes, E.J., Cegielski, A.C. & Aherne, F.X. (1998) Feeding Lactating Primiparous Sows to Establish Three Divergent Metabolic States: III. Milk Production and Pig Growth 1. *Journal of Animal Science*. 76 (4), pp. 1165–1171.

Pluske, J.R., Kerton, D.J., Cranwell, P.D., Campbell, R.G., Mullan, B.P., King, R.H., Power, G.N., Pierzynowski, S.G., Westrom, B., Rippe, C., Peulen, O. & Dunshea, F.R. (2003) Age, sex, and weight at weaning influence organ weight and gastrointestinal development of weanling pigs. *Australian Journal of Agricultural Research*. 54 (5), pp. 515–527.

Pluske, J., Payne, H., Williams, I. & Mullan, B. (2005) Early feeding for lifetime performance of pigs. *Recent Advances in Animal Nutrition in Australia*. 15, pp. 171–181.

Pluske, J. R., Kim, J., Hansen, C. F., Mullan, B. P., Payne, H. G., Hampson, D. J., Callesen, J and Wilson, R.H. (2007). Piglet growth before and after weaning in relation to a qualitative estimate of solid feed (creep) intake during lactation: a pilot study. *Archives of Animal Science*. 61 (6), pp. 469-480.

Pluske, J.R., Turpin, D.L. & Kim, J.C. (2018) Gastrointestinal tract (gut) health in the young pig. *Animal Nutrition.* 4 (2) pp.187–196.

Pollock, J., Gally, D.L., Glendinning, L., Tiwari, R., Hutchings, M.R. & Houdijk, J.G.M. (2018) Analysis of temporal fecal microbiota dynamics in weaner pigs with and without exposure to enterotoxigenic Escherichia coli. *Journal of Animal Science*. 96 (9), pp. 3777–3790.

Poroyko, V., White, J.R., Wang, M., Donovan, S., Alverdy, J., Liu, D.C. & Morowitz, M.J. (2010) Gut microbial gene expression in mother-fed and formula-fed piglets. *PLoS ONE*. 5 (8), E12459.

Poulsen, H.D. (1995) Zinc oxide for weanling piglets. *Acta Agriculturae Scandinavica A: Animal Sciences*. 45 (3), pp.59–167.

Poulsen, A.S.R., De Jonge, N., Sugiharto, S., Nielsen, J.L., Lauridsen, C. & Canibe, N. (2017) The microbial community of the gut differs between piglets fed sow milk, milk replacer or bovine colostrum. *British Journal of Nutrition*. 117 (7), pp. 964–978.

Qiao, J., Li, H., Wang, Z. & Wang, W. (2015) Effects of Lactobacillus acidophilus dietary supplementation on the performance, intestinal barrier function, rectal microflora and serum immune function in weaned piglets challenged with Escherichia coli lipopolysaccharide. *Antonie van Leeuwenhoek, International Journal of General and Molecular Microbiology*. 107 (4), pp. 883–891.

Quan, J., Cai, G., Ye, J., Yang, M., Ding, R., Wang, X., Zheng, E., Fu, D., Li, S., Zhou, S., Liu, D., Yang, J. & Wu, Z. (2018) A global comparison of the microbiome compositions of three gut locations in commercial pigs with extreme feed conversion ratios. *Scientific Reports*. 8 (1), 4536.

Quast, C., Pruesse, E., Yilmaz, P., Gerken, J., Schweer, T. & Yarza, P. (2013) The SILVA ribosomal RNA gene database project: Improved data processing and web-based tools. *Nucleic Acids Research*. 41, D590.

Quesnel, H., Brossard, L., Valancogne, A. & Quiniou, N. (2008) Influence of some sow characteristics on within-litter variation of piglet birth weight. *Animal*. 2 (12), pp.1842–1849.

Quiniou, N., Dagorn, J. & Gaudré, D. (2002) Variation of piglets' birth weight and consequences on subsequent performance. *Livestock Production Science*. 78 (1), pp.63–70.

Ramayo-Caldas, Y., Mach, N., Lepage, P., Levenez, F., Denis, C., Lemonnier, G., Leplat, J.J., Billon, Y., Berri, M., Doré, J., Rogel-Gaillard, C. & Estellé, J. (2016) Phylogenetic network analysis applied to pig gut microbiota identifies an ecosystem structure linked with growth traits. *ISME Journal*. 10 (12), pp. 2973–2977.

Rehfeldt, C. & Kuhn, G. (2006) Consequences of birth weight for postnatal growth performance and carcass quality in pigs as related to myogenesis. *Journal of Animal Science*. 84 (13, Electronic Supplement 1), E113-123.

Rehfeldt, C., Tuchscherer, A., Hartung, M. & Kuhn, G. (2008) A second look at the influence of birth weight on carcass and meat quality in pigs. *Meat Science*. 78 (3), pp. 170–175.

Reyer, H., Oster, M., McCormack, U.M., Muráni, E., Gardiner, G.E., Ponsuksili, S., Lawlor, P.G. & Wimmers, K. (2020) Host-microbiota interactions in ileum and caecum of pigs divergent in feed efficiency contribute to nutrient utilization. *Microorganisms*, 8 (4), 563.

Riedler, J., Braun-Fahrländer, C., Eder, W., Schreuer, M., Waser, M., Maisch, S., Carr, D., Schierl, R., Nowak, D. & Von Mutius, E. (2001) Exposure to farming in early life and development of asthma and allergy: A cross-sectional survey. *Lancet*. 358 (9288), pp. 1129–1133.

Risnes, K.R., Belanger, K., Murk, W. & Bracken, M.B. (2011) Antibiotic exposure by 6 months and asthma and allergy at 6 years: Findings in a cohort of 1,401 US children. *American Journal of Epidemiology*. 173 (3), pp. 310–318.

Rist, V.T.S., Weiss, E., Eklund, M. & Mosenthin, R. (2013) Impact of dietary protein on microbiota composition and activity in the gastrointestinal tract of piglets in relation to gut health: A review. *Animal*. 7 (7) pp.1067–1078.

Robert, S. & Martineau, G.P. (2001) Effects of repeated cross-fosterings on preweaning behavior and growth performance of piglets and on maternal behavior of sows. *Journal of Animal Science*. 79 (1), pp.88–93.

Romeo, A., Vacchina, V., Legros, S. & Doelsch, E. (2014) Zinc fate in animal husbandry systems. *Metallomic*s. 6 (11) pp.1999–2009.

Roselli, M., Finamore, A., Garaguso, I., Britti, M.S. & Mengheri, E. (2003) Zinc Oxide Protects Cultured Enterocytes from the Damage Induced by Escherichia coli. *Journal of Nutrition*. 133 (12), pp. 4077–4082.

Roy, C., Lavoie, M., Richard, G., Archambault, A. & Lapointe, J. (2016) Evidence that oxidative stress is higher in replacement gilts than in multiparous sows. *Journal of Animal Physiology and Animal Nutrition*. 100 (5), pp.911–919.

Roza, S.J., Steegers, E.A.P., Verburg, B.O., Jaddoe, V.W.V., Moll, H.A., Hofman, A., Verhulst, F.C. & Tiemeier, H. (2008) What is spared by fetal brain-sparing? Fetal

circulatory redistribution and behavioral problems in the general population. *American Journal of Epidemiology*. 168 (10), pp. 1145–1152.

Russell, S.L., Gold, M.J., Hartmann, M., Willing, B.P., Thorson, L., Wlodarska, M., Gill, N., Blanchet, M.R., Mohn, W.W., McNagny, K.M. & Finlay, B.B. (2012) Early life antibiotic-driven changes in microbiota enhance susceptibility to allergic asthma. *EMBO Reports*. 13 (5), pp. 440–447.

Rutherford, K.M.D., Baxter, E.M., D'Eath, R.B., Turner, S.P., Arnott, G., Roehe, R., Ask, B., Sandøe, P., Moustsen, V.A., Thorup, F., Edwards, S.A., Berg, P. & Lawrence, A.B. (2013) The welfare implications of large litter size in the domestic pig I: biological factors. *Animal Welfare*. 22 (2), pp. 199–218.

Sappok, M.A., Perez Gutierrez, O., Smidt, H., Pellikaan, W.F., Verstegen, M.W.A., Bosch, G. & Hendriks, W.H. (2015) Adaptation of faecal microbiota in sows after diet changes and consequences for in vitro fermentation capacity. *Animal*. 9 (9), pp. 1453–1464.

Saraf, M.K., Piccolo, B.D., Bowlin, A.K., Mercer, K.E., LeRoith, T., Chintapalli, S. V., Shankar, K., Badger, T.M. & Yeruva, L. (2017) Formula diet driven microbiota shifts tryptophan metabolism from serotonin to tryptamine in neonatal porcine colon. *Microbiome*. 5 (1), 77.

Schachtschneider, K.M., Yeoman, C.J., Isaacson, R.E., White, B.A., Schook, L.B. & Pieters, M. (2013) Modulation of Systemic Immune Responses through Commensal Gastrointestinal Microbiota. *PLoS ONE*. 8 (1), E53969.

Scharek-Tedin, L., Filter, M., Taras, D., Wrede, P. & Schmidt, M.F.G. (2009) Influence of an Enterococcus faecium probiotic on the development of Peyer's patches B cells in piglets. *Archives of Animal Nutrition*. 63 (5), pp. 343–355.

Scharek, L., Guth, J., Filter, M. & Schmidt, M.F.G. (2007) Impact of the probiotic bacteria Enterococcus faecium NCIMB 10415 (SF68) and Bacillus cereus var. toyoi NCIMB 40112 on the development of serum IgG and faecal IgA of sows and their piglets. *Archives of Animal Nutrition*. 61 (4), pp. 223–234.

Schmidt, B., Mulder, I.E., Musk, C.C., Aminov, R.I., Lewis, M., Stokes, C.R., Bailey, M., Prosser, J.I., Gill, B.P., Pluske, J.R. & Kelly, D. (2011) Establishment of normal gut

microbiota is compromised under excessive hygiene conditions. *PLoS ONE*. 6 (12), E28284.

Schokker, D., Zhang, J., Zhang, L.L., Vastenhouw, S.A., Heilig, H.G.H.J., Smidt, H., Rebel, J.M.J. & Smits, M.A. (2014) Early-life environmental variation affects intestinal microbiota and immune development in new-born piglets. *PLoS ONE*. 9 (6), 100040.

Schokker, D., Zhang, J., Vastenhouw, S.A., Heilig, H.G.H.J., Smidt, H., Rebel, J.M.J. & Smits, M.A. (2015) Long-lasting effects of early-life antibiotic treatment and routine animal handling on gut microbiota composition and immune system in pigs. *PLoS ONE*. 10 (2), E0116523.

Scholtens, P.A.M.J., Oozeer, R., Martin, R., Amor, K. Ben & Knol, J. (2012) The early settlers: Intestinal microbiology in early life. *Annual Review of Food Science and Technology*. 3 (1), pp. 425–447.

Shang, Q., Liu, H., Liu, S., He, T. & Piao, X. (2019) Effects of dietary fiber sources during late gestation and lactation on sow performance, milk quality, and intestinal health in piglets. *Journal of Animal Science*. 97 (12), pp. 4922–4933.

Shao, Y., Zhou, J., Xiong, X., Zou, L., Kong, X., Tan, B. & Yin, Y. (2020) Differences in gut microbial and serum biochemical indices between sows with different productive capacities during perinatal period. *Frontiers in Microbiology*. 10, 3047.

Shelton, N.W., Tokach, M.D., Nelssen, J.L., Goodband, R.D., Dritz, S.S., Derouchey, J.M. & Hill, G.M. (2011) Effects of copper sulfate, tri-basic copper chloride, and zinc oxide on weanling pig performance. *Journal of Animal Science*. 89 (8), pp. 2440–2451.

Si, J., Feng, L., Gao, J., Huang, Y., Zhang, G., Mo, J., Zhu, S., Qi, W., Liang, J. & Lan, G. (2020) Evaluating the association between feed efficiency and the fecal microbiota of early-life Duroc pigs using 16S rRNA sequencing. *AMB Express*. 10 (1), 115.

Siepert, B., Reinhardt, N., Kreuzer, S., Bondzio, A., Twardziok, S., Brockmann, G., Nöckler, K., Szabó, I., Janczyk, P., Pieper, R. & Tedin, K. (2014) Enterococcus faecium NCIMB 10415 supplementation affects intestinal immune-associated gene expression in post-weaning piglets. *Veterinary Immunology and Immunopathology*. 157 (1–2), pp. 65–77.

Simreń, M., Barbara, G., Flint, H.J., Spiegel, B.M.R., Spiller, R.C., Vanner, S., Verdu, E.F., Whorwell, P.J. & Zoetendal, E.G. (2013) Intestinal microbiota in functional bowel disorders: A Rome foundation report. *Gut*. 62 (1), pp.159–176.

Skok, J., Brus, M. & Škorjanc, D. (2007) Growth of piglets in relation to milk intake and anatomical location of mammary glands. *Acta Agriculturae Scandinavica A: Animal Sciences*. 57 (3), pp. 129–135.

Skok, J. & Škorjanc, D. (2014) Group suckling cohesion as a prelude to the formation of teat order in piglets. *Applied Animal Behaviour Science*. 154, pp. 15–21.

Snoeck, V., Huyghebaert, N., Cox, E., Vermeire, A., Saunders, J., Remon, J.., Verschooten, F. & Goddeeris, B.. (2004) Gastrointestinal transit time of nondisintegrating radio-opaque pellets in suckling and recently weaned piglets. *Journal of Controlled Release*. 94 (1), pp. 143–153.

Soliman, M.M., Ahmed, M.M., Salah-Eldin, A.E. & Abdel-Aal, A.A.A. (2011) Butyrate regulates leptin expression through different signaling pathways in adipocytes. *Journal of Veterinary Science*. 12 (4), pp. 319–323.

Song, D., Peng, Q., Chen, Y., Zhou, X., Zhang, F., Li, A., Huang, D., Wu, Q., Ye, Y., He, H., Wang, L. & Tang, Y. (2017) Altered gut microbiota profiles in sows and neonatal piglets associated with Porcine Epidemic Diarrhea Virus infection. *Scientific Reports*. 7 (1), 17439.

Song, Y., Malmuthuge, N., Steele, M.A. & Guan, L.L. (2018) Shift of hindgut microbiota and microbial short chain fatty acids profiles in dairy calves from birth to pre-weaning. *FEMS Microbiology Ecology*. 94 (3), fix179.

Spreeuwenberg, M.A.M., Verdonk, J.M.A.J., Gaskins, H.R. & Verstegen, M.W.A. (2001) Small intestine epithelial barrier function is compromised in pigs with low feed intake at weaning. *Journal of Nutrition*. 131 (5), pp. 1520–1527.

Stappenbeck, T.S., Hooper, L. V. & Gordon, J.I. (2002) Developmental regulation of intestinal angiogenesis by indigenous microbes via Paneth cells. *Proceedings of the National Academy of Sciences of the United States of America*. 99 (24), pp. 15451–15455.

Starke, I.C., Pieper, R., Neumann, K., Zentek, J. & Vahjen, W. (2013) Individual responses of mother sows to a probiotic Enterococcus faecium strain lead to different microbiota composition in their offspring. *Beneficial Microbes*. 4 (4), pp. 345–356.

Starke, I.C., Zentek, J. & Vahjen, W. (2015) Effects of the probiotic Enterococcus faecium NCIMB 10415 on selected lactic acid bacteria and enterobacteria in co-culture. *Beneficial Microbes*. 6 (3), pp. 345–352.

Stewart, C.J., Ajami, N.J., O'Brien, J.L., Hutchinson, D.S., Smith, D.P., Wong, M.C., Ross, M.C., Lloyd, R.E., Doddapaneni, H.V., Metcalf, G.A., Muzny, D., Gibbs, R.A., Vatanen, T., Huttenhower, C., Xavier, R.J., Rewers, M., Hagopian, W., Toppari, J., Ziegler, A.G., et al. (2018) Temporal development of the gut microbiome in early childhood from the TEDDY study. *Nature*. 562 (7728), pp. 583–588.

Stiemsma, L.T. & Michels, K.B. (2018) The Role of the microbiome in the developmental origins of health and disease. *Pediatrics*. 141 (4), E20172437.

Strathe, A. V., Bruun, T.S. & Hansen, C.F. (2017) Sows with high milk production had both a high feed intake and high body mobilization. *Animal*. 11 (1), pp. 1913-1921.

Sugiharto, S., Poulsen, A.S.R., Canibe, N. & Lauridsen, C. (2015) Effect of bovine colostrum feeding in comparison with milk replacer and natural feeding on the immune responses and colonisation of enterotoxigenic Escherichia coli in the intestinal tissue of piglets. *British Journal of Nutrition*. 113 (6), pp. 923–934.

Sulabo, R.C., Tokach, M.D., Dritz, S.S., Goodband, R.D., de Rouchey, J.M. & Nelssen, J.L. (2010) Effects of varying creep feeding duration on the proportion of pigs consuming creep feed and neonatal pig performance. *Journal of Animal Science*. 88 (9), pp. 3154–3162.

Sun, J., Du, L., Li, X.L., Zhong, H., Ding, Y., Liu, Z. & Ge, L. (2019) Identification of the core bacteria in rectums of diarrheic and non-diarrheic piglets. *Scientific Reports*. 9 (1), 18675.

Surek, D., Almeida, L.M., Panisson, J.C., Krabbe, E.L., Oliveira, S.G., Alberton, G.C. & Maiorka, A. (2019) Impact of birth weight and daily weight gain during suckling on the weight gain of weaning piglets. *Arquivo Brasileiro de Medicina Veterinaria e Zootecnia*. 71 (6), pp. 2034–2040.

Tan, Z., Yang, T., Wang, Y., Xing, K., Zhang, F., Zhao, X., Ao, H., Chen, S., Liu, J. & Wang, C. (2017) Metagenomic analysis of cecal microbiome identified microbiota and functional capacities associated with feed efficiency in landrace finishing pigs. *Frontiers in Microbiology*. 8, 1546.

Tan, Z., Dong, W., Ding, Y., Ding, X., Zhang, Q. & Jiang, L. (2019) Changes in cecal microbiota community of suckling piglets infected with porcine epidemic diarrhea virus. *PLoS ONE*. 14 (7), E0219868.

Taras, D., Vahjen, W., Macha, M. & Simon, O. (2006) Performance, diarrhea incidence, and occurrence of Escherichia coli virulence genes during long-term administration of a probiotic Enterococcus faecium strain to sows and piglets. *Journal of Animal Science*. 84 (3), pp. 608-617.

Te Pas, M., Jansman, A., Kruijt, L., van der Meer, Y., Vervoort, J., & Schokker, D. (2020). Sanitary conditions affect the colonic microbiome and the colonic and systemic metabolome of female pigs. *Frontiers in Veterinary Science*. 7, 585730.

Thompson, C.L., Wang, B. & Holmes, A.J. (2008) The immediate environment during postnatal development has long-term impact on gut community structure in pigs. *ISME Journal*. 2 (7), pp. 739-748.

Tiwari, U.P., Chen, H., Kim, S.W. & Jha, R. (2018) Supplemental effect of xylanase and mannanase on nutrient digestibility and gut health of nursery pigs studied using both in vivo and in vitro models. *Animal Feed Science and Technology*. 245, pp. 77–90.

Torres-Pitarch, A., Gardiner, G.E., Cormican, P., Rea, M., Crispie, F., O'Doherty, J. V., Cozannet, P., Ryan, T., Cullen, J. & Lawlor, P.G. (2020) Effect of cereal fermentation and carbohydrase supplementation on growth, nutrient digestibility and intestinal microbiota in liquid-fed grow-finishing pigs. *Scientific Reports*. 10 (1), 13716.

Town, S.C., Patterson, J.L., Pereira, C.Z., Gourley, G. & Foxcroft, G.R. (2005) Embryonic and fetal development in a commercial dam-line genotype. *Animal Reproduction Science*. 85 (3–4), pp. 301–316.

Town, S.C., Putman, C.T., Turchinsky, N.J., Dixon, W.T. & Foxcroft, G.R. (2004) Number of conceptuses in utero affects porcine fetal muscle development. *Reproduction*. 128 (4), pp. 443–454.

Trckova, M., Faldyna, M., Alexa, P., Sramkova Zajacova, Z., Gopfert, E., Kumprechtova, D., Auclair, E. & D'Inca, R. (2014) The effects of live yeast Saccharomyces cerevisiae on postweaning diarrhea, immune response, and growth performance in weaned piglets. *Journal of Animal Science*. 92 (2), pp. 767–774.

Turnbaugh, P.J., Bäckhed, F., Fulton, L. & Gordon, J.I. (2008) Diet-Induced Obesity Is Linked to Marked but Reversible Alterations in the Mouse Distal Gut Microbiome. *Cell Host and Microbe*. 3 (4), pp. 213–223.

Turnbaugh, P.J., Hamady, M., Yatsunenko, T., Cantarel, B.L., Duncan, A., Ley, R.E., Sogin, M.L., Jones, W.J., Roe, B.A., Affourtit, J.P., Egholm, M., Henrissat, B., Heath, A.C., Knight, R. & Gordon, J.I. (2009) A core gut microbiome in obese and lean twins. *Nature*. 457 (7228), pp. 480–484.

Twardziok, S.O., Pieper, R., Aschenbach, J.R., Bednorz, C., Brockmann, G.A., Fromm, M., Klingspor, S., Kreuzer, S., Lodemann, U., Martens, H., Martin, L., Richter, J.F., Scharek-Tedin, L., Siepert, B.F., Starke, I.C., Tedin, K., Vahjen, W., Wieler, L.H., Zakrzewski, S.S., et al. (2014) Cross-talk between host, microbiome and probiotics: A systems biology approach for analyzing the effects of probiotic enterococcus faecium NCIMB 10415 in piglets. *Molecular Informatics*. 33 (3), pp. 171–182.

Unger, S., Stintzi, A., Shah, P., Mack, D. & O'Connor, D.L. (2015) Gut microbiota of the very-low-birth-weight infant. *Pediatric Research*. 77, pp.205–213.

Vahjen, W., Taras, D. & Simon, O. (2007) Effect of the probiotic Enterococcus faecium NCIMB10415 on cell numbers of total Enterococcus spp., E. faecium and E. faecalis in the intestine of piglets. *Current Issues in Intestinal Microbiology*. 8 (1), pp. 1-7.

Vahjen, W., Pietruszyńska, D., Starke, I.C. & Zentek, J. (2015) High dietary zinc supplementation increases the occurrence of tetracycline and sulfonamide resistance genes in the intestine of weaned pigs. *Gut Pathogens*. 7 (1), pp. 1–8.

Valeriano, V.D.V., Balolong, M.P. & Kang, D.K. (2017) Probiotic roles of Lactobacillus sp. in swine: insights from gut microbiota. *Journal of Applied Microbiology*. 122 (3) pp. 554–567.

van Barneveld, R.J. & Hewitt, R.J.E. (2016) Reducing variation in pork production systems through maternal and pre- and post-weaning nutrition strategies. *Animal Production Science*. 56 (8), pp. 1248–1253.

Van Hees, H.M.J., Davids, M., Maes, D., Millet, S., Possemiers, S., Den Hartog, L.A., Van Kempen, T.A.T.G. & Janssens, G.P.J. (2019) Dietary fibre enrichment of supplemental feed modulates the development of the intestinal tract in suckling piglets. *Journal of Animal Science and Biotechnology*. 10 (1), pp. 183-193.

Vasdal, G. & Andersen, I.L. (2012) A note on teat accessibility and sow parity - consequences for newborn piglets. *Livestock Science*. 146 (1), pp. 91–94.

Vatanen, T., Franzosa, E.A., Schwager, R., Tripathi, S., Arthur, T.D., Vehik, K., Lernmark, Å., Hagopian, W.A., Rewers, M.J., She, J.X., Toppari, J., Ziegler, A.G., Akolkar, B., Krischer, J.P., Stewart, C.J., Ajami, N.J., Petrosino, J.F., Gevers, D., Lähdesmäki, H., et al. (2018) The human gut microbiome in early-onset type 1 diabetes from the TEDDY study. *Nature*. 562 (7728), pp. 589–594.

Vendt, N., Grünberg, H., Tuure, T., Malminiemi, O., Wuolijoki, E., Tillmann, V., Sepp, E. & Korpela, R. (2006) Growth during the first 6 months of life in infants using formula enriched with Lactobacillus rhamnosus GG: Double-blind, randomized trial. *Journal of Human Nutrition and Dietetics*. 19 (1), pp. 51–58.

Vigors, S., O'Doherty, J. V., Kelly, A.K., O'Shea, C.J. & Sweeney, T. (2016) The effect of divergence in feed efficiency on the intestinal microbiota and the intestinal immune response in both unchallenged and lipopolysaccharide challenged ileal and colonic explants. *PLoS ONE*. 11 (2), E0148145.

Vigors, S., O' Doherty, J. V. & Sweeney, T. (2020a) Colonic microbiome profiles for improved feed efficiency can be identified despite major effects of farm of origin and contemporary group in pigs. *Animal*. 14 (12), pp. 2472-2480.

Vigors, S., O'Doherty, J. V., Rattigan, R., McDonnell, M.J., Rajauria, G. & Sweeney, T. (2020b) Effect of a laminarin rich macroalgal extract on the caecal and colonic microbiota in the post-weaned pig. *Marine Drugs*. 18 (3), 157.

Vojinovic, D., Radjabzadeh, D., Kurilshikov, A., Amin, N., Wijmenga, C., Franke, L., Ikram, M.A., Uitterlinden, A.G., Zhernakova, A., Fu, J., Kraaij, R. & van Duijn, C.M. (2019)

Relationship between gut microbiota and circulating metabolites in population-based cohorts. *Nature Communications*. 10 (1), 5813.

Wan, J.J., Lin, C.H., Ren, E. Du, Su, Y. & Zhu, W.Y. (2019) Effects of early intervention with maternal fecal bacteria and antibiotics on liver metabolome and transcription in neonatal pigs. *Frontiers in Physiology*. 10, 171.

Wang, T., Yong, J.H., Shi, F., Ruo, J.X. & Hutz, R.J. (2005) Effects of intrauterine growth retardation on development of the gastrointestinal tract in neonatal pigs. *Biology of the Neonate*. 88 (1), pp. 66–72.

Wang, J., Chen, L., Li, D., Yin, Y., Wang, X., Li, P., Dangott, L.J., Hu, W. & Wu, G. (2008) Intrauterine growth restriction affects the proteomes of the small intestine, liver, and skeletal muscle in newborn pigs. *Journal of Nutrition*. 138 (1), pp. 60–66.

Wang, X., Wu, W., Lin, G., Li, D., Wu, G. & Wang, J. (2010) Temporal proteomic analysis reveals continuous impairment of intestinal development in neonatal piglets with intrauterine growth restriction. *Journal of Proteome Research*. 9 (2), pp. 924–935.

Wang, T., Liu, C., Feng, C., Wang, X., Lin, G., Zhu, Y., Yin, J., Li, D. & Wang, J. (2013a) IUGR alters muscle fiber development and proteome in fetal pigs. *Frontiers in Bioscience*. 18 (2), pp. 598–607.

Wang, M., Radlowski, E.C., Monaco, M.H., Fahey, G.C., Gaskins, H.R. & Donovan, S.M. (2013b) Mode of delivery and early nutrition modulate microbial colonization and fermentation products in neonatal piglets. *Journal of Nutrition*. 143 (6), pp. 795–803.

Wang, Z., Chai, W., Burwinkel, M., Twardziok, S., Wrede, P., Palissa, C., Esch, B. & Schmidt, M.F.G. (2013c) Inhibitory influence of Enterococcus faecium on the propagation of Swine Influenza A Virus in vitro. *PLoS ONE*. 8 (1), E53043.

Wang, J., Tang, H., Wang, X., Zhang, X., Zhang, C., Zhang, M., Zhao, Y., Zhao, L. & Shen, J. (2016a) The structural alteration of gut microbiota in low-birth-weight mice undergoing accelerated postnatal growth. *Scientific Reports*. 6 (1), 27780.

Wang, Y.B., Du, W., Fu, A.K., Zhang, X.P., Huang, Y., Lee, K.H., Yu, K., Li, W.F. & Li, Y.L. (2016b) Intestinal microbiota and oral administration of Enterococcus faecium

associated with the growth performance of new-born piglets. *Beneficial Microbes*. 7 (4), pp. 529–538.

Wang, H., Ji, Y., Yin, C., Deng, M., Tang, T., Deng, B., Ren, W., Deng, J., Yin, Y. & Tan, C. (2018) Differential analysis of gut microbiota correlated with oxidative stress in sows with high or low litter performance during lactation. *Frontiers in Microbiology*. 9, 1665.

Wang, X., Tsai, T., Deng, F., Wei, X., Chai, J., Knapp, J., Apple, J., Maxwell, C. V., Lee, J.A., Li, Y. & Zhao, J. (2019a) Longitudinal investigation of the swine gut microbiome from birth to market reveals stage and growth performance associated bacteria. *Microbiome*. 7 (1), 109.

Wang, W., Van Noten, N., Degroote, J., Romeo, A., Vermeir, P. & Michiels, J. (2019b) Effect of zinc oxide sources and dosages on gut microbiota and integrity of weaned piglets. *Journal of Animal Physiology and Animal Nutrition*. 103 (1), pp. 231–241.

Wang, Yang, Gong, L., Wu, Y. ping, Cui, Z. wen, Wang, Yong qiang, Huang, Y., Zhang, X. ping & Li, W. fen (2019c) Oral administration of Lactobacillus rhamnosus GG to newborn piglets augments gut barrier function in pre-weaning piglets. *Journal of Zhejiang University: Science B*. 20 (2), pp. 180–192.

Wang, M., Radlowski, E.C., Li, M., Monaco, M.H. & Donovan, S.M. (2019d) Feeding mode, but not prebiotics, affects colonic microbiota composition and volatile fatty acid concentrations in sow-reared, formula-fed, and combination-fed piglets. *Journal of Nutrition*. 149 (12), pp. 2156–2163.

Wang, Q., Sun, Q., Qi, R., Wang, J., Qiu, X., Liu, Z. & Huang, J. (2019e) Effects of Lactobacillus plantarum on the intestinal morphology, intestinal barrier function and microbiota composition of suckling piglets. *Journal of Animal Physiology and Animal Nutrition*. 103 (6), pp. 1908–1918.

Wang, M., Wu, H., Lu, L., Jiang, L. & Yu, Q. (2020) Lactobacillus reuteri promotes intestinal development and regulates mucosal immune function in newborn piglets. *Frontiers in Veterinary Science*. 7, 42.

Weary, D.M., Pajor, E.A., Thompson, B.K. & Fraser, D. (1996) Risky behaviour by piglets: A trade off between feeding and risk of mortality by maternal crushing? *Animal Behaviour*. 51 (3), pp. 619–624.

Wei, H.K., Xue, H.X., Zhou, Z.X. & Peng, J. (2017) A carvacrol-thymol blend decreased intestinal oxidative stress and influenced selected microbes without changing the messenger RNA levels of tight junction proteins in jejunal mucosa of weaning piglets. *Animal*. 11 (2), pp. 193–201.

Weishaar, R., Wellmann, R., Camarinha-Silva, A., Rodehutscord, M. & Bennewitz, J. (2020) Selecting the hologenome to breed for an improved feed efficiency in pigs—A novel selection index. *Journal of Animal Breeding and Genetics*. 137 (1), pp. 14–22.

Wellock, I.J., Fortomaris, P.D., Houdijk, J.G.M. & Kyriazakis, I. (2007) Effect of weaning age, protein nutrition and enterotoxigenic Escherichia coli challenge on the health of newly weaned piglets. *Livestock Science*. 108 (1–3), pp. 102–105.

Wesemann, D.R., Portuguese, A.J., Meyers, R.M., Gallagher, M.P., Cluff-Jones, K., Magee, J.M., Panchakshari, R.A., Rodig, S.J., Kepler, T.B. & Alt, F.W. (2013) Microbial colonization influences early B-lineage development in the gut lamina propria. *Nature*. 501 (7465), pp. 112–115.

Wijesiriwardana, D.A., Craig, J., Ringuet, M., Fothergill, L., O'Halloran, K., Turpin, D., Munoz, L., Collins, C., Furness, J.B., Pluske, J., Cottrell, J. & Dunshea, F.R. (2019) Characterisation of poor gastrointestinal tract development in gilt progeny. *Journal of Animal Science*. 97 (Supplement_2), p.113.

Williams, B.A., Verstegen, M.W.A. & Tamminga, S. (2001) Fermentation in the large intestine of single-stomached animals and its relationship to animal health. *Nutrition Research Reviews*. 14 (02), pp. 207-227.

Wolter, B.F. & Ellis, M. (2001) The effects of weaning weight and rate of growth immediately after weaning on subsequent pig growth performance and carcass characteristics. *Canadian Journal of Animal Science*. 81 (3), pp. 363–369.

Wu, G., Bazer, F.W., Cudd, T.A., Meininger, C.J. & Spencer, T.E. (2004) Maternal nutrition and fetal development. *Journal of Nutrition*. 134 (9) pp. 2169–2172.

Xian, L., Li, Y., Jiang, Z., Ma, J., Jin, L., Chen, L., Zhou, C., Zhang, J., Liu, Y., Zhu, L., Jiang, A. an, Tang, G., Xiao, J., Wang, J., Zeng, B., Li, M. & Li, X. (2014) Alterations in cecal microbiota of Jinhua piglets fostered by a Yorkshire sow. *Chinese Science Bulletin*. 59 (32), 4304–4311.

Xiao, L., Estellé, J., Kiilerich, P., Ramayo-Caldas, Y., Xia, Z., Feng, Q., Liang, S., Pedersen, A., Kjeldsen, N.J., Liu, C., Maguin, E., Doré, J., Pons, N., Le Chatelier, E., Prifti, E., Li, J., Jia, H., Liu, X., Xu, X., et al. (2016) A reference gene catalogue of the pig gut microbiome. *Nature Microbiology*. 1 (12), 16161.

Xiao, Y., Li, K., Xiang, Y., Zhou, W., Gui, G. & Yang, H. (2017) The fecal microbiota composition of boar Duroc, Yorkshire, Landrace and Hampshire pigs. *Asian-Australasian Journal of Animal Sciences*. 30 (10), pp. 1456–1463.

Xiao, Y., Kong, F., Xiang, Y., Zhou, W., Wang, J., Yang, H., Zhang, G. & Zhao, J. (2018) Comparative biogeography of the gut microbiome between Jinhua and Landrace pigs. *Scientific Reports*. 8 (1), 5895.

Ximenez, C. & Torres, J. (2017) Development of Microbiota in Infants and its Role in Maturation of Gut Mucosa and Immune System. *Archives of Medical Research*. 48 (8) pp. 666–680.

Xin, J., Zeng, D., Wang, H., Sun, N., Zhao, Y., Dan, Y., Pan, K., Jing, B. & Ni, X. (2020) Probiotic Lactobacillus johnsonii BS15 Promotes Growth Performance, Intestinal Immunity, and Gut Microbiota in Piglets. *Probiotics and Antimicrobial Proteins*. 12 (1), pp. 184–193.

Xu, R.J., Mellor, D.J., Birtles, M.J., Reynolds, G.W. & Simpson, H. V. (1994) Impact of intrauterine growth retardation on the gastrointestinal tract and the pancreas in newborn pigs. *Journal of Pediatric Gastroenterology and Nutrition*. 18 (2), pp. 231–240.

Yan, L., Jang, H.D. & Kim, I.H. (2011) Effects of varying creep feed duration on pre-weaning and post-weaning performance and behavior of piglet and sow. *Asian-Australasian Journal of Animal Sciences*. 24 (11), 1601–1606.

Yang, F., Hou, C., Zeng, X. & Qiao, S. (2015) The use of lactic acid bacteria as a probiotic in swine diets. *Pathogens*. 4 (1) pp.34–45.

Yang, H., Huang, X., Fang, S., He, M., Zhao, Y., Wu, Z., Yang, M., Zhang, Z., Chen, C. & Huang, L. (2017) Unraveling the fecal microbiota and metagenomic functional capacity associated with feed efficiency in pigs. *Frontiers in Microbiology*. 8, 1555.

Yang, J., Wang, C., Liu, L. & Zhang, M. (2020) Lactobacillus reuteri KT260178 supplementation reduced morbidity of piglets through its targeted colonization, improvement of cecal microbiota profile, and immune functions. *Probiotics and Antimicrobial Proteins*. 12 (1), pp. 194–203.

Yu, Y., Lu, L., Sun, J., Petrof, E.O. & Claud, E.C. (2016) Preterm infant gut microbiota affects intestinal epithelial development in a humanized microbiome gnotobiotic mouse model. *American Journal of Physiology - Gastrointestinal and Liver Physiology*. 311 (3), pp. 521–532.

Yu, T., Zhu, C., Chen, S., Gao, L., Lv, H., Feng, R., Zhu, Q., Xu, J., Chen, Z. & Jiang, Z. (2017) Dietary high zinc oxide modulates the microbiome of ileum and colon in weaned piglets. *Frontiers in Microbiology*. 8, 825.

Zak, L.J., Williams, I.H., Foxcroft, G.R., Pluske, J.R., Cegielski, A.C., Clowes, E.J. & Aherne, F.X. (1998) Feeding lactating primiparous sows to establish three divergent metabolic states: I. Associated endocrine changes and postweaning reproductive performance. *Journal of Animal Science*. 76 (4), pp. 1145–1153.

Zeissig, S. & Blumberg, R.S. (2014) Life at the beginning: Perturbation of the microbiota by antibiotics in early life and its role in health and disease. *Nature Immunology*. 15 (4) pp. 307–310.

Zeng, M.Y., Inohara, N. & Nuñez, G. (2017) Mechanisms of inflammation-driven bacterial dysbiosis in the gut. *Mucosal Immunology*. 10 (1) pp.18–26.

Zeng, Z.K., Urriola, P.E., Dunkelberger, J.R., Eggert, J.M., Vogelzang, R., Shurson, G.C. & Johnston, L.J. (2019) Implications of early-life indicators for survival rate, subsequent growth performance, and carcass characteristics of commercial pigs. *Journal of Animal Science*. 97 (8), pp. 3313–3325.

Zeyner, A. & Boldt, E. (2006) Effects of a probiotic Enterococcus faecium strain supplemented from birth to weaning on diarrhoea patterns and performance of piglets. *Journal of Animal Physiology and Animal Nutrition.* 90 (1-2), pp. 25–31.

Zhang, L., Xu, Y.Q., Liu, H.Y., Lai, T., Ma, J.L., Wang, J.F. & Zhu, Y.H. (2010) Evaluation of Lactobacillus rhamnosus GG using an Escherichia coli K88 model of piglet diarrhoea:

Effects on diarrhoea incidence, faecal microflora and immune responses. *Veterinary Microbiology*. 141 (1–2), pp. 142–148.

Zhang, L., Mu, C., He, X., Su, Y., Mao, S., Zhang, J., Smidt, H. & Zhu, W. (2016) Effects of dietary fibre source on microbiota composition in the large intestine of suckling piglets. *FEMS Microbiology Letters*. 363 (14), fnw138.

Zhang, L., Wang, Y., Kong, Y., Ahmad, H., Yan, R., Dong, L., Zhang, J. & Wang, T. (2018a) Effects of intrauterine growth retardation on growth, meat quality and muscle fiber composition of pigs. *Pakistan Journal of Zoology*. 50 (3), pp. 1137–1146.

Zhang, Z., Kwawukume, A., Moossavi, S., Sepehri, S., Nyachoti, M. & Khafipour, E. (2018b) Time series and correlation network analyses to identify the role of maternal microbiomes on development of piglet gut microbiome and susceptibility to neonatal porcine diarrhea. *Journal of Animal Science*. 96 (suppl_2), pp. 213–213.

Zhang, W., Ma, C., Xie, P., Zhu, Q., Wang, X., Yin, Y. & Kong, X. (2019a) Gut microbiota of newborn piglets with intrauterine growth restriction have lower diversity and different taxonomic abundances. *Journal of Applied Microbiology*. 127 (2), pp. 354–369.

Zhang, G., Wang, Yachun, Luo, H., Qiu, W., Zhang, H., Hu, L., Wang, Yajing, Dong, G. & Guo, G. (2019b) The association between inflammaging and age-related changes in the ruminal and fecal microbiota among lactating holstein cows. *Frontiers in Microbiology*. 10, 1803.

Zhao, W., Wang, Y., Liu, S., Huang, J., Zhai, Z., He, C., Ding, J., Wang, J., Wang, H., Fan, W., Zhao, J. & Meng, H. (2015) The dynamic distribution of porcine microbiota across different ages and gastrointestinal tract segments. *PLoS ONE*. 10 (2), E0117441.

Zhao, P.Y. & Kim, I.H. (2015) Effect of direct-fed microbial on growth performance, nutrient digestibility, fecal noxious gas emission, fecal microbial flora and diarrhea score in weanling pigs. *Animal Feed Science and Technology*. 200 (1), pp. 86–92.

Zhu, L., Liao, R., Tu, W., Lu, Y. & Cai, X. (2020) Pyrodextrin enhances intestinal function through changing the intestinal microbiota composition and metabolism in early weaned piglets. *Applied Microbiology and Biotechnology*. 104 (9), pp. 4141–4154.

Zhuang, L., Chen, H., Zhang, S., Zhuang, J., Li, Q. & Feng, Z. (2019) Intestinal microbiota in early life and its implications on childhood health. *Genomics, Proteomics and Bioinformatics*. 17 (1), pp.13–25.

Appendix

Appendix 3a

Table 1. Ingredient composition of gestation and lactation home milled mash diets, as fed to sows.

Item	Unit	Diet	
		Gestation	Lactation
Ingredient, g/kg complete feed			
Barley	g/kg	860	760
Soya bean meal	g/kg	100	150
Full fat soya bean	g/kg	0	50
Fishmeal	g/kg	0	25
Soya oil	ml/kg	9.1	18.2
Premix supplements, kg of complete feed	g/kg	31	27
Vitamin A	iu	12 000	12 000
Vitamin D3	iu	2 000	2 000
Vitamin E	mg/kg	50	50
Copper ($CuSO_4 \cdot 5H_2O$)	mg/kg	16	16
Manganese (H_2MnO_5S)	mg/kg	60	60
Zinc (ZnO)	mg/kg	100	100
Iron (FeH_2O_5S)	mg/kg	100	100
Iodine ($Ca(IO_3)_2$)	mg/kg	2	2
Selenium (Na_2SeO_3)	mg/kg	0	0.3
Calcium	%	1	1
Phosphorus	%	0	0.07
Magnesium	%	0.02	0
Sodium	%	0	0.14
Butylated hydroxytoluene (BHT)	mg/kg	1	1
Butylated hydroxyanisole (BHA)	mg/kg	0	0.03
Propyll Gallate	mg/kg	0	0.01
4a16 6-phytase	OTU/kg[1]	250	250
Composition, % as fed			
Digestible energy, MJ/kg	%	13.14	13.98
Crude protein	%	13.82	18.5
SID lysine[2]	%	0.62	0.95

[1]OTU is the enzymatic activity unit measure. One OTU defines the quantity of enzyme required to release 1µmol of inorganic phosphorus per minute from 5.1mM sodium phytate in pH 5.5 citrate buffer at 37°C, measured as the blue P-molybdate complex colour at 820nm (EFSA, 2011)[†].
[2]Standard ileal digestible lysine.

[†]EFSA Panel on Additives and Products or Substances used in Animal Feed (FEEDAP)., 2011. Scientific Opinion on the safety and efficacy of Optiphos® (6-phytase) as a feed additive for chickens and turkeys for fattening, chickens reared for laying, turkeys reared for breeding, laying hens, other birds for fattening and laying, weaned piglets, pigs for fattening and sows. EFSA Journal 9, 29.

Appendix 3b

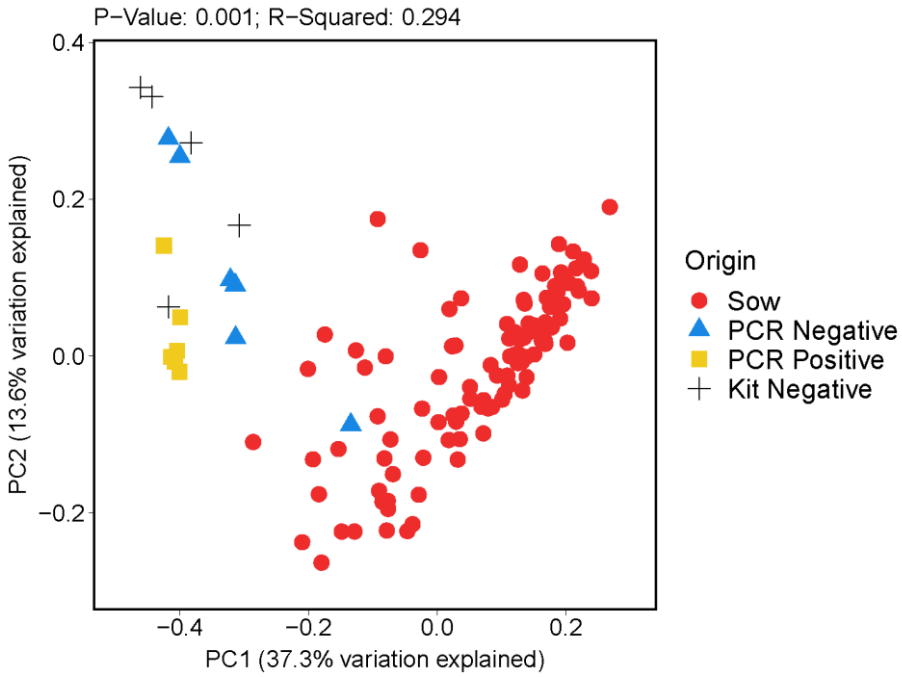
Table 2. Descriptive sow performance characteristics (Chapter 3) presented as mean values and standard deviation (SD) for each parity.

Performance parameters	Sow Parity			
	Primiparous (n = 13)	**SD**	**Multiparous (n = 16)**	**SD**
Born alive	13.23	1.691	13.69	2.750
Still born	0.54	0.776	0.25	0.112
Mummified	0.15	0.555	0.06	0.250
Average birthweight of biological litter (kg)	1.30	0.107	1.38	0.194
Average birthweight of reared litter after cross-fostering (kg)[1]	1.39	0.326	1.38	0.326
Average liveweight of reared litter day 8 (kg)	2.82	0.581	3.00	0.581
Average daily gain of reared litter birth - day 8 (kg)	0.18	0.049	0.20	0.049
Number of sows treated with antibiotics[2]	2.00	-	3.00	-
Reared litter size after cross-fostering	12.38	0.961	12.50	0.730
Number of reared piglets weaned	11.77	1.013	11.56	1.031

[1]Cross-fostering of piglets within experimental sow litters occurred within the first 24 hours post-partum in order to create uniform litters based on piglet birthweight and functional teat capacity, after cross-fostering had occurred piglets were classified as the *reared litter*.
[2]10ml intramuscularly of penicillin for 3 days given to sows with symptoms of mastitis, metritis and agalactia, or vulvar discharge.

Appendix 3c

a)



b)

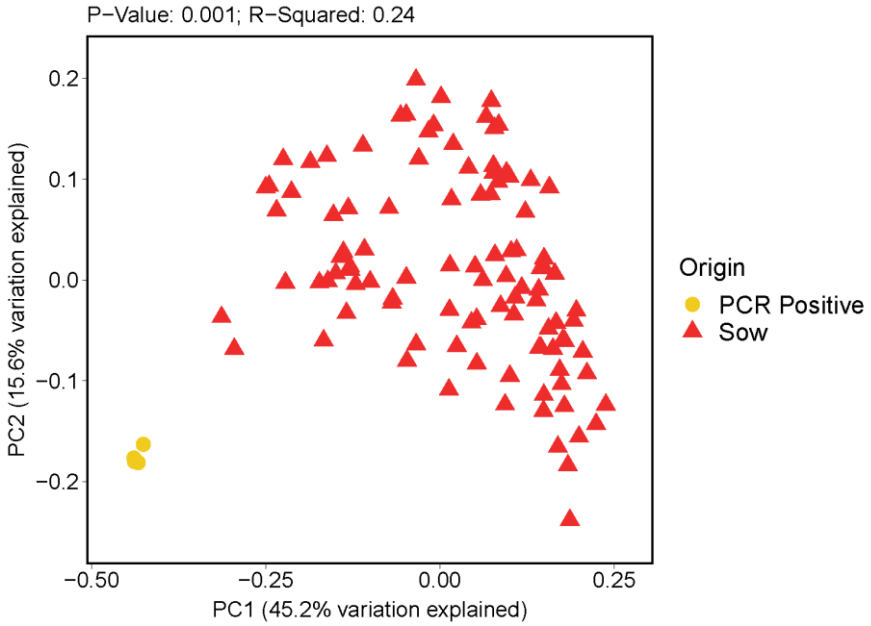


Figure 1. Principle coordinates analysis (PCoA) plots comparing the community composition of quality control samples to sow faecal microbiota samples using weighted UniFrac distances. (a) community composition of kit negatives, polymerase chain reaction (PCR) negatives, PCR positive and sow microbiota samples following rarefaction to 34 sequencing reads in the order to include all kit negatives for analysis. (b) community composition of PCR positive and sow microbiota samples after rarefying to 3 500 sequencing reads (the rarefaction depth which was used for the microbiota analysis). Rarefying to 3 500 sequencing reads removed all kit and PCR negatives ($\leq$ 751 sequencing reads).

Appendix 4a

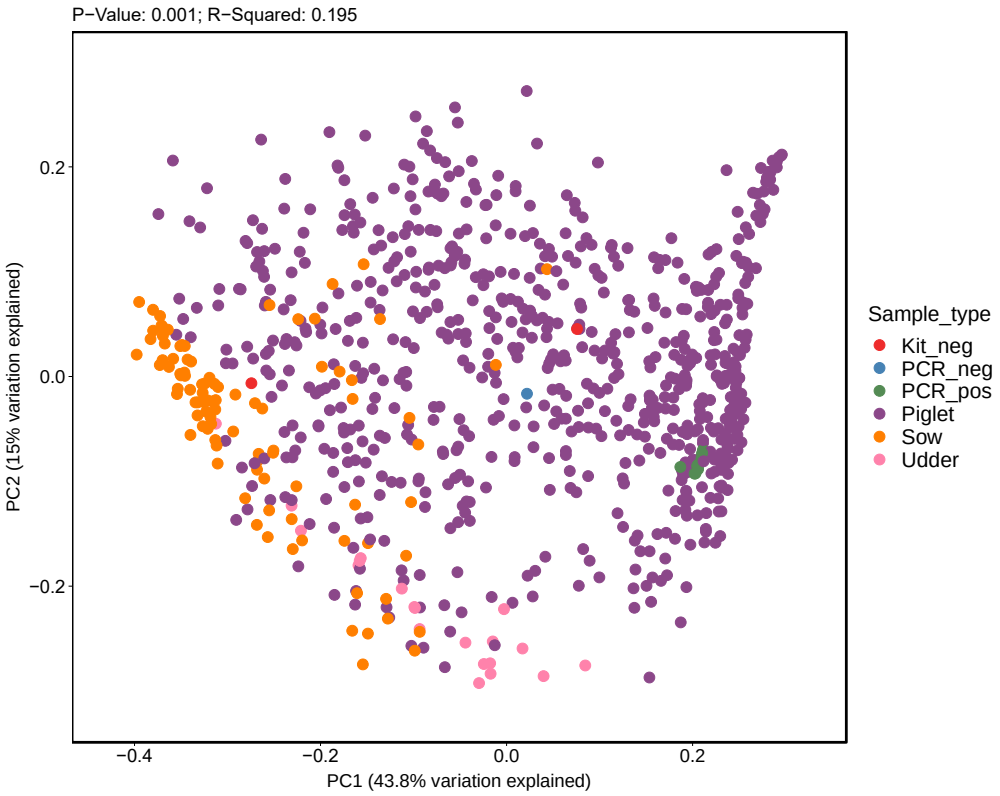


Figure 1. Post-rarefaction (4000 sequencing reads) weighted UniFrac distances between piglet faecal (Piglet), sow faeces (Sow), areolar skin (Udder) samples and DNA extraction kit negatives (Kit_neg), PCR negative (PCR_neg) and positive controls (PCR_pos).

Appendix 5a. PCR master mix and PCR thermo-cycle program

PCR master mix per 1 µl extracted DNA per sample

- 12.5 µl Taq master mix (DreamTaq Hot Start PCR Master Mix, ThermoFisher Scientific, UK)
- 1.25 µl F primer 27F-2 10pMol from stock
- 1.25 µl R primer 1492e-2 10pMol from stock
- 9 µl distilled water

PCR thermo-cycle program (Bio-Rad T100 Thermal Cycler, Bio-Rad, UK)

- 95ºC for 2 minutes
- 95ºC for 30 seconds
- 50ºC for 30 seconds

} x 29

- 72ºC for 1 minute
- 72ºC for 10 minutes
- 4ºC for ∞

Appendix 5b. ExoSAP-IT PCR master mix and thermo-cycle program

ExoSAP-IT PCR product clean-up is required to use up, thus remove, any unused primers or nucleotides.

PCR reagents

- 5 µl PCR product added to individual PCR tubes
- 2 µl ExoSAP-IT reagent (ThermoFisher Scientific, UK)
- Pipette to mix

PCR thermo-cycle program (Bio-Rad T100 Thermal Cycler, Bio-Rad, UK)

- 37ºC for 15 minutes
- 80 ºC for 15 minutes

Appendix 5c. Creating the growth curve values used to formulate future CFU/ml based on a spectrophotometer reading of PBS washed inoculum

1) Firstly, enter the Spectrophotometer readings for each cuvette 1:1 dilution (1= Neat 5 = 1/16) for each replicate into the red box.
2) Then enter the number of individual colonies (CFU) for each replicate into the blue box.
3) Based on the CFU for each plate and dilution calculate the number of colonise per ml of inoculum (**bold numbers** in green box)
4) Use the OD 600 (spectrophotometer readings) in the orange box to create an average OD value for each dilution.
5) Use the average CFU foreach rep and dilution (**bold numbers**) to calculate the average CFU/ml for the neat Eppendorph (pink box), for the rest of the Y axis CFU/ml values divide the value in the cell above by 2.

Eppendorf tube	OD 600		
	Rep 1	Rep 2	Rep 3
1	1.797	1.783	1.788
2	1.101	1.106	1.12
3	0.585	0.59	0.609
4	0.369	0.303	0.313
5	0.152	0.157	0.163

Eppendorf	X axis	Y axis
	OD average	CFU/ml
1 (neat)	1.79	4.86E+09
2 (1:1)	1.11	2.43E+09
3 (1:2)	0.59	1.21E+09
4 (1:8)	0.33	6.07E+08
5 (1:16)	0.16	3.04E+08

Eppendorf tube		Dilution	CFU		
			Rep 1	Rep 2	Rep 3
2	1/2.	-6	319	268	265
4	1/8.	-5	434	591	488

CFU/ml	Correct for -6 plating dilution	3.19E+08	2.68E+08	2.65E+08
	Correct for plating 100ul (to get ml)	3.19E+09	2.68E+09	2.65E+09
	Correct for 1/2 dilution in Eppendorf	**6.38E+09**	**5.36E+09**	**5.3E+09**
CFU/ml	Correct for -5 plating dilution	43400000	59100000	48800000
	Correct for plating 100ul (to get ml)	4.34E+08	5.91E+08	4.88E+08
	Correct for 1/8 dilution in Eppendorf	**3.47E+09**	**4.73E+09**	**3.9E+09**

The above steps create the growth curve values needed to calculate CFU/ml for a neat PBS washed inoculum as follows:

1. Generate the OD 600 value for the inoculum using a Spectrophotometer.
2. In excel use the formula to calculate CFU/ml:

	A	B	C
1	Eppendorf	X axis	Y axis
2		OD average	CFU/ml
3	1 (neat)	1.78933333	4.9E+09
4	2 (1:1)	1.109	2.4E+09
5	3 (1:2)	0.59466667	1.2E+09
6	4 (1:8)	0.32833333	6.1E+08
7	5 (1:16)	0.15733333	3E+08

=@TREND(B3:B7,A3:A7, ***INSERT OD 600 value****)*

3. To calculate the amount of inoculum needed to create a certain CFU/ml in a certain volume of PBS the following equation was used for neat CFU/ml calculations:
   *= Volume of final inoculum required (ml)*CFU/ml desired / CFU/ml in current inoculum.*
4. To calculate the amount of PBS dilution required was then simply:

   = Volume of final inoculum required – volume calculated in step 3.

Appendix 5d. Enterococcus faecium loss by freezing

In the experimental inoculum, *E. faecium* was suspended in PBS with no glycerol cryopreservative, as glycerol can induce diarrhoea in neonatal infants. Due to the lack of cryopreservative agent, it was important to establish how much *E. faecium* died as a result of freezing and crystal formation. This knowledge enabled the experimental inoculum to give the required ~5 x 10^8 CFU/ml viable *E. faecium* post-freezing to experimental piglets.
From the *E. faecium* BHI agar plate used to spike the growth curve broths in section 2.3.2, a single *E. faecium* colony was used to spike 30ml of sterile BHI broth, incubated at 37ºC and shaken at 170 rpm overnight, in triplicate. Following incubation, the 30ml cultured BHI centrifuge tubes (50ml Greiner centrifuge tubes, Sigma-Aldrich, UK) were spun down for 10 minutes at 3260rpm and 4ºC (Heraeus Megafuge 8R, Thermo Scientific, UK), the BHI broth was discarded whilst the *E. faecium* cells formed a pellet at the bottom of the tube. The *E. faecium* pellet was then re-suspended in 1ml of PBS follow by another 9ml PBS and vortexed to wash the *E. faecium* cells of any residual BHI broth. The suspension was spun down for 5 minutes at 3260rpm at 4ºC. The residual PBS and BHI broth were discarded and the washed *E. faecium* pellet was retained in the centrifuge tube. The washed *E. faecium* was then re-suspended in 10ml PBS. This wash step was necessary as pigs cannot consume processed animal protein by law in the UK (The Processed Animal Protein (England) Regulations 2001), thus cannot ingest BHI broth. The spectrophotometer was first blacked with 1ml PBS and then a reading from 1ml of the neat *E. faecium* PBS suspension was recorded for each of the three replicates. These reading were entered into the growth curve formula, firstly to generate the CFU/ml and secondly the amount of *E. faecium* suspension and PBS required to make 9ml of 5 x 10^8 CFU/ml for each of the replicates individually. The 9ml per replicate of 5 x 10^8 CFU/ml *E. faecium* was used to make 6 x 1.5ml syringes; 3 of these syringes were used to generate a 1:1 dilution series. The 1/2 and 1/8 dilutions were used to make a further 1:10 dilution series down to the -8, with 100µl of the -6 to -8 and -3 to -6 dilutions for the 1/2 and 1/8 dilutions, respectively, and these were spread on to BHI agar for raw CFU counts the

following day after overnight incubation at 37ºC (Labnet Mini Incubator, Labnet International Inc, UK) (**table 1**). The remaining 3 syringes per replicate were frozen at -80ºC for 2.5 hours; a short freeze was deemed adequate as the crystal formation damage and consequently cell death was of interest in this experiment. The syringes were then thawed on a bench at 21ºC and 1ml decanted into a 1.5ml Eppendorf tube, and the 1:1 and subsequent 1:10 dilution series were generated as described for the raw CFU counts, in order to establish the CFU counts after freezing. This experiment was repeated with different biological replicates to establish percentage loss of *E. faecium* due to freezing, the results are shown in **table 1**.

Table 1. Based on the number of colonies counted on BHI agar the concentration of Enterococcus faecium CFU/ml PBS was calculated prior to and after freezing test *E. faecium* inoculum for 2.5 hours.

Experimental replicate	Replicates (n)	Mean pre-freezing *E. faecium* CFU/ml	Mean post-freezing *E. faecium* CFU/ml	Mean percentage loss of viable *E. faecium*
1	7	$2.97x10^{11}$	$2.10x10^{10}$	92.9
2	6	$1.53x10^{11}$	$8.60x10^{10}$	43.8

To determine the longitudinal effect of freezing (-80ºC) on *E. faecium* viability once defrosted, syringes from batch 1 and 2 were removed after various time periods. Four replicates were used per batch and at each time point. *E. faecium* viability was determined by plate counts on Brain Heart Infusion agar (1.5%; Sigma Aldrich, UK). Firstly, the 1ml *E. faecium* treatment syringe was emptied into a 1.4ml Eppendorf tube and 500 µl removed and added to a new 1.5ml Eppendorf containing 500 µl PBS and mixed by pipetting and vortexing to create a 1:1 dilution. The 1:1 dilution was subjected to a serial 1:10 dilution down to the -8, by sequentially removing 100 µl from the 1:1 dilution and adding it to 900 µl PBS and pipetting up and down to mix. Following a 5 second vortex, 100 µl from the previous dilution was removed and added to 900 µl PBS and mixed by pipetting. The cycle was continued until a -8 dilution was reached. The -5 to -8 were used for plate counts; 100 µl from each dilution was pipetted onto BHI agar and spread. All experimental procedures were conducted over a flame to prevent contamination. The *E. faecium* agar plates were incubated at 37ºC for ~24 hours (GalaxyB, Scientific Laboratory Supplies, UK). For all syringes, the -6 dilution BHI agar plate was used to count individual colonies (**table 2**).

Table 2. The mean colony forming units per ml (CFU/ml) for experimental *Enterococcus faecium* inoculum prior to freezing and after different time periods of storage at -80ºC.

Batch	Period of storage at -80ºC				
	Pre-freezing	**24 hours**	**1 month**	**2 months**	**5 months**
1	$2.00x10^9$	$7.10x10^8$	$8.00x10^8$	$5.10x10^8$	$7.88x10^8$
2	$7.10x10^8$	$4.51x10^8$	$4.13x10^8$	NA	$6.35x10^8$

Appendix 5e. Similarity of piglet faecal and control samples

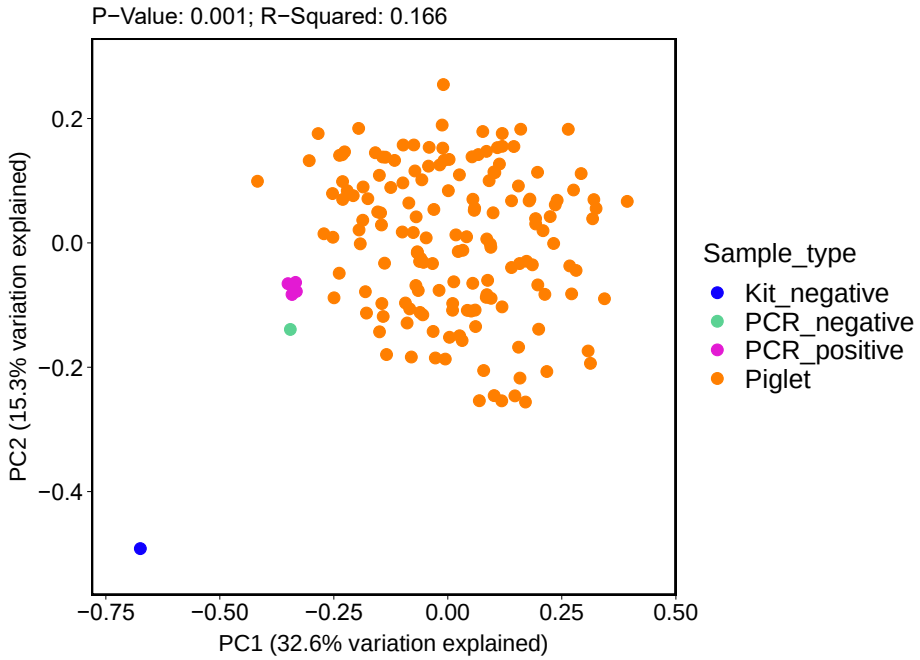


Figure 2. A Principal Coordinate Analysis plot of weighted UniFrac distances between piglet faecal samples (Piglet), DNA extraction kit negatives (Kit_negative), PCR negative (PCR_negative) and PCR positive (PCR_positive) samples not filtered after rarefying to 3350 sequence reads per sample.