ENERGY METABOLISM, DIGESTIVE AND ABSORPTIVE CAPACITY, AND GUT MICROBIOME OF NURSERY PIGLETS SELECTED FOR FEED EFFICIENCY

By

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ABSTRACT

Improving feed efficiency (FE) plays an essential role in the economic and environmental sustainability of the swine industry. Genetic selection based on estimated breeding values for feed conversion ratio (EBV_FCR) can effectively improve FE. This study investigated the growth performance, nutrient and energy digestibility, activity of hydrolyses, expression of nutrient transporters and tight junction proteins, and gut microbiome of nursery pigs selected for high and low feed efficiency. A total of 128 pigs weaned at 21±2 days were selected from parents with low or high EBV_FCR, representing low (n=64) and high (n=64) FE groups. Pigs were fed corn-soybean meal-based diets in a two-phase feeding program for four weeks under similar rearing conditions. The results revealed no differences in average daily feed intake, average body weight gain, feed conversion ratio (FCR), and energy and nutrient digestibility between the two groups. Moreover, the two groups had no differences in the maximal activity of alkaline phosphatase, sucrase, maltase, and maltase-glucoamylase ($P > 0.05$). *SGLT1, ASCT2, PepT1, EAAC1,* and *B^{0}AT1* mRNA abundances were not different between the two groups ($P > 0.05$). There were also no significant differences in claudin-1 and ZO-1 protein abundances in the jejunum between the two pig groups. The two pig groups had similar fecal microbial taxonomic composition and function. However, the high FE group had a higher relative abundance of *Lactobacillus* in the jejunum, and the low FE group was associated with higher relative abundances of *Prevotella*, *Blautia* and *Faecalibacterium* in the cecum and colon. The high-efficiency pig group had higher species evenness, and there was a trend ($P < 0.084$) for beta diversity difference, indicating that high-efficiency pigs might have a healthier gut environment. In conclusion, nursery pigs selected for high and low feed efficiency based on parents'

EBV_FCR did not differ in growth performance, nutrient digestibility and absorptive capacities, fecal microbiota composition and functions. Microbiota differences were present for the jejunum, cecum and colon of these two pig groups suggesting different functionality that need to be further investigated.

Keywords: feed efficiency, digestive enzymes, nutrient transporters, tight junction proteins, gut microbiota, swine

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FOREWORD

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The manuscripts are listed as follows:

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CONTRIBUTIONS OF AUTHORS

The study was designed by Dr. Chengbo Yang, Martin Nyachoti, and Huaigang Lei. Yujia Wu, Shunshun Jin, Dr. Paula Azevedo, and Haoxiang Xu conducted the animal trial. Yujia Wu and Dr. Paula Azevedo conducted the laboratory analysis, statistical analysis, and bioinformatics. The two manuscripts were prepared by Yujia Wu and Dr. Paula Azevedo and revised by Shunshun Jin, Xiaoya Zhao, Haoxiang Xu, Dr. Huaigang Lei, Argenis Rodas-Gonzalez, Martin Nyachoti, and Chengbo Yang.

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LIST OF ABBREVIATIONS

ADF	Acid detergent fiber
ADFI	Average daily feed intake
ADG	Average daily gain
ASCT2	Neutral amino acid transporter 2
ASV	Amplicon sequence variant
ATTD	Apparent total tract digestibility
B^0AT1	Neutral amino acid transporter 1
BW	Body weight
CLDN1	Claudin 1
CP	Crude protein
DM	Dry matter
EAAC1	Excitatory amino-acid carrier 1
EBV	Estimated breeding value
FCR	Feed conversion ratio
FE	Feed efficiency
FHP	Fasting heat production
GAPDH	Glyceraldehyde 3-phosphate dehydrogenase
GE	Gross energy
GIT	Gastrointestinal tract
IBW	Initial body weight
KEGG	Kyoto encyclopedia of genes and genomes
NDF	Neutral detergent fiber

OTU	Operational taxonomic unit
PCR	Polymerase chain reaction
PepT1	Polymerase chain reaction
PICRUSt	phylogenetic investigation of communities by reconstruction of unobserved states
QIIME	Quantitative insights into microbial ecology
RDP	Ribosomal database project
RFI	Residual feed intake
SCFA	Short chain fatty acids
SEM	Standard error of the mean
SGLT1	Na+-glucose cotransporter 1
V_{max}	Maximal enzyme activity
ZO1	Zonula occludens 1

LIST OF APPENDICES

1.0 CHAPTER 1 GENERAL INTRODUCTION

Feed is the most critical cost in the swine industry, which accounts for more than 70% of the total cost in swine production (McCormack et al., 2017). Feed efficiency (FE) is the body weight gain per unit of feed consumed, and FE can also be quantified as feed conversion ratio (FCR) or residual feed intake (RFI) among different studies. The FCR is the inverse of FE, i.e., the amount of feed intake divided by the body weight gain; therefore, high FE pigs have low FCR and RFI. Improving FE is one of the various approaches to reducing the feed cost of swine production, where selection for highly efficient breeds and or genotypes has proven to be of paramount importance.

Several studies have provided evidence of links between the microbial ecosystem and growth traits, including FE in pigs (e.g., Ramayo-Caldas et al., 2016; Gardiner et al., 2020). Gut microbiota is important for nutrient absorption, energy harvest and intestinal health. In addition, there is increasing evidence that specific microbiota taxa are linked to increased feed efficiency of pigs, as reviewed by Gardiner et al. (2020). Therefore, studying the gut microbiota and its relationship with FE provides a better understanding of how to improve FE through regulating the gut microbiota and, therefore, economically and environmentally benefiting the swine industry.

At the phylum level, *Bacteroidetes* and *Firmicutes* are the most abundant bacteria in pig's gastrointestinal tract (GIT). However, there are also differences in microbiome composition among the various regions of the GIT (Kim and Isaacson, 2015). Many factors affect the intestinal microbiota composition, including breed, age, sex, growth traits and diet (Wang et al., 2020; Homan et al., 2017). Besides, the living environment also causes a significant difference in fecal microbiome structure. For example, a study reported that pigs raised in an outdoor environment contain more *Lactobacillus* and pathogenic bacteria than pigs raised in a sanitized indoor

environment (Mulder et al., 2009; Luise et al., 2021). Many researchers have been studying the microbiota composition among pigs with different FE through next-generation sequencing and analyzed the gut microbial functionalities, which is a great resource of information when exploring the potential effect of gut microbiota on FE in swine. Despite the considerable research on the links between gut microbiota and FE in swine, the topic is still far from being fully understood. Some conflicting results exist and need further investigation.

Selection of pigs for feed efficiency based on estimated breeding value for feed conversion ratio is an approach the swine industry takes to improve the production and environmental sustainability of swine production. Although this improvement is seen in grower and finisher pigs, it is unknown if the same can be seen earlier in nursery pigs. Nursery pigs with different estimated breeding values for feed conversion ratio may have different growth performances and nutrient and energy digestibility. Gut microbiomes may also be different, and so the contribution of the gut microbiota to pig growth and energy utilization.

In chapter 4, we investigated the effects of the selection of pigs based on estimated breeding value for feed conversion ratio on pig's growth, nutrient and energy digestibility, and fecal microbiota of post-weaning pigs. Digestive and absorptive functions, capacity changes, and microbiota adaptations are often reported during weaning. However, it is unknown whether these changes are different between nursery pigs selected for feed efficiency, and this was investigated in chapter 5 on the same pigs in Chapter 4. Chapter 5 allowed further insights into the growth responses of the two pig groups selected for feed efficiency. Furthermore, besides the fecal microbiota analysis in Chapter 4, the analysis of microbiota at the various regions of the GIT in Chapter 5 allowed for a more detailed analysis of the gut microbiota changes of the two pig groups

selected for feed efficiency. Before Chapters 4 and 5, a review of current literature on swine growth performance and gut microbiota was done.

This review outlined the gut microbiome analysis method, summarized gut microbiota composition; summarized the factors affecting the swine gut microbiota community; outlined the microbiota diversity and functionalities of high and low FE pigs; and outlined the effect of gut microbiota on nutrient utilization by swine.

2.0 CHAPTER 2 LITERATURE REVIEW

2.1 Gut microbiome analysis in swine

2.1.1 The 16S rRNA gene

The 16S rRNA gene sequencing is one of the most used methods to identify gut microbiota. The 16S rRNA gene was first studied to distinguish bacteria and Archaea by Carl Woese in 1977. In the studies that followed, it was shown that the 16S rRNA gene could be found in all prokaryotes and each bacterial species has its unique sequence of the 16S rRNA gene (Van de Peer et al., 1996; McCabe et al., 1999). Also, researchers found that the similarity of the 16S rRNA sequence can indicate closeness of bacteria species. Therefore, the 16S rRNA gene sequence can be used for identifying bacteria into different species (Clarridge, 2004; Wang et al., 2007).

There are nine hypervariable regions in the 16S rRNA gene, and the full 16S rRNA sequence achieves better taxonomic accuracy. However, sequencing the whole gene is complex and expensive. Therefore, most research only uses one or multiple regions for sequencing (Johnson et al., 2019; Chakravorty et al., 2007). Different regions or combinations of regions would cause various accuracy levels. For example, fecal samples sequenced using V4 regions, compared with V3 to V4 regions, show more abundance of the genera *Bacteroide*s, *Lactobacillus*, and *Treponema* (Homan et al., 2017). Nevertheless, V2, V4 and V3-V4 regions are the most accurate and commonly used regions (Wang et al., 2007; Claesson et al., 2010).

Recently, high throughput DNA sequencing played a vital role in sequencing the 16S rRNA gene for its low cost and allowing for a deeper understanding of microbiome structure. Several high throughput sequencing methods exist, such as Roche 454, Illumina, SOLiD, or IonTorrent. Moreover, Roche 454 pyrosequencing is one of the most popular methods for it has longer DNA reads and higher accuracy (Kim & Isaacson, 2015; Schuster, 2007).

Typically, high throughput DNA sequencing groups 16S rRNA gene into operational taxonomic units (OTUs) to improve accuracy because OTUs removed the extraneous sequences that would cause an error during PCR amplification (Johnson et al., 2019). Usually, the studies use 95% identity at the genus level and 97% at the species level (Schloss & Handelsman, 2005). Nevertheless, recent advances have made it possible to analyze the high-throughput marker-gene sequencing data without resorting to OTUs and, therefore, independent from clusters of sequencing reads that differ by less than a fixed dissimilarity threshold (Chong et al., 2020). Furthermore, according to Callahan et al. (2017), new methods are now available that control errors sufficiently such that amplicon sequence variants (ASVs) can be resolved precisely, down to the level of single-nucleotide differences over the sequenced gene region.

Some bioinformatic tools, such as Ribosomal Database Project Classifier (RDP Classifier), Quantitative Insights into Microbial Ecology (QIIME) and Mothur, and databases, like Ribosomal Database Project, Greengenes, SILVA and ExTaxon, could help us to analyze and understand the high throughput DNA sequencing data and improve microbiota composition accuracy (Kim & Isaacson, 2015).

2.2 Gut microbiota composition and diversity

Microbial composition analysis is a valuable tool when comparing the effects of different treatments such as diet, age, and breed on the microbial community and its interactions with the host. This analysis is often based on microbiota abundances, which can be used to define microbial communities in terms of microbial diversity assessments such as richness and evenness. Diversity defines the number of different species and the number of individuals of each species present in a community. In 16S rRNA data, related sequences are clustered at 97% similarity into defined Operational Taxonomic Units (OTUs). Accurate estimation of diversity relies on the use of these

OTUs. Diversity within a community, i.e., alpha diversity, is the total number of species (richness), the abundance of the species (evenness) or measures that consider both richness and evenness. It is often calculated based on various diversity indices such as Chao1 (Chao, 1984), Simpson index (Simpson, 1949) and Shannon index (Shannon, 1948). Richness indexes such as Chao1, consider only the presence or absence of data for each taxon. In contrast, richness or evenness and richness indices such as Simpson index and Shannon index, respectively, are based on the relative abundances of each taxon. The variation in diversity of microorganisms from one community to another, i.e., beta-diversity, is often based on phylogenetic distances between bacteria. It compares microbial communities (between samples) based on the distance or dissimilarity between each sample pair. In this case, a distance of dissimilarity matrix is created based on the beta diversity calculated for every pair of samples. Visual representation of these matrices is then done by ordination-based methods such as Nonmetric Multidimensional Scaling (NMDS) or Principal Coordinate Analysis (PCoA), which are then used for the visual representation of the distances matrix in 2D or 3D dimensional plots. Beta diversity indices include Bray-Curtis measures (Bray and Curtis, 1957) of similarity and UniFrac distances (Lozupone et al., 2007). The Bray-Curtis measures considers the compositional similarities or dissimilarities between two communities based on counts of bacteria in each site. UniFrac distances are different from Bray Curtis as it incorporates phylogenetic distances between observed organisms in the computation. UniFrac distances can be either unweighted (qualitative) or weighted (quantitative). In the first case, the analysis is qualitative as it considers only the presence/absence of OTUs and their phylogenetic distances. In contrast, the weighted UniFrac distances are a quantitative measure of diversity as it considers the abundances of OTUs besides their phylogenetic distances. Therefore, Alpha and beta diversity indices are important when comparing microbial diversity within and between microbial

communities that vary either in time or space, e.g., different sites of the GIT tract, or simply to test treatment effects of, for example, breed, age or diets on the microbial community.

2.2.1 Gastrointestinal tract site differences

Several studies have shown that microbiota composition varies among the various regions of the GIT of pigs (e.g., Gardiner et al., 2020; Homan et al., 2017). Gut microbiota composition in different GIT regions could be affected by the host digestion and secretions, which leads to various substrate availabilities for microbial fermentation (Gardiner et al., 2020). Also, different substrates would result in different conditions in the various gut regions, including pH, redox potential, digestion time and antimicrobial activities, which could also cause different microbial activities and interactions with the host (Broom & Kogut, 2018). Greese et al. (2019), using PcoA analysis, reported distinct clusters of microbial numbers and diversity between the large and small intestines. They suggested that these microbial differences could be due to shifts in the physicochemical conditions and differences in substrate availability between the large and small intestines. Several studies have shown that *Firmicutes* and *Bacteroidetes* are the most abundant bacteria at the phylum level of GIT and fecal microbiota of pigs, and they account for 90% of the total populations of the present microbiota at the nursery stage (Crespo-Piazuelo et al., 2019; Quan et al., 2018). At the Genus level, *Prevotella*, *Clostridium*, *Alloprevotella*, and *Ruminococcus* are the dominant gut microbiota of pigs (Homan et al., 2017). Despite the similarity of dominant phyla and genera in the gut microbiome of pigs, apparent differences among different GIT sites, particularly between small and large intestines, exist. For example, Yang et al. (2016) compared the gut microbiota composition of Laiwu pigs from three different gastrointestinal sites: jejunum, ileum, and cecum. Their results showed that cecum contains more complex microbiota taxa with a higher diversity than jejunum and ileum, and jejunum and ileum have similar microbiota composition. *Firmicutes*

and *Bacteroidetes* were the most abundant phyla; *Prevotella* was the most abundant genus in cecum; *Firmicute* was the major phylum, and *Clostridium* was the major genus in the ileum. In their study, *Firmicutes* accounted for 65.8 % of relative abundance in the ileum, 51.7% in the cecum, and *Bacteroidetes* accounted for 37.6% in the cecum (Yang et al. 2016).

In another study with Duroc × (Landrace × Yorkshire) (DLY) pigs by Quan et al. (2020), similar results were found where cecum and colon had similar microbiota composition, and *Bacteroidetes* and *Firmicutes* were the primary phyla in cecum and colon, while *Firmicutes* and *Proteobacteria* were the primary phyla in the ileum. Also, Tan et al. (2017) reported that *Prevotella*, *Bacteroides*, and *Lactobacillus* were the major genera in the cecum of Landrace finishing pigs. Cecum and colon had similar microbiota taxa, while ileum had lower alpha diversity than cecum and colon of Duroc pigs (Quan et al., 2018; Quan et al., 2020). Physicochemical differences between the various sections of the GIT, particularly the small and large intestine, are believed to be the primary drivers of the microbial variability between these GIT sections. Also, higher variability of bacterial community observed between the small intestine samples than in the large intestine samples could explain the above differences between GIT sections (Crespo-Piazuelo et al., 2018). According to these studies, a lower number of microorganisms present in the small intestine, the continuous influx of new bacteria from food and the shorter transit time in the small intestine could cause a potentially less stable bacterial community in the small intestine compared to the hindgut. Regardless of the driver for these differences, one needs to be considered that different GIT sections have different microbial ecosystems, which is particularly important when considering only fecal samples as a representation of the gut microbiota. In dairy cattle, fecal microbiota was similar and a good representative of cecal microbiota (Plaizier et al., 2017). In chickens, the comparison of fecal and cecal microbiota revealed qualitative similarities but

quantitative differences (Stanley et al., 2015). In swine, feces have a 75 % similarity of microbiota composition with the large intestine (Zhao et al., 2015). Therefore, fecal sampling has the advantage that it is a non-invasive technique and can be sampled repeatedly to study gut microbiota, and it is a good representative of the large intestine in pigs.

2.2.2 Functional metagenomics

In 16 rRNA sequencing, only one marker gene is targeted, and this cannot provide direct information on a microbial community's functional capacities. Nevertheless, the functional potential of a community can be indirectly inferred from the 16 rRNA sequencing data. This inference relies on statistical methods available from packages, such as Phylogenetic Investigation of Communities by Reconstruction of Unobserved States (PICRUSt) (Langille et al., 2013) or Tax4FUN (Aßhauer et al., 2015), which allow estimating the metabolic composition of a metagenome from its taxonomic profile. They use reference databases like the Kyoto Encyclopedia of Genes and Genomes (KEGG), which consist of KEGG Orthology (KO) families directly associated with KEGG pathways. These databases group genes into pathways which are basically lists of genes participating in the same biological processes. These methods are predictive and therefore estimate the potential functionality of a community, not the measured functionality.

Nevertheless, they are handy tools for estimating the metabolic potential of a microbial community. This potential is the sum of all metabolic capacities of all present microorganisms in one community, and often the fundamental metabolic pathways are not possible to measure as many of these bacteria are not culturable in media. In these cases, culture-independent approaches such as the ones described above are crucial.

2.2.2.1 Gastrointestinal tract site differences of functionality

Several studies have shown that the small intestine is mainly associated with enzymatic hydrolysis and starch digestion, whereas the large intestine plays an important role in non-starch polysaccharides fermentation by the gut microbiome (Serena et al., 2008; Quan et al., 2018). According to the study of Yang et al. (2016), the microbiota in the small intestine was mainly related to the metabolism of small molecule nutrients. In contrast, the microbiota in the cecum has a better capacity for the metabolism of more complex molecules such as xylan, pectin, and cellulose. Quan et al. (2020) also reported that the cecum and colon have better ability for polysaccharide metabolism than the ileum. The cecum is an essential gut region for microbial fermentation, and it was found to be necessary for fiber fermentation, particularly with high fiber diets (He et al., 2018; Pu et al., 2020).

2.3 Factors affecting intestinal microbiota community

Several factors can affect the bacterial community composition, structure, and functions at one point in time or space and over time and across different regions of the GIT. These include intrinsic factors such as animal genetics, age, and sex and extrinsic factors such as diets or environment (Wang et al., 2020; Homan et al., 2017).

2.3.1 Breed Effects

The breed is one of the factors that influence the intestinal microbiota community. Crespo-Piazuelo et al. (2019) studied the relationship between the pig's genome and its gut microbiome, and they found that 39 genes could regulate the gut microbiota composition of pigs. Camarinha-Silva et al. (2017) mentioned that the variations of immunoglobulin and antimicrobial compounds that are produced in the intestinal lumen could be one of the reasons for pig's genome modulating the gut microbiome.

A study analyzed the fecal microbiome compositions of three 15-week-old purebred pigs: Duroc, Landrace, and Yorkshire, and this study showed apparent differences in fecal microbiota between those three breeds (Pajarillo et al., 2014). According to their study, *Bacteroidetes* and *Firmicutes* are the predominant phyla in the three breeds but have different abundance levels. There were significant differences among the three breeds regarding fecal microbiota; Landrace contains the most diverse microbiome composition. *Catenibacterium*, *Blautia*, *Dialister*, and *Sphaerochaeta* were found to vary among breeds (Pajarillo et al., 2014). Also, another study indicated that the gut microbiome composition was different among three breeds of Duroc, Landrace, and Large White (Bergamaschi et al., 2020). The Landrace pigs had the highest alpha diversity of fecal microbiota, and the genera *Catenibacterium, Clostridium*, and *Bacteroides* mainly drive the differences among the three breeds (Bergamaschi et al., 2020).

In addition to the fecal microbiota, the microbiome compositions vary among the various gastrointestinal sections between different breeds. At the genus level, *Clostridium and SMB53* were the significant genera in jejunum and ileum; *Prevotella*, *Treponema*, *Ruminococcus*, and *Faecalibacterium* were the leading genera in the cecum of Laiwu pigs (Yang et al., 2016). In the study by Quan et al. (2020) with DLY pigs, ileum was dominated by the genera *Clostridium, Clostridioides*, and *Escherichia*; cecum contained the genus *Treponema* primarily; and *Prevotella, Clostridium*, and *Treponema* were the most abundant genera in the colon. Another study of Jinhua and Landrace pigs reported that *Lactobacilli* and *Clostridia* were the most frequent genera in the ileum, cecum, and colon (Xiao et al., 2018). These variations among pig breeds need to be considered when comparing gut microbiota structures and functions among studies.

2.3.2 Age and growth trait effects

Age is an essential factor that impacts the intestinal microbiota community. In the study of Frese et al. (2015), microbiome composition remained similar during the first week after birth. However, from liquid sow milk to a solid feed diet, the piglet's fecal microbiome composition considerably changed during the weaning transition (Luise et al., 2021; Frese et al., 2015). Alpha diversity was increased from pre-weaning to post-weaning, and the relative abundance of bacteria shifted heavily. For example, *Enterobacteriaceae* decreased by 42%, whereas *Prevotellaceae* increased by 143% (Luise et al., 2021; Frese et al., 2015). Jurburg and Bossers (2021) also reported microbial taxa changes in nursery stage pigs. They found that the microbial community's taxonomic richness increased linearly with host age in pigs' first month of life, during which the host microbiome is most susceptible to external influences, including the diet and environment. Luise et al. (2021) reported that alpha diversity increased post-weaning compared to pre-weaning, which is recognized as an indicator of excellent stability and microbial maturity (Chen et al., 2017). Wang et al. (2019) studied the fecal microbiota composition from the pig's lactation stage to the finishing stage, and it was found that alpha diversity increased over the whole period. Also, the microbiota taxa seem to change significantly through pig's different growing stages, especially from lactation to nursery and the growing stage, while 89% of the microbiome at the finishing stage was derived from the growing stage (Wang et al., 2019).

Furthermore, there are stage-associated bacteria that only appear in the specific growth stages. For example, *Prevotella stercorea* and *Escherichia coli* were abundant at lactation, and *Clostridium butyricum* only appeared at the growing and finishing stage (Wang et al., 2019). Ke et al. (2019) also reported growth stage-associated bacteria. In their study, the gut microbial

community of pre-weaning piglets was dominated by *Fusobacterium* and *p-75*. However, *Prevotella* and *Treponema* were the dominant bacteria for pigs at 80, 120, and 240 days.

Pigs with different growth traits also have different gut microbiota compositions. For example, a study shows that the alpha diversity of fecal microbiota in post-weaning pigs is negatively related to back fat thickness (Lu et al., 2018). On the other hand, high fatness pigs were found to contain more fatness-associated taxa, for example, *Escherichia spp.* (Yang et al., 2016). Other than fatness, Wang et al. (2019) stated that *Turicibacter* and *Clostridium butyricum* were positively associated with pig's body weight. Moreover, the gut microbiota community also has close relationships with FE, and many studies looked for the microbial biomarkers for promoting FE (e.g., Quan et al., 2020; Gardiner et al., 2020; Wang et al., 2019; McCormack et al., 2017; Yang et al., 2017).

2.3.3 Sex effects

Sex influences FE in pigs. One may expect differences in the microbiota profiles due to sex (Zhou et al., 2015). Female pigs were observed to have higher fecal microbiota diversity than males (He et al., 2019). Moreover, these authors reported that the female pigs contained more *Treponema* and *Bacteroides*, and male pigs had a higher relative abundance of *Veillonellaceae*, *Roseburia*, *Bulleidia*, and *Escherichia*. According to Xiao et al. (2016), 25 genera and 41 species differed between male and female pigs, but there were not many differences between female and castrated males. Also, Verschuren et al. (2017) found 18 OTUs and six genera, which mainly belong to *the Methanobacteria* family and *Bifidobacterium* genera, that significantly differ between sexes on the fecal microbiota of pigs at 8 to 9 weeks of age. These authors also reported a lower abundance of some OTUs belonging to the *Lactobacillus* genus in low FE than high FE gilts, but the reverse was observed for boars (Verschuren et al., 2017). Sex hormones could be the

reason that female pigs and male pigs have different gut microbiota. He et al. (2019) explored that the interactions between gut microbiota and androgens-related metabolites could regulate gut microbiota composition and help male pigs have better ability of carbohydrate and protein metabolism. In other words, androgen would be one of the reasons that male pigs had better feed efficiency and a greater energy harvest/metabolism ability. However, the sex effects on weaning and post-weaning pigs were not significant, which might be because the sex hormones are mainly produced with the onset of puberty (Mach et al., 2015; Verschuren et al., 2017).

2.3.4 Diet effects

Diet is another factor that affects swine's gut microbiota composition. The fact that the gut microbiota of piglets shifts after the weaning transition from sow's milk to solid plant-based feeds is a clear example of the diet effect on gut microbiota. When switching from liquid food to solid food, only 8% of the dominant bacteria derived from the lactation stage carries on to the nursery stage (Wang et al., 2019). Another example of diet effect on gut microbiota includes probiotics. Some feed additives such as probiotics can be used to promote the growth performance and gut health of pigs, and they can also affect the gut microbiome communities. For example, some species of *Lactobacillus* can release antimicrobial bacteriocins, which influence the gut microbiota (Kim and Issacson, 2015). In addition, *Lactobacillus* can suppress other bacteria by lactic acid production and create an acidified environment (Sami et al., 1997) which will be detrimental to pathogenic bacteria such as *E.coli*.

2.3.4.1 Dietary fiber

Gut microbiota plays a vital role in the host's nutrient digestion and energy harvest, especially in processing the indigestible components of dietary polysaccharides. Total dietary fiber (TDF) refers to these dietary polysaccharides, and it is the sum of a wide range of carbohydrates

known as non-starch polysaccharides, including pectins, cellulose, hemicellulose, beta-glucans fructans as well as oligosaccharides and starch that are resistant to hydrolysis in the small intestine (Jarrett and Ashworth, 2018). Dietary fiber content seems to be a significant driver of gut microbiota compositions (e.g., Heinritz et al., 2016, Pu et al., 2020). Moreover, dietary fiber is a critical ingredient with a prebiotic effect and could affect the gut microbiota. Fiber fermentation could promote the growth of cellulolytic bacteria, which produce volatile fatty acids (VFAs) and lower the pH of the gastrointestinal tract (Williams et al., 2001; Bouhnik et al., 2004). In this environment, the pathogenic bacteria would be inhibited, while the beneficial bacteria to the host would be promoted in the low pH condition. When pigs were fed a high-fiber diet, Bach Knudsen et al. (1991) observed that gut microbial activity improved by 5.5 times. In the study of Wang et al. (2019), corn neutral-detergent fiber (NDF) was the primary determinant of the swine gut microbiota. In the study by Quan et al. (2020), the high carbohydrate diet was linked to a greater relative abundance of *Prevotella*, a group of bacteria with an essential role in the process of complex dietary polysaccharides (Ellekilde et al., 2014). Moreover, several species of this genus are known to contribute to the utilization of plant cell-wall material through their ability to hydrolyze xylans and pectins and to utilize breakdown products from plant cell-wall degradation (Flint et al., 2014).

2.3.4.2 Microbial fermentation - short chain fatty acids and volatile fatty acids

The gut microbiota community could be influenced by the characteristics of the substrate used for microbial fermentation (Jha & Berrocoso, 2015). It has been reported that bacteria ferment most non starch polysaccharides (NSP) in the large intestine. The short SCFAs such as acetate, propionate, and butyrate are produced from this fermentation and utilized by the epithelium and host body tissues (Serena et al., 2008; Quan et al., 2017). Fiber fermentation products are estimated

to contribute up to 15% to growing-finishing pig's energy requirement (Dierick et al., 1989). Zhao et al. (2018) found that dietary corn bran or wheat bran which are high in fiber, may enhance the growth performance of weaned piglets via altering gut microbiota and improving butyrate production. Besides providing energy to the host, SCFAs have anti-inflammatory effects by stimulating the pathogen-recognition receptors on the mucus membrane and leading to an immune reaction (Broom & Kogut, 2018).

2.4 Gut microbiome diversity impact on growth and feed efficiency

2.4.1 Microbial composition diversity in pigs of low or high feed efficiency

Evidence is accumulating that there are possible links between gut microorganisms and the feed efficiency of pigs. Commonly higher microbial diversity is regarded as beneficial for the growth performance of the pigs and associated with higher FE (Gardiner et al., 2020). Quan et al. (2020) compared the cecum microbiota composition of high FE pigs with low FE pigs, and the results show that high FE pigs have higher richness and evenness. Generally, as mentioned earlier, Firmicutes and Bacteroidetes are the most abundant phyla in the gut. Moreover, Quan et al. (2020) found that *Firmicutes* were more abundant in the cecum of high FE pigs, and most species from *Bacteroidetes* were more numerous in low FE pigs.

As the researchers tested the gut microbiome composition in pigs with very different FE, many potential biomarkers were explored for high FE pigs. Moreover, those potential biomarkers for high FE are more likely to appear in high FE pigs. For example, Weishaar et al. (2019) pointed out OTUs of *Oscillibacter*, *Prevotella*, *Corynebacterium*, *Lachnospiraceae*, *Anaerovibrio*, and *Clostridia* have a significant impact on FE. Similarly, Quan et al. (2019) found OTUs from *Streptococcus* genera and the *Lachnospiraceae*, *Erysipelotrichaceae*, *Ruminococcaceae*, *Coriobacteriaceae*, *Peptococcaceae*, *Prevotellaceae*, and *Enterobacteriaceae* families could be

potential biomarkers of high FE pigs. They also described that *Lachnospiraceae* and *Prevotellaceae* families and the *Escherichia-Shigella* and *Streptococcus* genera were more numerous in high FE pigs (Quan et al., 2019). Moreover, *Faecalibacterium*, a butyrate producer, was found to be more abundant in high FE pigs (Bergamaschi et al., 2020).

Some microbiome taxa were found less abundant in high FE pigs. Generally, most studies observed that low FE pigs have a higher relative abundance of *Prevotella* than high FE pigs, which could be a biomarker of low FE pigs (Tan et al., 2017; Yang et al., 2017). However, one study found that *Prevotella* was more abundant in the high FE pigs (Quan et al., 2018). The reason for these conflicting results regarding *Prevotella* is unknown, but it could be due to differences in pig breed, age, or diets like fiber source and contents among studies. In addition, some species of *Streptococcus* are pathogenic, which could cause inflammation of the intestinal mucosa, and it was also explored to be inversely associated with FE (McCormack et al., 2017; Kaakoush, 2015; Gardiner et al., 2020).

2.4.2 Microbial functionality diversity in pigs of low or high feed efficiency

Carbohydrate metabolism and amino acid metabolism are the major functionalities of gut microbiota in pigs, and many studies found that different FE groups have some significant differences in the microbial functionalities (Quan et al., 2020; Wang et al., 2019; McCormack et al., 2017; Yang et al., 2017). Of note, according to the previous studies, most taxa, which are more abundant in high FE pigs, promote nutrient digestion and energy harvest (Gardiner et al., 2020).

Quan et al. (2018) found that the different microbiota compositions of high FCR and low FCR pigs were related to the polysaccharides and protein metabolisms in the ileum, cecum, and colon. Quan et al. (2019) reported that high FE pigs have more fecal microbiota that has more substantial capacity to break down dietary cellulose, polysaccharide, and protein. Tan et al. (2017)

proposed that modulating the transport pathway of protein synthesis substrates could be the primary influence factor of the gut microbiota to the host. Moreover, they also found that genes linked to pyruvate and butyrate metabolism are more abundant in the high FE pigs. Furthermore, it was shown that high FE pigs had more bacterial chemotaxis and flagellar arrangements, providing a better environment for bacteria growth (Tan et al., 2017). Yang et al. (2017) observed that low RFI pigs showed a higher abundance of the metabolic pathway of glycine, serine, and threonine, which indicates that the bacteria in the gut may improve FE by enhancing protein biosynthesis and through SCFAs generated by digesting dietary polysaccharides.

Similarly, McCormack et al. (2017) also identified that high FE pigs had higher numbers of metabolic pathways related to amino acid biosynthesis. According to these authors, the bacterial metabolite isobutyric acids was found at a higher concentration in the ilea of pigs that were more feed efficient. Isobutyric acid is an end product of protein fermentation, and increased concentrations could be indicative of better utilization of dietary protein by the microbiota (McCormack et al., 2017). Jiang et al. (2021) also reported that the gut microbiome of low RFI and, therefore, high FE pigs had a high abundance of the pathways related to amino acid metabolism and biosynthesis but a low abundance of the pathways associated with monosaccharide metabolism and lipopolysaccharide biosynthesis which is related to inflammation and therefore, would contribute to a lower FE. These changes are also behind differences in functionality and metabolism with pig's age. For example, the study by Ke et al. (2019) showed that the utilization of simple carbohydrates and lactose, which are predominant in pre-and post-weaning nursery pigs, are changed to the digestion and utilization of complex dietary polysaccharides at the ages of 80, 120, and 240 days.

2.5 Effect of gut microbiota on nutrient utilization in swine

2.5.1 Effect of gut microbiota on growth responses

Studies explored that gut microbiota could positively or negatively affect swine growth and body weight (BW). Evidence supports the view that *Prevotella* is related to a more excellent post-weaning BW and average daily gain (ADG) (Ramayo-Caldas et al., 2016). According to Han et al. (2017), pigs with higher body weight have higher fecal microbiome diversity during the nursery stage. They also found that feces of heavier pigs contain more *Bacteroides* and *Anaerotruncus*, two genera containing pathogenic species. Wang et al. (2019) mentioned that *Turicibacter* could improve the growth performance by having a beneficial function on swine microbiome immunological interactions. In addition, *Clostridium butyricum*, *Streptococcus*, and *Lactobacillus* were found to be correlated to higher body weight and better growth performance (Wang et al., 2019). Furthermore, it was reported that *Escherichia/Shigella/Brenneria* have negative impacts on ADG, whereas *Lactococcus* has a positive influence on ADG (Torres-Pitarch et al., 2020). Also, another study (Mach et al., 2015) showed that *Bacteroides* in fecal samples affect the pig's body weight negatively. Moreover, *Lactococcus* was reported to be negatively associated with ADG and carcass weight (Han et al., 2017; Torres-Pitarch et al., 2020).

2.5.2 Effect of gut microbiota on energy harvest and energy efficiency

Gut microbiota impacts the host's energy harvest and energy utilization efficiency, which is one of the most important mechanisms for influencing the FE of pigs. Many bacteria associated with energy harvest and nutrient digestibility are related to carbohydrate degradation, which provides SCFA as a practical energy source for the host (Gardiner et al., 2020). For example, *Christensenellaceae*, *Treponema*, *Methanobrevibacter, and Actinobacillus* are carbohydrate degraders, and they are more abundant in high FE pigs and associated with fiber digestibility

(Gardiner et al., 2020). Moreover, *Prevotella* plays an essential role in dietary fiber degradation and producing monosaccharides and SCFAs that could be utilized by the host (Ellekilde et al., 2014; Ramayo-Caldas et al., 2016).

Some bacteria involved in butyrate synthesis also promote energy harvest and FE, for example, *Ruminococcus*, *Butyricicoccus*, *Roseburia*, and *Lachnospiraceae* (Gardiner et al., 2020). *Clostridium butyricum* was associated with butyric acid production and promoted immune system function and nutrient and energy digestibility (Meng et al., 2010; Wang et al., 2019). *Lactobacillus* benefited the host energy harvest by enhancing the epithelial barrier function and depressing the epithelial permeability (Wang et al., 2019).

2.6 Research gaps and future study directions

The complexity of the intestinal microbiome is reflected in both the community structure and functional capacity due to their dynamic nature and compositional variability. Most of the peer-reviewed studies have focused on the relationship between gut microbiome and growth and FE of pigs at the growing and finishing stages, while the biomarkers for high FE pigs at the nursery stage need to be further investigated. Due to the numerous potential influencing variables, the relationship between diet, gut microbiota composition, their interactions, and metabolite production is hard to know and predict (Morrison & Preston, 2016; Gardiner et al., 2020). Furthermore, when looking at the effect of a particular factor on these responses and microbiota-host interaction, it is essential to ensure all the other factors are kept constant while only the factor in question varies. For example, it is paramount to use identical diets when exploring pig genotypes' effects on the microbiota/host relationships. Most studies looking at the genotype effects on these relationships are based on breed differences and comparison of extreme cases of FE within the same genotype. No one to our knowledge has compared the effect of selecting FE

based on Estimated Breeding Value for (FE) on the microbiota/FE relationships, which needs to be investigated when all the other factors such as diet, age, sex, environment, and health are kept similar between the treatment groups. Also, there might be some differences between high and low FE pigs due to low bacteria counts, but it is hard to detect. Therefore, better-improved analysis methods are needed, for example, by considering Amplicon Sequence Variant (ASVs) rather than OTUs when analyzing 16S rRNA gene sequencing data. Traditionally, raw 16S rRNA amplicon sequencing data are converted into OTUs, clusters of reads that meet a 97% similarity threshold. Nowadays, it is generally recommended to convert row reads to high-resolution ASVs, which can be identified based on their unique biological sequences and therefore facilitate meta-analysis across studies (Chong et al., 2020). Moreover, there are some conflict findings associated with some bacteria related to FE, e.g., *Prevotella*. Moreover, the reason behind it needs to be investigated in a more explanatory, mechanistic way in studies where the factors affecting the relationship between gut microbiota and FE are controlled and tested without biases.

3.0 CHAPTER 3 HYPOTHESES AND OBJECTIVES

3.1 Hypothesis

Nursery pigs selected for high and low efficiency could have different growth performance, digestibility and energy metabolism and can be explained by the differences in:

1) Nutrient absorption capacity;
2) Membrane barrier integrity; and
3) Composition and functionality of their gut microbiota.

3.2 Objectives

The overall objective was to test the possible link among nutrient absorption capacity, membrane barrier integrity, gut microbiome, and pigs selected for high and low FE. The specific objectives were to:

1) Determine the growth performance, digestibility, and energy metabolism of nursery pigs selected for feed efficiency;
2) Determine digestive enzyme activity, gene expression of nutrient transporters, and protein expression of tight junction proteins; and
3) Characterize the composition and predicted functionality of the gut microbiome in pigs selected for feed efficiency and how these relate to pigs' performance.

4.0 CHAPTER 4 MANUSCRIPT I

FEED EFFICIENCY AND FECAL MICROBIOME OF NURSERY PIGS WITH HIGH OR LOW ESTIMATED BREEDING VALUE FOR FEED CONVERSION RATIO

4.1 Abstract

Improving feed efficiency (FE) is key to the economic and environmental sustainability of the swine industry. Genetic selection based on estimated breeding value for feed conversion ratio (EBV_FCR) aims at improving FE. It is unclear which factors most contribute to the phenotypic improvement of FE in pigs with lower EBV_FCR, which was investigated in the present study. Major contributing factors investigated included nutrients and energy digestibility, energy metabolism measurements, and differences in the fecal microbiota composition and functionality. A total of 128 pigs weaned at 21±2 days of age (initial body weight, IBW = 6.87 ± 0.34 kg (±SD)) of different EBV_FCR were randomly assigned to 32 pens with four pigs per pen. Pigs were fed the same corn/ soybean-based diet in two feeding phases of 2 weeks each and under similar rearing conditions. The results revealed that there were no differences in average daily feed intake (ADFI), average body weight gain (ADG), or feed conversion ratio (FCR = feed/gain) between pig groups. Digestibility and energy metabolism were also not different between the two pig groups apart from a lower urinary gross energy excretion for the pigs with the highest EBV_FCR. Pigs of the two FE groups had similar fecal microbial taxonomic composition and function. Alpha and beta diversity were also not different between the two pig groups, but there was a trend for a higher beta diversity when ASV counts and phylogenetic relationships were considered. We identified five genera potentially associated with swine FE variation in the fecal microbiota through LEfSe analysis. Predicted functionality did not differ between pig groups, and carbohydrate and amino acid metabolism dominated the metabolism of pigs regardless of FE according to the KEGG annotation.

At the post-weaning nursery stages, genotype differences in EBV_FCR did not translate to phenotypic differences in growth performance and nutrient and energy utilization. Also, genotype differences in the selection of pigs based on EBV_FCR did not affect microbiota composition and function. Therefore, it is possible that diet rather than genetics based on EBV_FCR was the primary driver for the growth performances and microbiome responses at nursery stage.

Keywords: Feed efficiency, Digestibility, Energy metabolism, Fecal microbiota, Predicted functionality, Swine

4.2 Introduction

Feed cost accounts for up to 60-70% of the total costs in the swine industry (McCormack et al., 2017). Therefore, optimizing feed conversion into pig body weight gain, i.e., feed efficiency, is key for the industry's sustainable economic and environmental development. Selection of pigs with a high Estimated Breeding Value (EBV) for Feed Efficiency (FE, gain: feed) is one of the various approaches to improve FE in the swine industry and lower industry costs. Genetic evaluations by gentic companies aim to select pig breeding lines with better FE values. Often what is measured is the EBV for FCR (feed: gain). A negative EBV for the dam and sire lines, such as EBV_FCR = -0.14, is desirable as it indicates that the FCR of the offspring will be 0.14 lower than the average population FCR.

Higher FE of grower pigs of low EBV for FCR compared to the high EBV_FCR pigs were associated with lower feed intake while the same body weight gain was obtained (Verschuren, 2021). Higher fecal digestibility was also reported by this author in grower pigs of different EBV_FCR and associated with lower FCR and lower residual feed intake (RFI). Verschuren et al. (2021) concluded that part of the observed variation in feed efficiency traits was associated with variation in fecal digestibility values, but the results differed between young and older animals. According to Verschuren et al. (2021), the study was conducted with grower and finisher pigs. Weaning is one of the most stressful events in pigs' life and often leads to intestinal and immune system dysfunctions resulting in poor growth performance and mortality, as reviewed by Wei et al. (2021). It is not known if similar results by Verschuren et al. (2021) would be observed between post-weaning pigs of different EBV_FCR, and this needs to be investigated. In particular, it is unknown if variations in feed intake, digestibility, or variations in the efficiency of energy

utilization would be behind higher FE of pigs with lower EBV_FCR at post-weaning and in particular in the nursery stage pigs, and this needs to be investigated.

Gut microbiota plays an important role in swine physiology and homeostasis, especially in energy harvest, nutrient digestion, and intestinal health, and is likely to affect FE (McCormack et al., 2017). Investigating the microbial taxa and functional capacity of the gut microbiome associated with FE can provide important knowledge to improve pig FE in the swine industry (McCormack et al., 2017; Yang et al., 2017, Pu et al., 2020). Several biological factors affect gut microbiota diversity in pigs, such as age, breed, and genotype (Wang et al., 2019; Gardiner et al., 2020). Moreover, several studies have reported gut microbiome differences between pigs of different FE. Gardiner et al. (2020) reported FE-associated species. According to these studies, bacteria of the family *Christensenellaceae* and genus *Actinobacillus*, *Treponema*, *and Methanobrevibacter* are associated with high FE pigs. Aliakabari et al. (2021) also reported that some genera genetically correlated with production and FE traits. These authors reported that *Streptococcus* and *Desulfovibrio* genus were negatively correlated with residual feed intake (RFI) and positively correlated with FE. These studies focused on grower and finisher pigs. It is unclear if differences exist in the gut microbiome of post-weaning nursery pigs of different EBV_FCR. At weaning, nursery pigs are at a higher risk of dysbiosis as gut microbiota goes through many changes due to the introduction of solid feed and pig's physiological stage changes (Wang et al., 2019). Also, it is unclear if differences in EBV_FCR would affect the gut microbiota profile and its relationships with FE at this stage, which needs to be investigated.

The fast development of next-generation sequencing technologies has allowed for culture-independent, high-throughput profiling of microbial communities. Lately, 16S rRNA gene sequencing has been undoubled useful to characterize complex microbial communities' taxonomic

composition and phylogenetic diversity (Chong et al., 2020; Carporaso et al., 2010). The present study hypothesized that nursery pigs selected from parents with high or low EBV for FCR present differences in FCR and could be explained by differences in nutrient digestibility, energy utilization efficiencies, and the composition and functionality of their gut microbiota. Therefore, our objectives were to: a) determine growth performance, digestibility, and energy utilization efficiencies of pigs with parents' EBV for low or high feed efficiency; and b) to characterize the composition and predicted functionality of the fecal microbiome in pigs with low or high feed efficiency based on EBV_FCR and their impact on pig performance of nursery pigs.

4.3 Materials and Methods

The experiment and all measurements were approved by the Animal Care Committee of the University of Manitoba (AC#F21-002), and pigs were handled according to the guidelines described by the Canadian Council on Animal Care (CCAC, 2009).

4.3.1 Animals and experimental design

The study included a total of 128 piglets (IBW = 6.87 $\pm$ 0.34 kg ($\pm$SD) obtained from the Glenlea Research Station. Piglets were from a crossbred of Large White × [Large White × Landrace], from Topigs, Norsvin (Oak Bluff, Manitoba, Canada). The EBVs of individual pigs were estimated using the ssGBLUP method in MIXBLUP (Ten Napel et al., 2018). A 25K single-nucleotide polymorphism chip was used to create the genomic relationship matrix and the EBVs for FCR were estimated by Topigs Norsvin Research Center (Beuningen, The Netherlands), using data collected over the past ten years.

The piglets were housed at the TK Cheng Centre at the University of Manitoba (4 animals per 2.88 m^2) with one feeder in each pen and unlimited access to water. From weaning at day 21, piglets were fed four times a day on a pre-starter diet (NE of 2.58 Mcal/kg: 1.35% SID lysine),

and from day 36 to day 49, piglets were fed four times a day on a starter diet (NE of 2.44 Mcal/kg: 1.20% SID lysine). The high and low efficiency piglets were selected from parents with a low and high estimated breeding value for FCR, respectively. Briefly, high efficiency piglets were obtained by inseminating low EBV_FCR sows (-0.12±0.011) with semen from low EBV_FCR boars (-0.29±0.041), and low efficiency piglets were obtained by inseminating high EBV_FCR sows (0.0±0.027) with semen from high EBV_FCR boars (0.033±0.045). All EBV_FCRs were provided by Topigs Norsvin based on their routine genetic evaluation.

4.3.2 Experimental diets

The experimental diet was a corn-soybean meal-based diet in a 2-phase feeding program for four weeks. Ingredients were selected based on relevance to the swine industry in Manitoba (Canada) and followed the TN Tempo breeding line requirements (Topigs Norsvin).

Table 4.1 Composition of experimental diets, as-fed basis (g/kg).

	Pre-starter (7-11 kg)	Starter (11-25 kg)
Ingredient %		
Corn	257.42	418.38
Wheat	200	225
Soybean meal	152	201
Canola meal	0	25
Fish meal	55	20
Dried whey powder	110	35
Vegetable oil	29.5	9
Oat groats	100	0
Barley	0	25
Hamlet HP300[1]	40	15
NUPRO[2]	30	0
Betaine	2	0
Zinc oxide	3.47	0
Limestone	4	7
Dicalcium phosphate	0	3.0
Salt	3.75	4.25
Copper sulfate	0.5	0.5
Vitamin premix[3]	1.5	1.5
Mineral premix[4]	1	1
Choline chloride (70%)	0.8	0.5

L-Lysine-HCl	4	4.25
DL-Methionine	2.23	1.8
L-Threonine	1.52	1.66
L-Tryptophan	0.76	0.54
L-Valine	0.45	0.52
Phytase[5]	0.1	0.1
Calculated Composition (as is basis)		
Dry Matter %	89.53	87.68
NE Swine Calc Mcal/kg	2.58	2.44
Crude Protein %	21.88	19.75
Crude Fat %	6.13	3.77
Crude Fiber %	1.86	2.4
ADF %	2.01	3.22
NDF %	6.39	9.47
SID[6] Lysine %	1.35	1.2
SID[6] Methionine %	0.53	0.45
SID[6] Tryptophan %	0.28	0.24
SID[6] Threonine %	0.88	0.78
Available Calcium %	0.89	0.76
Available Phosphorus %	0.81	0.67
SID Lys/NEc Ratio	5.23	4.91
Analyzed Composition (as is basis)		
Dry matter %	89.3	88.6
Crude protein %	22.3	20.8

Crude fat %	5.47	3.33
Crude fibre %	1.64	2.15
ADF %	5.14	5.65
NDF %	10.43	11.91
Ash %	5.42	5.05
Ca %	0.59	0.57
P %	0.65	0.59

Hamlet HP300[1]: from Hamlet Protein.

NUPRO[2]: from Alltech.

Vitamin premix[3] and mineral premix[4], from DSM, provided per kg of diet: Vitamin A, 9,000,000 IU; Vitamin D_3, 500,000 IU; 25-OH-D3, 16.67 mg; 25-OH-D3, 666,800 IU; Vitamin E, 90,000 IU; Vitamin K, 2,667 mg; Vitamin B_1, 2,667 mg; Vitamin B_2, 7,350 mg; Vitamin B_3, 33,333 mg; Vitamin B_5, 30,000 mg; Vitamin B_6, 3,000 mg; Vitamin B_{12}, 36,667 mcg; Vitamin B_9, 1,113 mg; Vitamin C, 66,667 mg; Vitamin B_7, 200,000 mg; Fe, 140,000 ppm; Cu, 25,000 ppm; I, 1,000 ppm; Se, 300 ppm; Mn 75,000 ppm; Zn, 130,000 ppm.

Phytase[5]: from AB Vista.

SID[6]: standardized ileal digestible.

4.3.3 Performance measurements and sampling

At the beginning of the trial and the end of the pre-starter and starter feeding phases, piglets were individually weighed, and feed intake per pen (experimental unit) for the two weeks of each phase was measured, and FCR (feed/gain) for each feeding phase and the overall feeding trial was calculated. At the end of the feeding trial, eight piglets (BW = 16.01 $\pm$ 0.57kg) from each group were randomly selected and moved into individual metabolic crates (1.8 x 0.6 m) to determine the total tract digestibility of crude protein, dry matter, gross energy, crude fat, and crude fiber. Room temperature was controlled to 25 ± 2ºC. The screen collected feces under the crate, and the stainless-steel tray collected urine under the screen. The pigs stayed in crates for ten days of adaptation and six days of collection. Pigs had unlimited access to water and were fed once daily at 6 am. Feed amounts offered were set to 550 kcal ME/kg $BW^{0.60}$ to be close to *ad libitum* (Noblet et al., 1994), and pigs were weighted every five days to adjust the feed amount.

After the adaptation period, the pig's movements were restricted, and a marker (ferric oxide) was mixed with the feed on day 1 and day 6 of collection days. Feces and urine samples were collected according to the SOPs from CLAMS (University of Manitoba) once the marker first appeared until the marker added on day 6 disappeared to determine DE and ME. Urine samples were mixed with 3N HCl to reduce nitrogen loss during collection. The fecal and urine samples were weighted and stored in a -20ºC freezer.

Following the digestibility measurements, 12 pigs (BW = 25.98 ± 1.76 kg) were transferred into indirect calorimetry chambers for energy metabolism measurements. Pigs were randomly divided into 4 groups of 3 pigs per group and transferred into respiration chambers (122cm × 61cm × 91cm; Columbus Instruments, Columbus, OH). After being fed at 6 am, pigs were weighed and transferred into the chambers at 8 am. While in the chambers, pigs had free access to water. The

temperature inside the chambers was controlled to 27ºC ± 2ºC via air conditioning, and the lights were turned on at 8 am and turned off at 8 pm. Carbon dioxide (CO_2) production and oxygen (O_2) consumption were measured to determine 24 h heat production (HP) and 12h fasting heat production (FHP). Urine was collected from the stainless-steel urine tray under the chamber for 24h of fed state and subsequent 12 h of fasting state. Urine samples were weighed and filtered through glass wool, and a 5% aliquot was stored in a -20ºC freezer for GE and N analysis. The accuracy of the calorimetry chambers was assessed by burning ethanol (99-100%) inside each chamber. The RQ values ranged from 0.684 to 0.738, considered acceptable and comparable to the RQ ratio of ethanol combustion of 0.667 (Benedict and Tompkins, 1916). The first 3 pigs were transferred directly from the metabolic crates within 1 hour of consuming their daily feed allowance. The next set of 3 pigs was transferred every 2 days within 1h of feed consumption.

At the end of the feeding trial, fecal samples from pigs in each pen were collected by grab sampling and stored at -80^{o}C for microbiome analysis. Also, 8 pigs from each group were anaesthetized with Ketamine: xylazine (20:2 mg/kg BW) and killed by a captive bolt gun. 10cm of middle jejunum, duodenum, ileum, cecum, colon, and the digesta of these gut sections were collected and immediately snap frozen in liquid nitrogen. At the same time, the spleen, heart, lung, kidneys, liver, and stomach were collected for organ weight, and the length of the large and small intestine were measured. The tissue and digesta samples were stored at -80^{o}C for further analysis.

4.3.4 Chemical Analysis

Diets, urine, and fecal samples were analyzed by a commercial laboratory, Central Testing Lab (Winnipeg, MB, Canada). Before chemical analysis, the fecal samples were dried in a forced-air drying oven at 60^{o}C for 7 days, pooled for each pig, and finely ground. Diets and fecal samples were analyzed for dry matter, GE, N, Crude fat, starch, NDF, ADF, AIA, and Ca and P. The

moisture (AOAC 930.01), crude protein (AOAC 990.03), Ash (AOAC 942.05), crude fat (AOAC AM5-04), Ca and P (AOAC 985.01), crude fiber (AOAC Ba6a-05) and AIA (AOAC 942.05) were determined according to the methods of the Association of Official Analytical Chemists International (AOAC International, 2006). The starch content was measured using the amyloglucosidase/alpha-amylase method (AOAC 996.11) using a starch megazyme starch kit. The ADF and NDF contents were analyzed according to the method by Goering and van Soest (1970) using an ANKOM 200 Fiber Analyzer (A200, ANKOM Technology, Macedon, NY) with an alpha-amylase (product A3306, Sigma-Aldrich, St. Louis, MO).

The GE was determined using a bomb calorimeter (model 6400, Parr Instruments Co, Moline, IL) calibrated using benzoic acid as a standard. For the GE of urine, 0.5 g of cellulose was dried at 100^{o}C for 24h, and a 2 mL of urine sample was added to it and dried at 50oC for 24 h and weighed to estimate the DM content of urine. GE of the cellulose with urine was subtracted by the GE of cellulose to estimate the GE of urine.

4.3.5 Calculations

Apparent total tract digestibility (ATTD) of DM, GE, CP and energy were calculated using the following formula:

$$\text{ATTD (\%)} = \frac{[1-(N_f \times AIA_d)]}{N_d \times AIA_f} \times 100$$

where N_f = nutrient concentration in feces (percentage of DM); N_d = nutrient concentration in diet; AIA_f = acid insoluble ash concentration in feces; AIA_d = acid insoluble ash concentration in diet (McCarthy et al., 1974).

HP and FHP and RE were calculated using the following equations (Noblet et al 1994):

HP (FHP) = 3.866 x O_2 +1.200 x CO_2 – 1.431 x urinary N excretion, where HP is in kCal, O_2 and CO_2 in L and urinary N excretion in grams.

RE = ME-HP

Where RE, ME and HP are in kcal/day.

DE = $\frac{GE_i - GE_f}{DMI}$

ME = $\frac{GE_i - GE_f - GE_u}{DMI}$

NE = $\frac{RE + FHP}{DMI}$

Where, GE_i, GE_f and GE_u are the gross energy intake (i), faeces (f) and urine (u) and DMI is the DM intake in kg.

RQ was calculated as the ratio of the CO_2 production and the O_2 consumption (Noblet et al., 2001).

The relative organ weight was calculated as:

Relative organ weight (%) = 100 x [organ weight (kg)/total pig weight (kg)]

4.3.6 Microbiome Analysis

4.3.6.1 DNA extraction and 16S rRNA sequencing

The fecal DNA was extracted using the QIAamp® Fast DNA Stool Mini Kits (Qiagen Ltd., Germany) according to the manufacturer's instructions, and bead-beating was included to lyse the microbial cells. The quantity and quality of extracted DNA were measured using a NanoDrop2000 spectrophotometer (Thermo Fisher Scientific, Waltham, MA, USA) and agarose gel electrophoresis, respectively. The V4 region of the 16S rRNA gene was amplified with universal primers 515F (GTGYCAGCMGCCGCGGTAA) and 806R (GGACTACNVGGGTWTCTAAT). Illumina MiSeq 250-bp paired-end reads were used to obtain the full-length reads of the V4 region. The single multiplexing step was executed using a 12 bp index. The PCR products were pooled, purified, and loaded into the Illumina MiSeq cartridge. Reads were joined using EA-Util's fastq-join script with default parameters, then screened to exclude sequences that contained one or more

base calls with a Phred quality score of less than 20. A Phred quality score of 20 or higher indicated an accuracy of 99%.

4.3.6.2 Microbial data analysis

Raw sequences were analyzed using the latest version of the QIIME2 platform (version 2021.8) as previously described by Wang et al. (2019). Initial reads were quality filtered, denoised, assembled, and chimeric sequences were removed using DADA2 (Callahan et al. 2016), which generates unique amplicon sequence variants (ASVs). We used the SILVA (version 138) reference database classifier to classify bacterial features with a threshold of 99% sequence similarity. Alpha and beta diversities were calculated in QIIME2. To examine the effects of pigs' FE on fecal microbiota, we performed a permutational multivariate analysis of variance (PERMANOVA, with 999 Monte Carlo permutations) based on Bray-Curtis, Unweighted Unifrac distances, and Weighted Unifrac distances matrices with the web-based tool MicrobiomeAnalyst (Chong et al., 2020). Differentially abundant features between pig groups were identified using linear discriminant analysis (LDA) and effect size (LEfSe) analysis (Segata et al., 2011). Only taxa with average relative abundances greater than 0.01% were included in LEfSe. Bar plots and heatmaps were visualized using the MicrobiomeAnalyst http://www.microbiomeanalyst.ca (Chong et al., 2020). The predicted metagenomes and function of the gut microbiota were inferred using an R package Tax4FUN (Aßhauer et al., 2015) available through MicrobiomeAnalyst. The predicted genes and their function were aligned to the Kyoto Encyclopedia of Genes and Genomes (KEGG) database, and the differences between pig groups were compared through the software STAMP http://kiwi.cs.dal.ca/Software/STAMP (Parks and Beiko, 2010). Two-side Welch's t-test and Benjamini-Hochberg FDR correction were used in two-group analysis.

4.3.6.3 Statistical analysis

Growth performance data were analyzed using PROC MIXED in SAS 9.4 (SAS Inst. Inc., Cary, NC, United States). The pen was the experimental unit. The covariate effects of weaning age, initial body weight (IBW), and sex (ratio of castrated males/females) were tested and their effects were not significant and therefore were removed from the model. A *P* value of 0.05 was considered statistically significant, while a *P* value of 0.1 indicates a trend for differences. A pen was the experimental unit for the individual body weight data (BW), while pigs were the model observational units. In this case, the mixed model included a random statement that the pen was nested within treatment. For the body weight gain (ADG), feed intake (ADFI), and FCR (feed: gain) data, a pen was again the experimental unit, but because there were no observational units (pigs), the random statement of a pen (treat) was removed. For organ weight and length data, digestibility, and indirect calorimetry data, the mixed procedure of SAS was also used, but, in this case, the individual pig was the experimental unit as previously described by Koo and Nyachoti (2021). Unpaired T-test analysis was performed to compare performance and organ measurements as well as digestibility and energy metabolism between the 2 pig groups. For the microbiome data, all parametric data were analyzed using an unpaired Student's t-test, while nonparametric data were analyzed using the Mann-Whitney U test or Kruskal-Wallis test. *P* values for group comparisons were adjusted with a false discovery rate (FDR) according to Benjamin and Hochberg (1995). The corrected *P* values below 0.05 were considered statistically different. Data were expressed as means and standard error of the mean (SEM).

4.4 Results

4.4.1 Growth performance and organ measurements

The covariate effects of initial body weight (IBW), weaning age, and sex was not significant on performance parameters and therefore removed from the model. Body weight gain (ADG), feed intake (ADFI), and FCR (feed/gain) were statistically not different between pigs with high and low EBV_FCR (Table 4.2). In addition, there were no differences in the relative organ weights nor the length of the small and large intestines between the two pig groups (Table 4.3). There was, however, a trend for a larger heart (P = 0.0649, Table 4.3) and larger lungs (P = 0.0834, Table 4.3) for the pig group with the lower EBV_FCR, i.e., the higher FE group.

4.4.2 Digestibility and energy metabolism

ATTD of nutrients and of gross energy were not different between the two pig groups (Table 4.4). ATTD of crude protein, no fiber carbohydrates and of GE were higher than 88% regardless of pig group.

The energy balance of nursery pigs selected for FE is summarized in Table 4.5. Similar ATTD for GE between pig groups resulted in a similar DE value (kcal/kg) of the diet and similar fecal GE excretions (kcal/d, P = 0.2508). Urinary GE excretion rates, on the other hand, were statistically higher in the high efficiency pigs versus the low efficiency pigs (Table 4.5, P =0.0065). Despite this difference, the ME value (Kcal/kg) of the diet was statistically not different between the two pig groups (Table 4.5, P = 0.3303).

The measured respiratory quotient (RQ) values for fed and fasting states were higher than 1.2. HP and FHP were on average 86.4 kcal/kg $BW^{0.6}$/day, regardless of pig group. Low FE group had a numerically higher FHP (75.3 vs. 65.9 kcal/kg $BW^{0.6}$/day, P=0.1133) but statistically not different than FHP of high FE group. This difference in FHP resulted in a NE 17% lower for the

low FE group compared to the high FE group but this difference was again statistically not different (2374 vs. 1973 kcal/kg, P=0.2819).

Table 4.2 Growth performance summary of nursery pigs fed the corn/soybean meal-based diet (n = 8 for each group). LSMeans for the high-efficient pigs (HE) and low-efficiency pigs (LE) were calculated, and unpaired T-test were used to test for significant differences between pig groups.

BW, kg	HE	LE	SEM	*P* value
BW, 21d	7.0	6.7	0.15	0.0545
BW, 28d	8.4[a]	8.2[b]	0.09	0.0067
BW, 42d	16.5	16.1	0.34	0.3678
BW, 56d	27.3	27.8	0.50	0.4662
ADG, g/d				
Day 28-42	575.1	567.0	13.63	0.6791
Day 42-56	781.0	813.2	13.34	0.0974
Day 28-56	683.8	682.0	18.08	0.8892
ADFI, g/d				
Day 28-42	888.5	903.1	22.50	0.6493
Day 42-56	1323.1	1354.8	48.66	0.6492
Day 28-56	1105.8	1128.9	63.83	0.6123
FCR, feed:gain				
Day 28-42	1.55	1.59	0.024	0.2078
Day 42-56	1.70	1.67	0.062	0.7723
Day 28-56	1.61	1.66	0.055	0.5637

BW = body weight; ADG = average daily gain; ADFI = average daily feed intake; (n = 16 for each group).

Table 4.3 Relative organ weights and organ length of nursery pigs from two groups with different EBV_FCR (n = 8 for each group) measured at the end of feeding phase 2.

Relative organ Weight (%)	HE	LE	SEM	*P* value
Spleen	0.23	0.23	0.018	1.0000
Heart	0.60	0.52	0.030	0.0649
Lungs	1.21	1.05	0.061	0.0834
Kidneys	0.54	0.55	0.018	0.9226
Liver	3.22	3.47	0.100	0.1244
Stomach	0.75	0.74	0.047	0.8967
Small Intestine	4.11	3.70	0.280	0.3198
Large Intestine	2.13	2.02	0.210	0.7294
Organ length (m)				
Small Intestine	18.0	17.4	0.60	0.5050
Large Intestine	4.0	3.2	0.35	0.1422

Table 4.4 ATTD of experimental diets fed to the nursery pigs. AIA was used as the digestibility marker.

Item	HE	LE	SEM	*P* value
ATTD, %				
Dry matter	89.92	89.40	0.864	0.5554
Crude protein	89.40	88.70	1.174	0.5620
Crude fat	62.21	62.66	3.853	0.9093
Crude fiber	47.63	43.50	5.182	0.4410
Non-fiber carbohydrates	95.06	94.68	0.451	0.4094
NDF	69.36	68.38	2.136	0.6539
ADF	70.05	69.70	2.562	0.8927
Ash	75.86	75.61	1.674	0.8821
GE	90.06	89.56	0.904	0.5930

ATTD = apparent total tract digestibility; NDF = neutral detergent fiber; ADF = acid detergent fiber.

Table 4.5 Energy balance of nursery pigs fed the experimental diets (n=8).

Item	HE	LE	SEM	*P* value
Energy Balance, kcal/d				
GE intake	3060	3049	136.73	0.9356
Fecal GE excretion	848	1296	367.48	0.2508
Urinary GE excretion	151[a]	73[b]	22.79	0.0065
Energy value, kcal/kg				
DE	2882	2345	423.63	0.2335
ME	2685	2249	426.54	0.3303
NE	2374	1973	352.12	0.2819
HP, kcal/kg $BW^{0.6}$/day	86.3	86.6	3.7215	0.9478
RQ, fed-state	1.75	1.71	0.0936	0.7294
FHP, kcal/kg $BW^{0.6}$/day	65.9	75.3	5.3880	0.1133
RQ, fasting- state	1.50	1.40	0.1155	0.4068
Energy efficiency				
ME/DE	0.93	0.95	0.01789	0.2897
NE/ME	0.88	0.89	0.03582	0.7513

HP = heat production; FHP = fasting heat production; RQ = respiration coefficient; DE = digestible energy; ME = metabolizable energy; NE = net energy. Different superscript letters indicate statistical differences, $P < 0.05$.

4.4.3 Taxonomic classification and diversity of fecal microbiota related to host feed efficiency

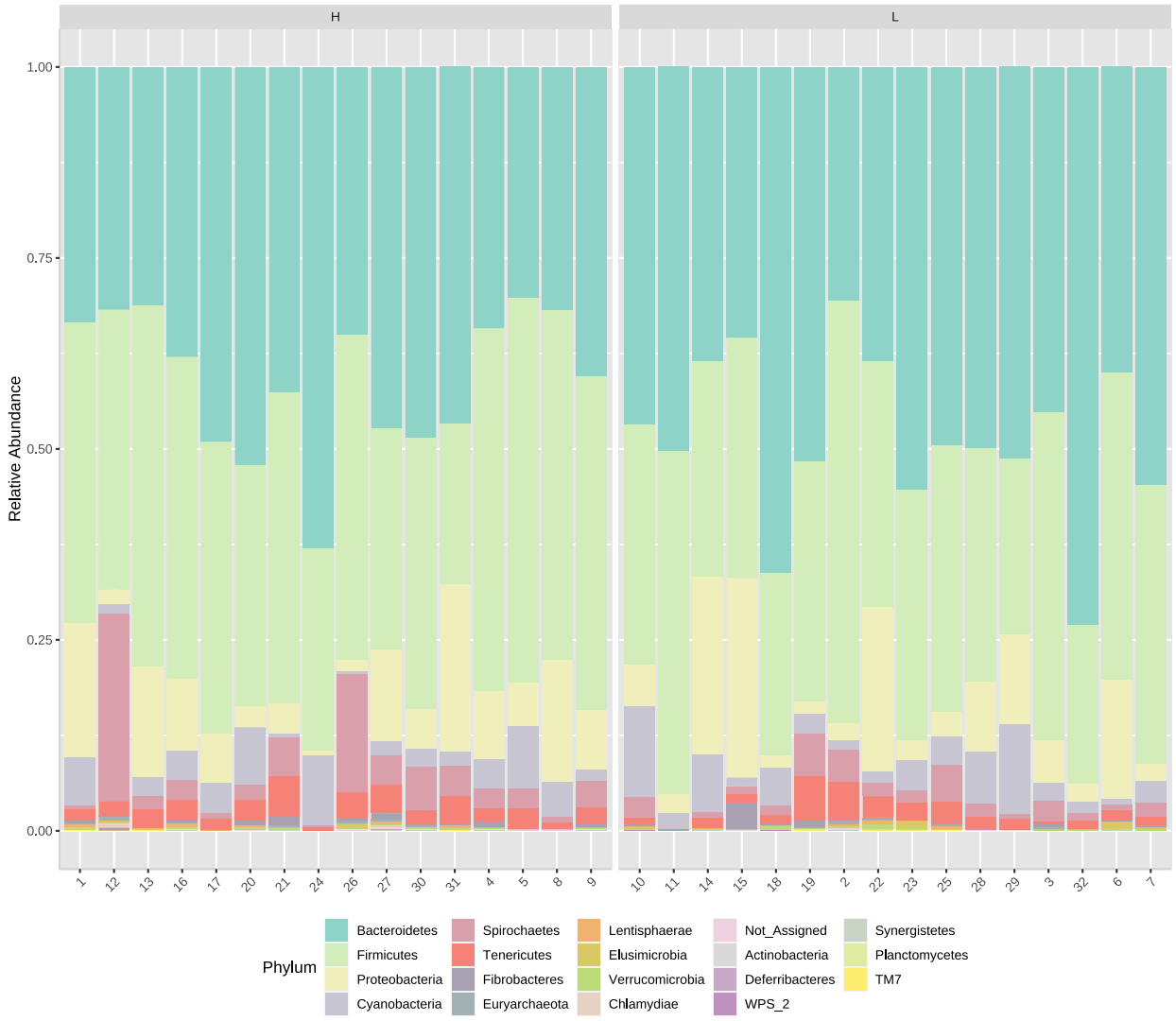
At 56 days of age, fecal samples from each pen were collected for 16S rRNA sequencing. The number of sequences ranged from 70,575 to 193,788 (130,988 ± 22,996 sequences on average). After quality control with DADA2, we obtained 120,528 (±21,059) reads per sample. With a 99% identity cut-off, the total number of ASVs was 1,960. *Bacteroidetes* and *Firmicutes* (Figure 4.11A) were the two most abundant phyla regardless of the pig EBV_FCR group. *Prevotella* (Figure 4.1B) was the prevalent genus in both pig groups; Statistical significances for abundant phyla and genera were reported in Appendix tables (1 and 2). At the phylum level, *Bacteroidetes* (45%) and *Firmicutes* (36%) were by far the most dominant phyla, followed by *Proteobacteria* (9%) and *Cyanobacteria* (4%). Unclassified bacteria accounted for 32% of the total reads in both pig groups at the genus level due to the targeted amplification sequencing limitations. Genus *Prevotella* (36%), V2 (33%), and *Roseburia* (4%) were the most abundant in both pig groups. *Lactobacillus*, *Ruminococcus*, and *Streptococcus* accounted for less than 4% of the total reads. At the feature level, no statistical differences in species richness (how many ASVs are in a sample) between the 2 pig groups were observed (Figure 4.2A, $P = 0.18351$). Also, no statistical differences in species dominance (Simpson index, Figure 4.2B, $P = 0.10997$) between the 2 pig groups nor statistical differences in species richness and evenness between the 2 pig groups (Shannon index, Figure 2C, $P = 0.15961$) were observed.

At the feature level, none of the beta-diversity measures investigated were statistically different between the two pig groups (Figure 4.3, A, B, C). When only ASVs counts were considered, i.e., beta-diversity based on Bray-Curtis dissimilarities, there were no pig group differences in diversity (Figure 4.3A, PERMANOVA $P < 0.332$).

When considering phylogenetic distances between observed organisms and the presence/absence of ASVs, there were also no significant diversity differences between the 2 pig groups (Unweighted Unifrac, PERMANOVA $P < 0.93$, Figure 3B). Also, when the phylogenetic distances and the abundance of ASVs were taken into account, there were no diversity differences between the 2 pig groups. However, there was a trend for a higher beta diversity on fecal samples from pigs with low EBV_FCR (Weighted Unifrac, PERMANOVA $P < 0.083$, Figure 3C). Heatmap of bacterial genus did not show any major enrichments between high and low FE pigs or statistical differences when DFR adjusted *P* values were considered (Appendix table 1). *Prevotella* was associated with low-efficiency pigs (high EBV_FCR), while *Chlamydia*, *Lachnospira*, *Streptococcus*, and *V4* were associated with high-efficiency pigs (Appendix table 3); These five genera could be considered to be suitable biomarkers for distinguishing between high and low FE pigs ($-2 \leq$ LDA score ≥ 2).

The genus *Prevotella*, *Streptococcus*, and *V4* were distinguishable as potential biomarkers for FE, with LDA scores > 4 for the first genus and < -4 for the other two genera. *Prevotella* was associated with the LE group, while *V4* and *Streptococcus* were higher in HE pigs. These significant genus differences disappeared when the FDR-adjusted *P*-value was considered.

(A)


H
L
Relative Abundance
1.00
0.75
0.50
0.25
0.00
1
12
13
16
17
20
21
24
26
27
30
31
4
5
8
9
10
11
14
15
18
19
2
22
23
25
28
29
3
32
6
7
Phylum
Bacteroidetes
Firmicutes
Proteobacteria
Cyanobacteria
Spirochaetes
Tenericutes
Fibrobacteres
Euryarchaeota
Lentisphaerae
Elusimicrobia
Verrucomicrobia
Chlamydiae
Not_Assigned
Actinobacteria
Deferribacteres
WPS_2
Synergistetes
Planctomycetes
TM7

(B)

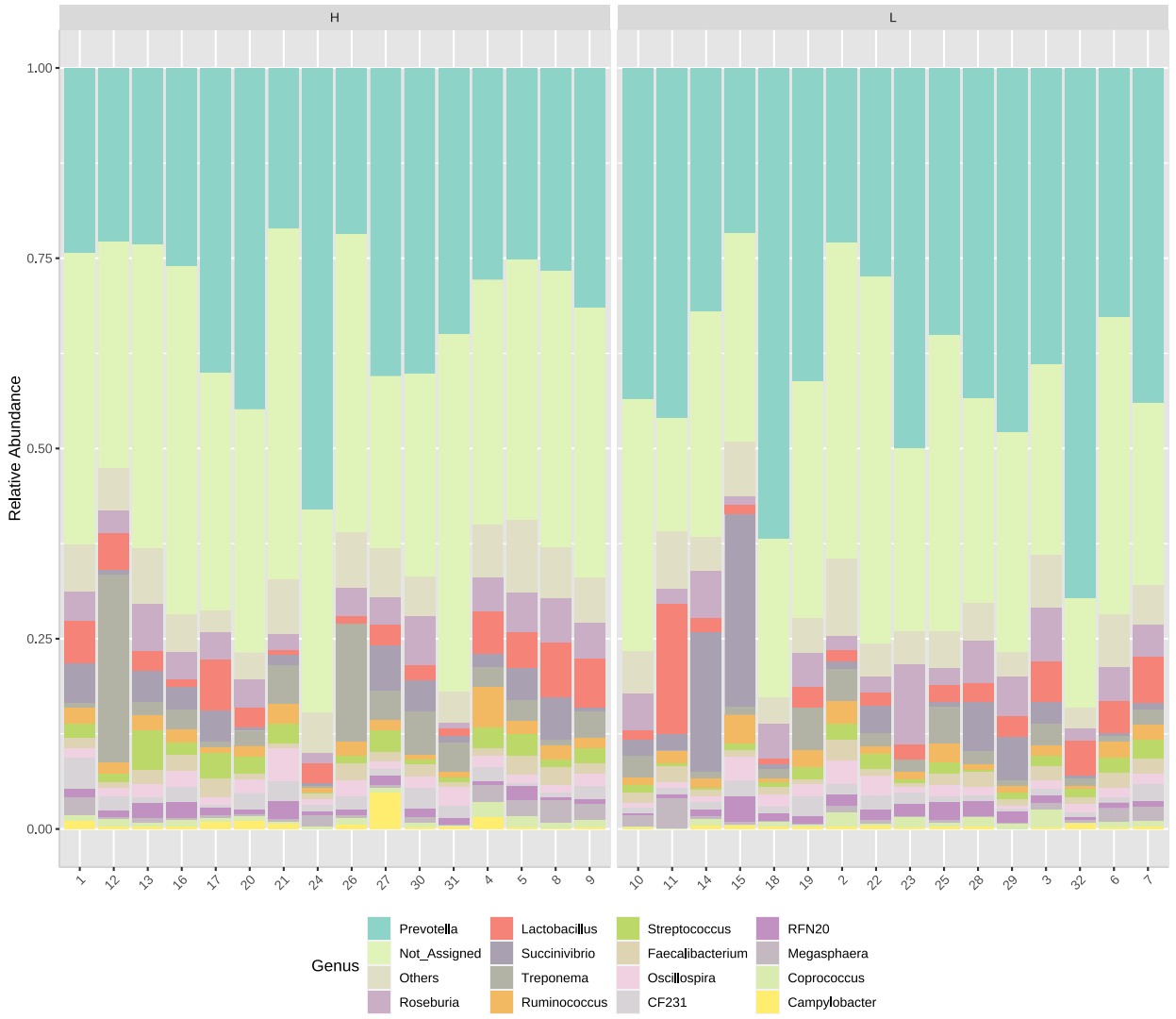
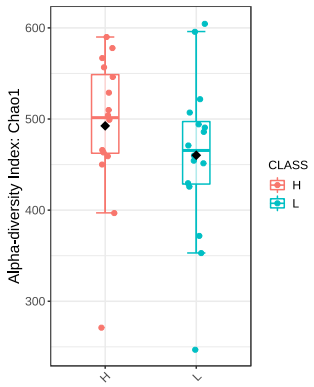
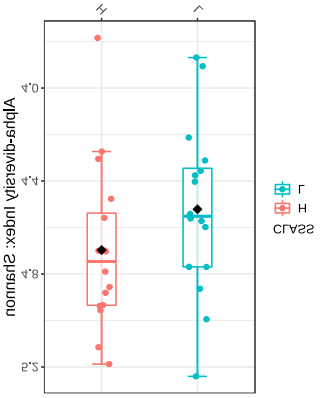


Figure 4.1 Relative abundance of bacteria of the most abundant taxa in the feces of nursery pigs at the phylum (A) and genus (B) levels.

(A)



(B)



(C)

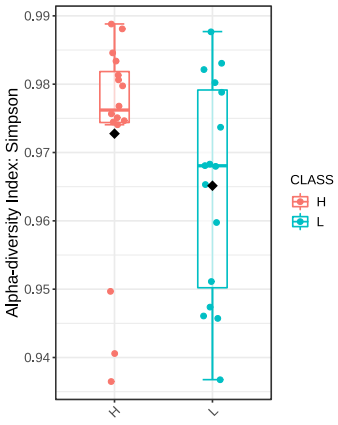
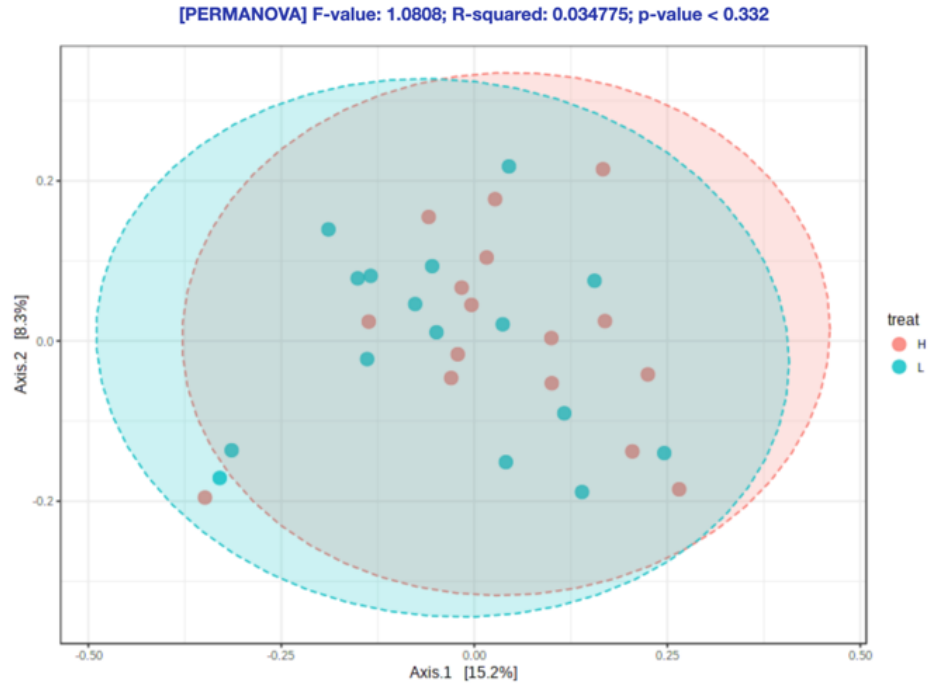
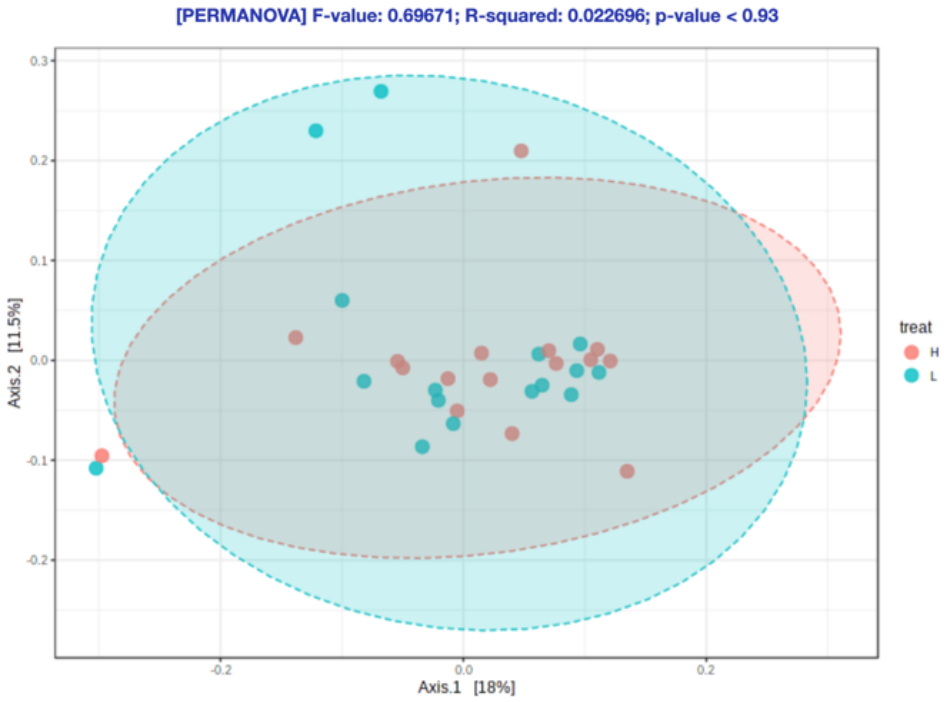


Figure 4.2 Alpha diversity in relation to host feed efficiency when bacteria aggregated at the feature level, Chao1 (A), Shannon index (B) and Simpson index (C)

(A)


[PERMANOVA] F-value: 1.0808; R-squared: 0.034775; p-value < 0.332
Axis.2 [8.3%]
Axis.1 [15.2%]
treat
H
L

(B)


[PERMANOVA] F-value: 0.69671; R-squared: 0.022696; p-value < 0.93
Axis.2 [11.5%]
Axis.1 [18%]
treat
H
L

(C)

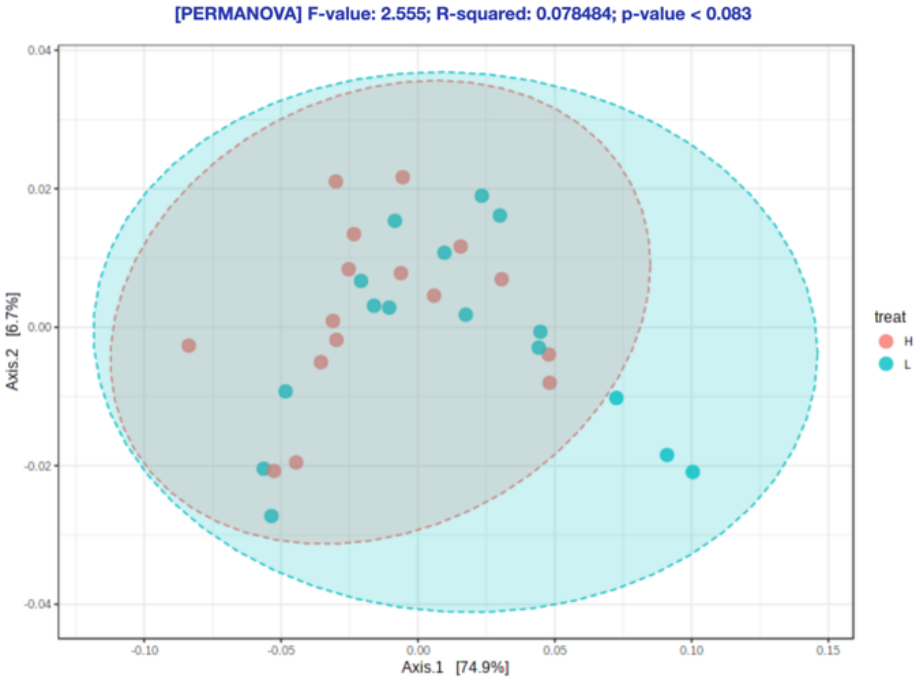


Figure 4.3 Beta diversity in relation to host feed efficiency at the feature level. Bray-Curtis dissimilarity (A), Unweighted Unifrac distances (B) and weighted Unifrac distances (C). Axes represent the two dimensions explaining the greatest proportion of variances in the communities for each analysis.

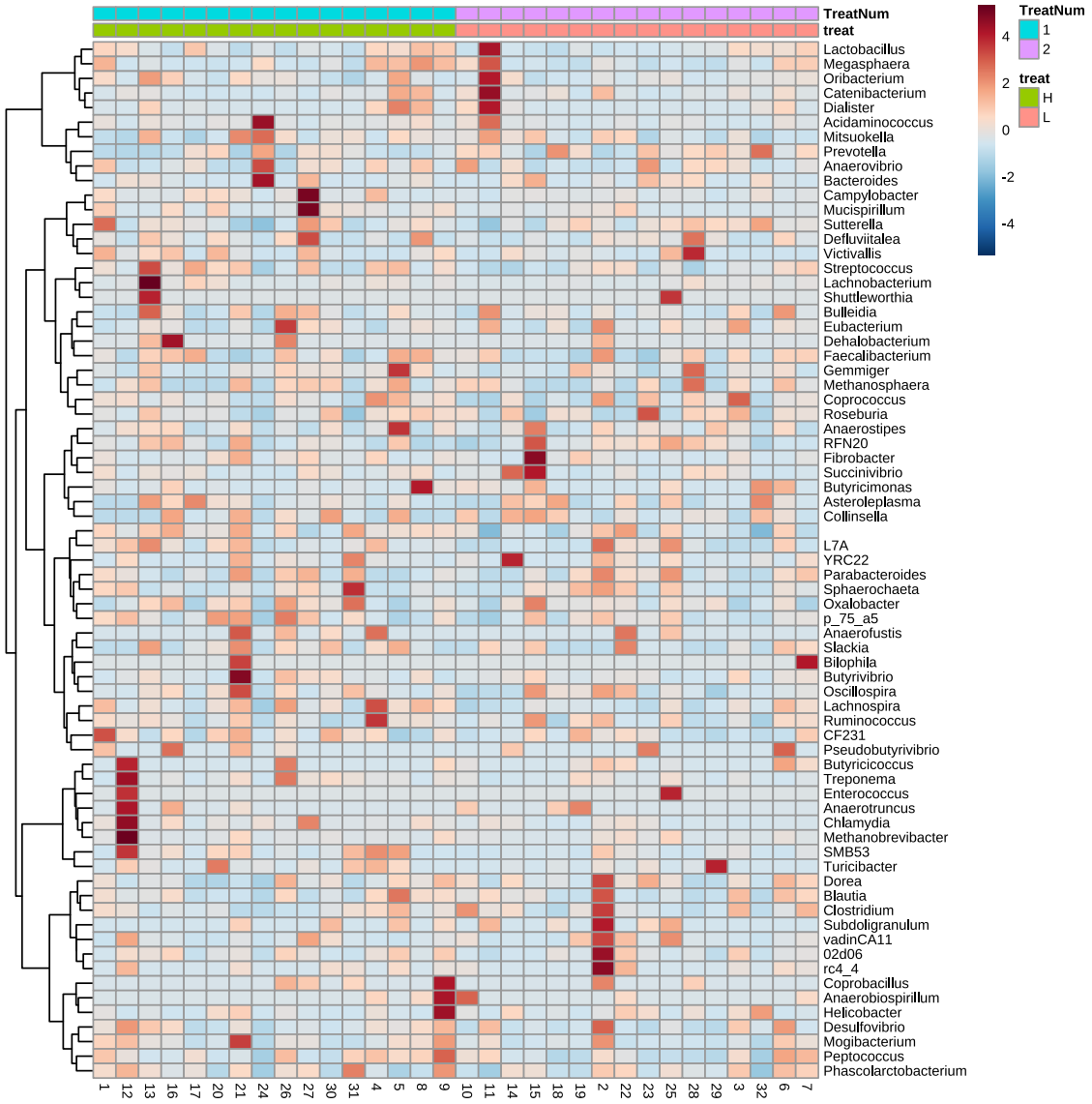
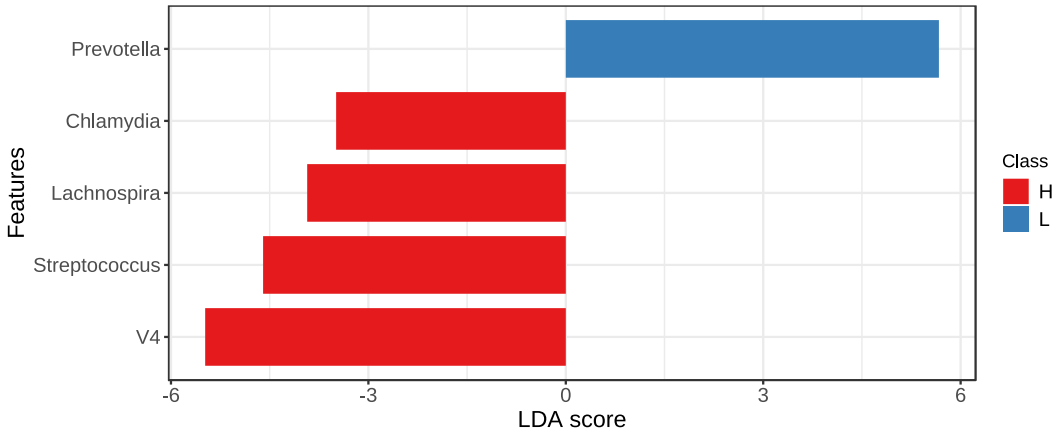


Figure 4.4 Heatmap of correlation coefficients in relation to pigs feed efficiency.

(A)


Prevotella
Chlamydia
Lachnospira
Streptococcus
V4
Features
-6
-3
0
3
6
LDA score
Class
H
L

(B)

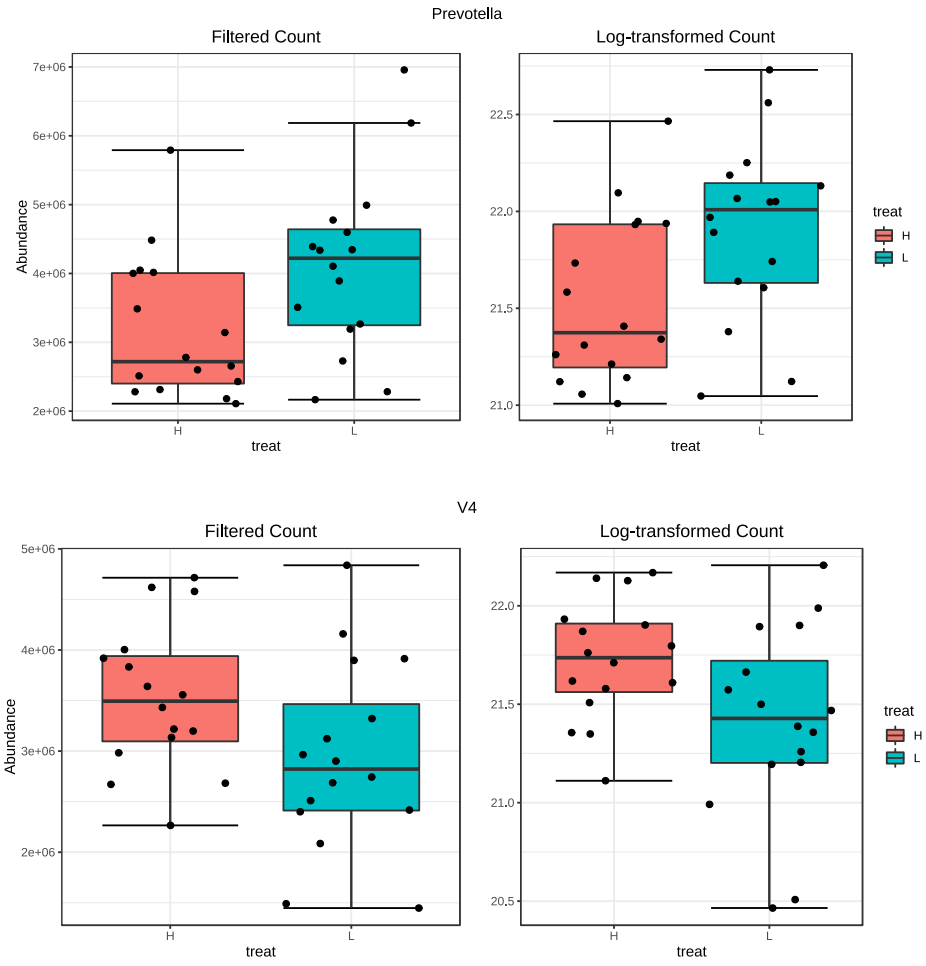
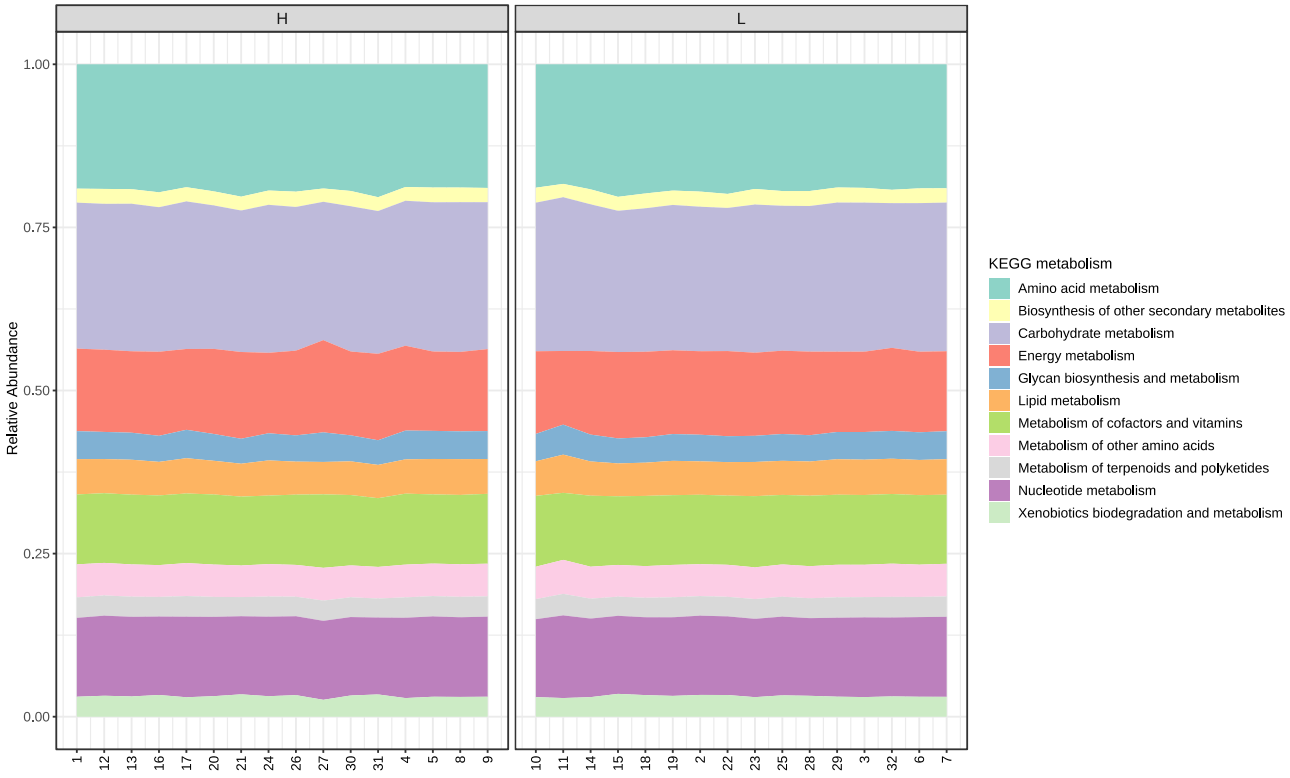


Figure 4.5 LEfSe analysis showing differential abundant taxa between pig groups at the genus level in relation to host feed efficiency (A) and boxplots for *Prevotella* and *V4* (B). Histogram of a linear discriminant analysis (LDA) score (threshold ≥ 2) in fecal samples of nursery pigs of different EBV_FCR. H-piglets selected from parents with low EBV_FCR, L-piglets selected from parents with high EBV_FCR.

(A)


H
L
Relative Abundance
1.00
0.75
0.50
0.25
0.00
1
12
13
16
17
20
21
24
26
27
30
31
4
5
8
9
10
11
14
15
18
19
2
22
23
25
28
29
3
32
6
7
KEGG metabolism
Amino acid metabolism
Biosynthesis of other secondary metabolites
Carbohydrate metabolism
Energy metabolism
Glycan biosynthesis and metabolism
Lipid metabolism
Metabolism of cofactors and vitamins
Metabolism of other amino acids
Metabolism of terpenoids and polyketides
Nucleotide metabolism
Xenobiotics biodegradation and metabolism

(B)

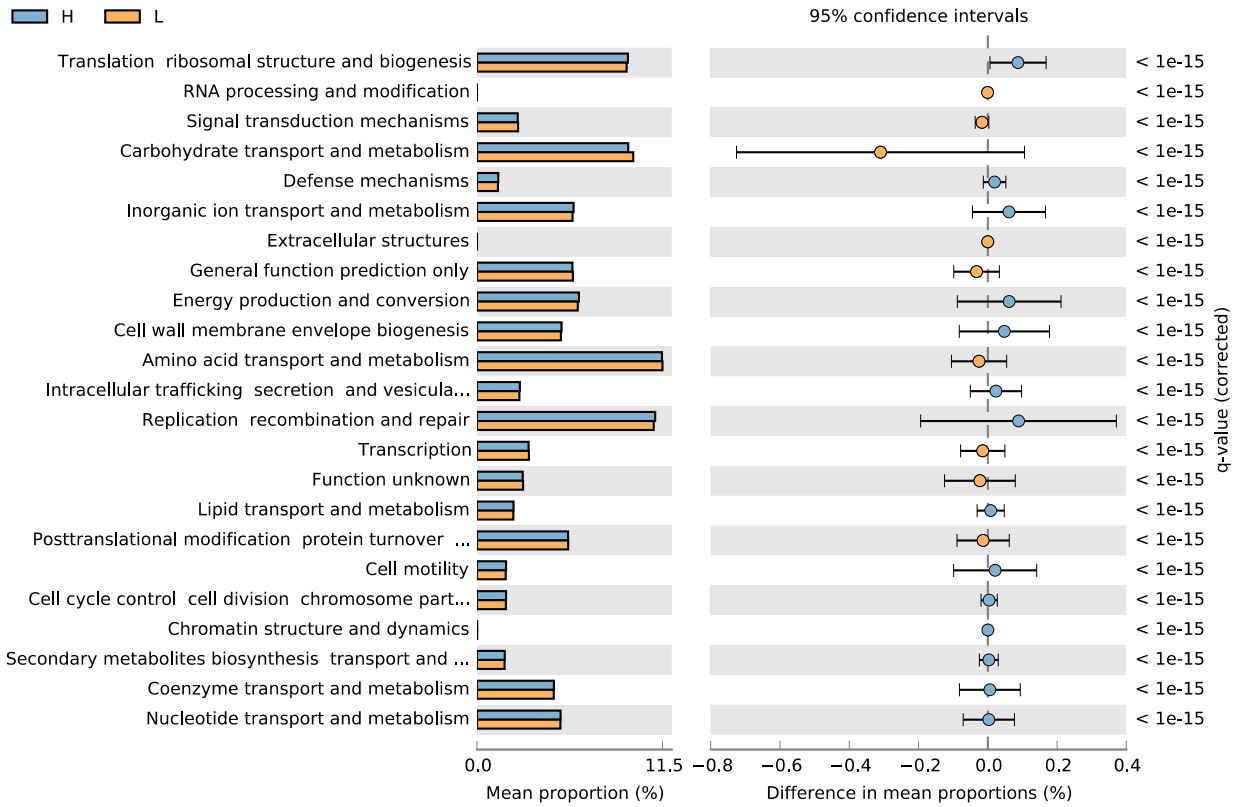


Figure 4.6 Predicted functionality of fecal microbiota related to host feed efficiency in terms of KEGG metabolism (A) and COG functional pathways (B). The samples are represented on the X-axis and separated based on metadata (pig FE group). The relative abundances of the various metabolism categories across all the samples are represented on the Y-axis.

4.4.4 Predicted functionality of fecal microbiota related to host feed efficiency

The R package, Tax4FUN was used to evaluate the functional profiles of the pig fecal microbiota for the two pig groups based on EBV_FCR. The results indicated that 11 pathways, including carbohydrate, amino acids, and energy metabolism were not different between the two pig groups (Figure 5A, B, Supplementary Table S4) and that it was dominated by carbohydrate and amino acid metabolism.

4.5 Discussion

4.5.1 Growth performance, digestibility and energy metabolism

Feed efficiency is a complex measure influenced by genetic and environmental factors, including nutrition, management, and animal physiological and health status (Patience et al., 2015). In order to investigate the effects of genetics, in particular, different genotypes based on EBV_FCR on feed efficiency without confounding effects of biotic factors like breed, sex, age, and environmental factors like diet and management on FE was the goal of the present study. Nutrition and management were kept similar between the two pig groups of different EBV_FCR. Similar feeding, growth, and mortality responses and the absence of post-weaning diarrhea observed during the 56 days of the trial between the two pig groups also suggest that physiological and health status were similar.

Host physiology was also similar, including nutrient digestibility and energy harvesting. Indeed, nutrient digestibility and energy metabolism were not different between pig groups, except for the low-efficiency pigs' lower urinary GE excretion, which is unclear but could be due to lower protein turnover rates in low-efficiency animals compared to high-efficiency ones (McBride and Kelly, 1990). On the other hand, Gabler (2012) reported that low RFI, indicative of higher FE, skeletal muscle had greater calpastatin activity, lower calpain, and ubiquitin-proteasome activity

compared to that of high RFI, i.e., less efficient pigs, suggesting that selection for improved FE and low RFI may select for reduced protein degradation and turnover. Low-efficiency pigs could require more nitrogen in the large intestine for fermentation, therefore it would be beneficial for these pigs to excrete less urea and energy in the urine and, therefore, a lower urinary GE loss. In the future, protein turnover rates in muscle from pigs with high and low EBV_FCR should be investigated. Despite possible differences in the urinary GE excretion, pig genotype did not affect body weight gain or growth rate. It is possible that there were differences in terms of lean tissue gain between the two pig groups, but neither carcass composition nor lean tissue growth was investigated in this study. Maximum lean growth potential and fat deposition rates vary as a function of feed intake among breeds and sex (Noblet et al., 1993). Measuring growth rates and body composition like body back fat content and comparing the residual feed intake (RFI) of these two pig groups should be considered in the future. Pigs with lower RFI are more efficient (Patience et al. 1995). RFI will add a deeper understanding of the feed efficiency differences between the two pig groups as it is the difference between the measured feed intake and the estimated feed intake based on the animal's rate of gain and body back fat content (Kennedy et al., 1993). The ratio of lean and fat body gain is affected by pig sex. Males have a higher potential for lean tissue growth than females or castrated males due to the hormone testosterone (Patience et al., 1995). In the present study only castrated males and females were included. The ratio of castrated males to females among pens did not affect growth performances as the covariate effect of castrated males/females ratio was not significant ($P > 0.05$) on growth performances. This is most likely because of the growth stage studied. At the nursery and growing stage, there is a linear lean (protein) accretion response to energy intake regardless of sex (Noblet et al., 1994). As at these stages pigs have not reached their sexual maturation, the lean growth is not affected by sexual

hormones but rather a linear function of energy intake. Alternatively, protein intake by the low EBV_FCR group could be higher than the amount required for lean tissue gain, leading to a greater urinary N excretion in the HE pigs (Le Goff and Noblet, 2001). Nevertheless, the energy value of the diet in terms of ME (kcal/kg) was similar for both pig groups.

Vigors et al. (2016) reported that pigs of high and low FE had differences in nutrient digestibility. These studies reported a negative correlation for ATTD of DM, GE, and N with FCR. They suggested that changes in small intestinal absorptive processes lead to increases in nutrient digestibility, affecting FE in pigs. Harris et al. (2012) also found higher total tract digestibility of dry matter, nitrogen, ash, and gross energy in high FE pigs compared to low FE pigs. In agreement with these studies, the ATTD of GE observed in the present study was also higher in the high-efficiency group but only numerically. Statistically, there were no differences in GE digestibility or fecal GE excretion rates between the two pig groups. The diet used was one possible contributor to the lack of performance differences, digestibility, and energy metabolism differences. Diets used in the two feeding phases were formulated based on the nutrient requirements of the TN Tempo breeding line (Topigs, Norsvin) and included premium ingredients, highly digestible and of high nutrient value for nursery pigs such as Nupro, betaine, phytase, as well as highly digestible soy protein supplement Hamlet HP300. These premium diets may have masked the potential differences in growth performance and energy utilization efficiency between the two pig groups of different EBV_FCR. It is also possible that the genetic differences between the two groups have not been enough to result in phenotypic differences in terms of growth and FE till later at the grower-finishers stages. This was the case in the follow up study with these pigs at the grower and finisher stages (Jin et al., *in press*). Harris et al. (2012) reported that selection for low RFI alters nutrient utilization, energy digestibility, and improved nitrogen and phosphorus balance. These

effects were, however, observed with grower pigs of extreme RFI. These improvements were not seen in our study, which could be due to the growth stage or the gap in EBV_FCR values was not enough to see phenotypic differences in growth performance and FE at the nursery stage.

The RQ values obtained in this study were unrealistically high. For example, in a fed status, RQ would vary from 1.0 when glucose is metabolized for energy to a maximum of 1.4 -1.6 when organic acids such as malic acid or tartaric acid would be used for energy and based on complete oxidation of the nutrient to CO_2 (Van Milgen et al., 1997, 2000). RQ value of mixed diets fed to growing pigs is slightly greater than 1.0 during the fed state and drops below 0.8 during fasting (Agyekum et al., 2016) but never higher than 1.2. Moreover, this value linearly increased with increasing carbohydrate metabolism and linearly decreased as the metabolism of lipids and proteins increased (Kim et al., 2018). In the present study, RQ was, on average higher than 1.70 in fed status and higher than 1.40 in the fasting status. These extremely high RQ values are most likely due to compromised oxygen values, as some moisture was observed in the input air line from the chamber to the oxygen sensor.

The sensor is an electrochemical sensor; therefore, the measured oxygen values were compromised by moisture reaching the sensor. If either glucose or malic acid or tartaric acids were metabolized as energy sources, the HP would range from 696 to 914 kcal/d and the FHP from 307 to 454 kcal/d, and the measured HP of 553 to 706 kcal/d could be underestimated, and the same with the FHP of 211 to 263 kcal/d. These values are lower than expected for pigs fed a well balance diet. Also, FHP of less than 90 kcal/$kg^{0.6}$/d is around two times below the average FHP values reported in NRC (2012) for pigs of more than 190 kcal/$kg^{0.6}$/d. In the future, indirect calorimetry of pigs of similar age, diet, and genotype should be repeated carefully to the input air drying capacity of Aquabsorb powder in the air tubing line to the oxygen sensor.

Visceral organs represent a small proportion of the overall body weight but account for a large portion of the animal energy expenditure due to being involved in metabolically expensive processes such as protein synthesis and degradation, for example (Noblet et al., 1999, Nyachoti et al., 2000). Although no statistical differences were observed in visceral organs between the two pig groups, there was a trend for a larger heart and lungs in the pig group with lower EBV_FCR, the high FE group. These results suggest that HE pigs would be anatomically better equipped for higher metabolic and growth rates, which, however, could not be confirmed by the metabolic rates measured as HP and FHP results were unreliable in the present study.

Although no growth performance nor nutrient and energy digestibility and utilization differences were observed between the two pig groups at the nursery stage, differences may appear as pigs grow more significantly, which was investigated in a companion study.

4.5.2 Microbiome in relation to feed efficiency

Gut microbiota plays an important role in swine physiology and homeostasis, especially in energy harvest, nutrient digestion, and intestinal health, and is likely to affect FE (McCormack et al., 2017). Investigating bacterial taxa/functionality associations with production traits and FE will highlight strategies to improve both productivity and environmental sustainability of swine production. There is no doubt that a link exists between microbiome and growth and FE. Gardiner et al. (2020) summarized several examples evidencing this link between the microbial taxa and functional capacity of gut microbiome and growth, body composition traits, and FE. According to their review, bacterial taxa involved in nutrient processing and energy harvest and those with anti-inflammatory effects are consistently linked with improved productivity. However, studies have a remarkable inconsistency (Maltecca et al., 2020; Gardiner et al., 2020). The reason could be that the link between FE and gut microbiota is affected by several factors, including biological and

external factors to the pig, such as diet and management. In particular, breed, sex, age, and gut site location are among the most important biological factors affecting the link between FE and the gut microbiome (Gardiner et al., 2020; Bergamaschi et al., 2020). All these factors present a challenge when identifying bacterial taxa associated with pig production traits.

Nevertheless, some trends can be reported. For example, evidence is emerging for associating various taxa like *Treponema, Roseburia,* and *Lactobacillus* in the large intestine with a leaner phenotype and improved FE. (Gardiner et al., 2020). In most of the studies reviewed by Gardiner et al. (2020), different FE pig groups were the result of the selection of pigs with extreme growth and body composition traits and, in particular, extreme residual feed intake (RFI) values. In our study, pig genotype selection was based on estimated breeding values for FCR.

Furthermore, we focused on post-weaning nursery pigs. Most of the earlier studies focus on growing-finishers pigs, and it is crucial to investigate if similar bacterial taxa/functionality associations of older pigs apply to nursery post-weaning pigs. Weaning is often associated with the disruption of the ecological balance of the gut microbiota community (Wei et al., 2021). This dysbiosis favors pathogens colonization on epithelial cells of the GIT and often results in inflammation and post-weaning diarrhea. It is therefore important to investigate if piglets of high EBV_FCR, and therefore less efficient, have different bacterial taxa/function associations that would make them more predisposed to gut dysbiosis and compromised growth performance than pigs with low EBV_FCR. Despite genetic selection differences from other studies, and consistent with previous studies (Lamendella et al. 2011; Yang et al. 2017; Wang et al. 2021), *Firmicutes* and *Bacteroidetes* were the most abundant phyla in the fecal microbiota of pigs in our study. An earlier study by Wang et al. (2019) also reported that *Firmicutes, Bacteroidetes,* and *Actinobacteria* were the most abundant phyla across each stage in the life of a pig. At the genus

level, *Prevotella*, *Runinococcaceae*, and *Lactobacillus* were reported as the core bacteria for fecal samples collected at 80, 120, and 240 days (Ke et al. al., 2019). In the present study, the genus *Prevotella* (36%), V2 (33%), and *Roseburia* (4%) were the most abundant in both pig groups. However, *Lactobacillus*, *Ruminococcus*, and *Streptococcus* accounted for less than 4% of the total reads from fecal samples collected when pigs were 56 days old, regardless of the pig group.

Differences between our study and the study by Ke et al. (2019) could be due to breed, genotype age as well as diet differences. No differences in fecal microbiota composition between the two pig groups may be caused by the diet used. Verschuren et al. (2018) did not find significant associations between FE and fecal microbiota composition when grower-finisher pigs were fed a corn/soybean meal-based diet. However, these authors found significant associations between FE and fecal microbiota when a wheat/barley/by-products diet was used instead, showing that the relationship between FE and fecal microbial composition is diet dependent. Another possible reason for no microbiota differences between the two pig groups in our study is that sex was not separated in each pen. Verschuren et al. (2018) reported sex effects on the relationship between FE and fecal microbiota composition. Despite that, the sex effect on the fecal microbiota of nursery pigs has not been reported. Males have higher growth rates, and there are body lean/fat deposition differences between males and females as pigs grow. However, these differences are most likely not significant at the nursery stage when feed is mainly used for maintenance and lean growth (Siebrits et al., 1986; Elbert et al., 2020) and are not significantly involved in sex maturation and fat deposition, but this needs further investigation.

Wang et al. (2021) reported that pigs with lower FCR had greater alpha diversity in their gut microbiota. In the present study, there were no differences in alpha diversity in feature level between pig groups, but high-efficiency pigs had a higher alpha diversity of Shannon index at the

genus level. There were also no beta diversity differences between the two pig groups except for a trend for higher beta diversity of the low EBV_FCR when the phylogenetic distances and the abundance of ASVs were taken into account. i.e., Weighed unifrac distances. This trend somehow agrees with the findings by (Gardiner et al., 2020).

In general, a greater bacterial diversity of more feed-efficient pigs was observed across studies. Nevertheless, studies may be inconsistent due to the pigs' age group, fecal samples or gut section, and sample sizes used. Wang et al. (2019) reported that swine gut microbiome from birth to market revealed staged and growth performance associated with bacteria. The present study focused on nursery post-weaning pigs, while the study by Wang et (2021) was on finishing pigs. Wang et al. (2019) also reported that alpha diversity, including community richness and diversity, showed an overall increasing trend with pig age. Si et al. (2020) reported a higher alpha diversity for the high FE pigs than the low FE pigs. However, these findings must have been age-related as these authors used grower-finisher pigs. Sciellour et al. (2019) reported that pig fecal microbiota evolved strongly from 52 to 99 days of age with an increased abundance of *Streptococcaceae* and a decreased abundance of *Lactobacillaceae*. These authors also found that at 52 days of age, two enterotypes were dominated by *Lactobacillus* or *Prevotella-Sarcina*. The *Ruminococcus*-dominated enterotype was associated with a higher ADG from 22 to 28 days and a lower ADG from 29 to 70 days of age. Therefore, their study demonstrated that the enterotypes strongly depend on the pig's age.

LEfSe analysis found that *Prevotella* was higher in low-efficiency pigs. Wang et al. (2021) also reported that *Prevotella* abundance was significantly higher in pigs with high FCRs (and thus low FE). The relative abundance of *Prevotella* is closely related to the consumption of plant polysaccharides, and it is enriched in the presence of plant polysaccharides (Ivarsson et al., 2014).

Therefore, these bacteria could be potential biomarkers for low FE (Tan et al., 2017). Other fecal microbiota potentially linked with porcine FE in our study included *Chlamyd*ia, *Lachnosphira*, *Streptococcus*, and *V4*, which were higher in the pigs with the lower EBV_FCR. Although these bacterial taxa are potential biomarkers of high FE (Yang et al., 2017), the results must be considered with caution as statistical significance was no longer observed when FDR-adjusted p values were considered. Furthermore, the absence of differences when the false discovery rate (DFR) was considered suggests that no specific associated taxa were observed with the FE.

Study shows that KEGG orthology related to nitrogen metabolism, amino acid metabolism, and transport system was positively associated with porcine feed efficiency (Yang et al., 2017; Quan et al.,2020; Jiang et al., 2021). In the present study, the KEGG orthology related to carbohydrate metabolism and amino acid metabolism were dominant over other KEGG pathways, but there were no significant differences between the two pig groups with different EBV_FCR. Similar to our study, Lamendella et al. (2011) found that *Prevotella spp*. and the function term of carbohydrate metabolism dominated the swine fecal metagenome regardless of FE. Several studies have found that pigs that are more feed efficient are likely to have fecal microbiota with higher levels of amino acid metabolism (e.g., Si et al., 2020). Our study did not find any significant differences between the two pig groups in terms of protein and amino acid metabolism. The lack of differences in the predicted functionalities in this study agrees with no differences in the microbiota composition of pigs with different EBV_FCR. This may be due to diet effects, or the EBV_FCR gap between the two pig groups was insufficient to cause microbiome differences at the nursery stage.

The fact that differences were not observed in microbiota composition and predicted functionality from fecal samples does not mean that the same will be valid for different gut sections

or pig ages, which needs further investigation (Quan et al., 2018; Gardiner et al., 2020). In a follow-up companion paper, microbiota composition and function will be addressed for different gut sections of the same pigs from the present study. Several studies have highlighted the importance of gut microbiome composition in shaping lean tissue growth and fat deposition in mammals (Gardiner et al., 2020; Tiezzi et al., 2021). Therefore, when looking at the genetic effects on FE and how this relationship is affected by gut microbiota, it is also essential to consider body composition traits besides growth traits and RFI.

4.6. Conclusion

The selection of pig genotypes with low EBV_FCR did not translate into better growth performance and feed efficiency than pigs with high EBV_FCR. Nutrient and energy digestibility, as well as energy utilization efficiency, also did not differ. These performance results were also supported by no differences in fecal microbiota composition and function. A high-quality diet with highly digestible ingredients may have overridden the effect of genotype on the growth performance and feed efficiency of the post-weaning pigs. The effects of selection based on EBV_FCR on FE with a commercial quality diet should be investigated in the future.

BRIDGE TO CHAPTER 5

According to the results from chapter 4, the piglets selected for feed efficiency based on parents' EBV_FCR had similar growth performance, nutrient and energy digestibility, and fecal microbiota composition at the nursery stage. To gain further insights into the potential difference between the two pig groups, the digestive and absorptive capacity and gut microbiome of various gut sections between the two pig groups were investigated.

5.0 CHAPTER 5 MANUSCRIPT II

DIGESTIVE AND ABSORPTIVE CAPACITY THROUGHOUT THE GASTROINTESTINAL TRACT OF NURSERY PIGS FROM PARENTS WITH DIFFERENT ESTIMATED BREEDING VALUE FOR FEED EFFICIENCY

5.1 Abstract

Digestive and absorptive functions and capacities changes and microbiota adaptations are often reported during weaning. However, it is unknown whether these changes are different between nursery pigs from parents selected for feed efficiency (FE) based on different estimated breeding values for feed conversion ratio (EBV_FCR). Therefore, this study investigated the activity of hydrolyses, the gene expression of nutrient transporters, and the expression of thigh junction proteins. Furthermore, digesta samples from duodenum, jejunum, ileum, colon, and cecum were collected from 56-day post-weaned pigs and subjected to microbiota analysis using 16S rRNA gene profiling. Enzyme activity kinetics showed no differences in the maximal activity rates of alkaline phosphatase, sucrase, maltase, and maltase-glucoamylase between the two pig groups. In addition, real time-PCR analyses showed that Na+-glucose co-transporter 1 (SGLT1), System ASC amino acid transporter 2 (ASCT2), Peptide transporter 1 (PepT1), Excitatory amino acid carrier 1 (EAAC1) and sodium-dependent neutral amino acid transporter (B^oAT1), mRNA abundances were not affected by pig group. There were also no significant differences in claudin-1 and ZO-1 protein abundances on the jejunum membrane barrier, suggesting no barrier integrity differences between the two pig groups.

Taxonomic analysis at the phylum level revealed that Firmicutes dominated in jejunum and ileum while *Firmicutes* and *Bacteriodetes* were the dominant phyla in the cecum and colon of pigs regardless of genotype. At the genus level, *Lactobacillus* was the most abundant genus in jejunum

and ileum regardless of pig group. *Lactobacillus* dominated the cecum and colon of HE pigs, while *Prevotella*, *Streptococcus*, and *Blautia* were the dominant genera in the large intestine of LE pigs. Richness and diversity were higher in the large intestine than in the small intestine, regardless of the pig group. Microbiota differences at the phylum and genus level and diversity indices suggest that different functional compositions exist between these two pig groups, which needs further investigation.

Keywords: digestive enzymes, nutrient transporters, tight junction proteins, digesta microbiota

5.2 Introduction

Nutritional changes during weaning and the introduction of solid feed with plant-based ingredients are often associated with small intestine adaptations to hydrolyze larger amounts of disaccharides such as sucrose, maltose as well as different sources of starch, including amylopectin and amylose (Lackeyram et al., 2012; Fan et al., 2002). With these solid food nutrients, digestive and absorptive function changes are often reported with weaning. In particular, changes in gut physiology activity and expression patterns of digestive enzymes and nutrient transporters are often associated with weaning (Wellington et al., 2021). Marion et al. (2005) reported increased maltase and sucrase activities in the small intestine associated with weaning. It is unknown if changes in activity and expression of these digestive enzymes in post-weaning piglets are different between pig lines selected for differential FE, and this needs to be investigated. Verschuren et al. (2018) concluded that at least at grower-finisher stages, higher efficiency pigs are associated with better digestive and absorptive functions. However, their study looked at digestibility measurements while differences in digestive enzyme activities or nutrient transporters expression were not investigated. Digestibility differences were not observed by Wu et al. (2022, *in press*) when comparing two pig lines selected for FE based on EBV_FCR, but digestive enzyme activities or nutrient absorption capacity were not measured, and this needs further investigation. Lackeyram et al. (2012) and Fan et al. (1999) reported different activities of specific carbohydrate enzymes among different sections of the small intestine. According to these studies, the maximal specific activity of alkaline phosphatase (ALP) was higher in the duodenum, intermediate in the jejunum, and lowest in the ileum.

Phenotypic growth and FCR responses to their genotype by these two pig groups may also be due to potential differences in the gut microbiota. According to Aliakbari et al. (2021), part of

the variability of the gut microbial community is under genetic control and has genetic relationships with FE, including bacterial diversity indicators. These genetic relationships offer promising perspectives for selecting feed efficiency using gut microbiome composition in pigs (Aliakbari et al., 2021).

Several studies analyzed the link between host genetics, microbiota data, and feed efficiency (e.g., Gardiner et al., 2020; Maltecca et al., 2020; McCormack et al., 2017). In these studies, differences between the intestinal microbiota of groups of animals chosen for their phenotypic residual feed intake (RFI) or FE were reported, suggesting a link between microbial community and FE at the phenotypic level (McCormack et al., 2017, Gardiner et al., 2020). These links are affected by several biotic and abiotic factors. Moreover, these links differ from the GIT section (Adhikari et al., 2019; Quan et al., 2018; Yang et al., 2016.; Gresse et al., 2019). As the review by Gardiner reveals, alpha and beta diversity responses in pig groups of different residual feed intake (RFI) or FE were quite variable among different sections of the GIT. Also, functional capacities of the localized microbiota varied according to gut locations and from mucosal to luminal samples (Mu et al., 2017), and these variations need to be investigated between pig groups from parents of different EBV_FCR.

To better understand the digestive and absorptive functions of the small intestine in post-weaning piglets, the activity and expression patterns of alkaline phosphatase, sucrase, maltase, and maltase-glucoamylase were compared between two pig groups from parents of different EBV_FCR at different sections of the small intestine. Moreover, the expression of nutrient transporters, SGLT1, ASCT2, PepT1, EAAC1, and B^oAT1, were also compared between the two pig groups. Furthermore, in this study and to provide a more comprehensive overview of gut microbiota residing in GIT, we investigated the gut microbiota associated with digesta from

different sections of the small and large intestines of the two pig groups selected for feed efficiency.

5.3 Materials and methods

5.3.1 Animals, diets and experimental design

The experiment and all measurements were approved by the Animal Care Committee of the University of Manitoba (AC#F21-002), and pigs were handled according to the guidelines described by the Canadian Council on Animal Care (CCAC, 2009). The animals, treatment groups, diets, and experimental design used in the present study were described previously by Wu et al. (2022, in press). Briefly, the study included 128 piglets (IBW = 6.87 $\pm$ 0.34 kg ($\pm$SD) obtained from the Glenlea Research Station.

Piglets were from a crossbred of Large White × [Large White × Landrace], from Topigs, Norsvin (Oak Bluff, Manitoba, Canada). The piglets were housed at the TK Cheng Centre at the University of Manitoba (4 animals per 2.88 m^2) with one feeder in each pen and *ad libitum* access to water. From weaning at day 21, piglets were fed 4 times a day a pre-starter diet (NE of 2.58 Mcal/kg: 1.35% SID lysine), and from day 36 to day 49, piglets were fed 4 times a day a starter diet (NE of 2.44 Mcal/kg: 1.20% SID lysine). The high and low efficiency piglets were selected from parents with a low and high estimated breeding value for FCR, respectively. Briefly, high efficiency piglets were obtained by inseminating low EBV_FCR sows (-0.12$\pm$0.011) with semen from low EBV_FCR boars (-0.29$\pm$0.041), and low efficiency piglets were obtained by inseminating high EBV_FCR sows (0.0$\pm$0.027) with semen from high EBV_FCR boars (0.033$\pm$0.045). All EBV_FCRs were provided by Topigs Norsvin based on their routine genetic evaluation. EBV_FCR were provided by Topigs Norsvin based on their routine genetic evaluation.

5.3.3 Hydrolyses activity

The maximal rate of digestive enzymes, including Alkaline phosphatase (ALP), sucrase, maltase, and maltase-glucoamylase, of jejunum, duodenum, and ileum were measured. Enzyme activities are expressed in nmol per mg of protein per min. In addition, alkaline phosphatase (EC 3.1.3.1.) activity was assayed according to established procedures (Hübscher and West, 1965).

Potassium fluoride (2.0 mM) was used to inhibit acid phosphatase activity (Hübscher and West, 1965). The maximal activity rate of jejunal tissue homogenate alkaline phosphatase activity was measured at 37^{o}C for 15 minutes in a final volume of 1 mL containing jejunum tissue homogenate (386.0 μg of protein), 2.0mM KF, 4.0mM MgCl, and 8.0 mM p-nitrophenyl 2 phosphate at pH 7.4. Incubations were stopped by adding 1 mL of 0.25 M NaOH, and a spectrophotometer measured P-nitrophenol at 400 nm. Enzyme activities for sucrase (EC 3.2.1.48), maltase (EC 3.2.1.20), and maltase-glucoamylase (EC 3.2.1.20) were conducted according to previously established procedures adapted from (Koldovsky and Dahlqvist, 1969). Maximal activity rates of sucrase, maltase, and maltase-glucoamylase in the jejunal tissue homogenates (217.0 μg) samples were carried out at 37^{o}C for 30, 20, and 20 min, respectively. Substrate concentrations were 75 mM of sucrose, 75 mM of maltose, and 125 mM of amylose. The pH was maintained at 6.0 for these enzymes, and the glucose liberated by these three enzymes was measured using the Point Scientific Glucose oxidase reagent set, and absorbance was read at 500 nm.

5.3.4 Real-time polymerase chain reaction

Total RNA was extracted using Trizol (Invitrogen, Carlsbad, CA, U.S.) according to the manufacturer's instructions. The total RNA was dissolved in 20 μL RNase-free water and stored at −80 ℃. The RNA concentration was determined using a NanoDrop 2000 Spectrophotometer

(Nano-Drop Technologies, Wilmington, DE, U.S.), with purity (A260/A280) between 1.8 and 2.0. About 1 µg of total RNA from each sample was converted into cDNA using the iScript cDNA Synthesis Kit (Bio-Rad) according to the manufacturer's instructions.

The primers were designed with Primer-Blast (https://www.ncbi.nlm.nih.gov/tools/primer-blast/index.cgi?LINK_LOC=BlastHome) and synthesized by Integrated DNA Technologies, Inc (Table 1). The qPCR was performed to quantify the target genes, such as nutrient transporters. The target genes included System ASC amino acid transporter 2 (ASCT2), excitatory amino acid carrier 1 (EAAC1), Na^+-glucose co-transporter 1 (SGLT1), peptide transporter 1 (PepT1), and sodium-dependent neutral amino acid transporter (B^0AT1). The GAPDH gene was used as the housekeeping gene. The relative changes in gene expression levels of tight junction proteins, enzymes, and nutrient transporters normalized against GAPDH were determined using the $2^{-\Delta\Delta CT}$ method (Livak and Schmittgen, 2001).

Table 5.1. Primers used.

Gene	Amplicon	Sequence (5' to 3')	Reference
CLDN1	220	GGTTGCTTGCAAAGTGGTGTT	Omonijo et al., 2018
ZO1	200	GATCCTGACCCGGTGTCTGA	Omonijo et al., 2018
SGLT1	153	GAGCTGGATGAGGTCCAAA ATCGCCATACCCTTCTG	Yang et al., 2011
PepT1	143	TTCCCATCCATCGTGACATT AGGCCCAGTACATGCTCAC	Omonijo et al., 2018
B^0AT1	102	CATAAATGCCCCTCCACCGT CCAAGGTCCAGGTTTTGGGT	Yang et al., 2011
EAAC1	168	GGGCAGCAACACCTGTAATC GCCAGCAAGATTGTGGAGAT	Omonijo et al., 2018
ASCT2	206	GAGCTGGATGAGGTTCCAAAC ACCTGTCTGTCCACGTTGT	Yang et al., 2011

CLDN1: Claudin-1, ZO-1: zonula occudens-1; SGLT1: Na^+-glucose cotransporter 1; PepT1: Peptide transporter 1; ASCT2: Neutral amino acid transporter 2; EAAC1: Excitatory amino acid transporter 1; B^0AT1: Neutral amino acid transporter.

5.3.5 Western blot

Proteins were extracted from jejunum tissues using RIPA Lysate Buffer (Sigma-Aldrich). Moreover, the protein concentrations were determined by the BCA Protein Assay Kit according to the manufacturer's protocol (Fisher Scientific). The protein was denatured by adding loading buffer (mixture of Laemmli protein buffer and 2-mercaptoethanol) at 95 ^{0}C for 5 to 10min. Then, 30 µg samples were loaded and separated by electrophoresis in gradient protein gels (Bio-Rad).

Proteins were then transferred onto nitrocellulose membranes (Bio-Rad, Hercules, CA, USA). The membrane was then blocked for 2 h with 5% skim milk in Tris-buffered saline (TBS) at room temperature (RT) and was subsequently immunolabeled in primary antibodies diluted in TBS overnight at 4 °C. Primary antibodies to rabbit polyclonal anti-ZO-1 (1:2000 dilution), anti-claudin-3 (1:1500 dilution) and mouse monoclonal anti-β-actin (1:4000 dilution) from Invitrogen by Thermo Fisher Scientific was used. After washing for 3×10 min with TBST, the blots were incubated with horseradish peroxidase-conjugated goat anti-mouse (1:500 dilution) or goat anti-rabbit IgG (1:5000 dilution) as a secondary antibody for 1 hour at RT, respectively. The antigen-antibody complex was visualized with a Clarity Max ECL Western Blotting Substrate (Bio-Rad), and immunoreactive proteins were visualized using the ChemiDoc^{TM} MP imaging system (2.4.0.03, Bio-Rad). The protein bands were analyzed by Image Lab 6.0 software (Bio-Rad). The β-actin was used as the internal control. Values of target protein were represented as the ratio of the optical density of the protein bands to the density of the respective β-actin band.

5.3.6 Microbiome analysis

5.3.6.1 DNA extraction and 16S rRNA sequencing

Digesta DNA was extracted using the DNeasy PowerSoil Pro kit (Qiagen Ltd., Germany) according to the manufacturer's instructions, and bead-beating was included to lyse the microbial cells. The quantity and quality of extracted DNA were measured using a NanoDrop2000 spectrophotometer (Thermo Fisher Scientific, Waltham, MA, USA) and agarose gel electrophoresis, respectively. The V4 region of the 16S rRNA gene was amplified with universal primers 515F (GTGYCAGCMGCCGCGGTAA) and 806R (GGACTACNVGGGTWTCTAAT).

The full-length readings of the V4 region were obtained using Illumina MiSeq 250-bp paired-end reads. In addition, a 12 bp index was utilized to carry out the single multiplexing phase.

The PCR products were combined, purified, and put into the cartridge of the Illumina MiSeq. Reads were joined using EA-Util's fastq-join script with default parameters, then screened to exclude sequences that contained one or more base calls with a Phred quality score less than 20. A Phred quality score of 20 or higher indicated an accuracy of 99%.

5.3.6.2 Microbial data analysis

Raw sequences were analyzed using the latest version of the QIIME2 platform (version 2021.8), as previously described by Wang et al. (2019). Initial reads were quality filtered, denoised, assembled, and chimeric sequences were removed using Dada2 (Callahan et al. 2016), which generates unique amplicon sequence variants (ASVs). We used the SILVA (version 138) reference database classifier to classify bacterial features with a 99% sequence similarity threshold. Alpha and beta diversities were calculated in QIIME2. To examine the effects of pigs' FE and tissue origin on tissue microbiota, we performed a permutational multivariate analysis of variance (PERMANOVA, with 999 Monte Carlo permutations) based on Bray-Curtis, Unweighted Unifrac distances, and Weighted Unifrac distances matrices with the web-based tool MicrobiomeAnalyst (Chong et al., 2020). Differentially abundant features between pig groups and tissues were identified using linear discriminant analysis (LDA) effect size (LEfSe) analysis (Segata et al., 2011). Only taxa with average relative abundances greater than 0.01% were included in LEfSe. Bar plots were visualized using the MicrobiomeAnalyst http://www.microbiomeanalyst.ca (Chong et al., 2020).

5.3.7 Statistical analysis

All statistical analyses were performed using the SAS program (SAS 9.4 software: SAS Inst. Inc., Cary, NC). Enzyme activity and gene expression data were analyzed using PROC MIXED in a complete randomized design with a pig as the experimental unit for all analyses. The

statistical model included genotype based on EBV_FCR as fixed effect and pig as a random effect. Treatment means were calculated using the LSMEANS statement, and differences among means were separated using the PDIFF option with Tukey's adjustment. Statistical significance and tendency were defined at $P < 0.05$ and $0.05 \leq P < 0.10$, respectively.

For the microbiome data, all parametric data were analyzed using an unpaired Student's t-test, while nonparametric data were analyzed using the Mann-Whitney U test or Kruskal-Wallis test. P values for group comparisons were adjusted with a false discovery rate (FDR) according to Benjamin and Hochberg (1995). The corrected P values below 0.05 were considered statistically different. Data were expressed as means and standard error of the mean (SEM).

5.4 Results

5.4.1 Enzyme activity

The present study measured the maximal transport rate (nmol × mg^{-1} protein × min^{-1}) of alkaline phosphatase, sucrase, maltase, and maltase-glucoamylase. Table 2 summarizes the kinetic parameters measured in duodenal, jejunal, and ileum tissue homogenates. Enzyme affinity was not tested, as only the case with the enzymes saturated with the substrate was considered. There were no differences in the maximal transport rate of the enzymes studied between the two pig groups.

Table 5.2 Enzyme activity of sucrase, maltase, maltase-glucoamylase (MGAM) and alkaline phosphatase (ALP) (nmol/mg per min) in the duodenum, jejunum and ileum (n = 8 for each group).

Gut section	Enzymes	LS Mean HE	LS Mean LE	SEM	*P* value
Duodenum	sucrase	3.5	2.8	1.0265	0.6384
	maltase	115.7	95.2	13.993	0.3190
	MGAM	22.8	14.2	5.251	0.2644
	ALP	29.4	26.5	2.666	0.4396
Jejunum	sucrase	25.0	27.1	5.340	0.7808
	maltase	163.5	174.9	19.813	0.6925
	MGAM	54.4	38.9	10.275	0.3044
	ALP	19.9	19.9	1.405	1.0000
Ileum	sucrase	12.4	11.9	2.022	0.8620
	maltase	83.0	90.5	10.318	0.6141
	MGAM	4.2	6.8	1.6136	0.3056
	ALP	16.5	16.8	1.880	0.8960

5.4.2 Gene expression of nutrient transporters

Real-time RT-PCR analysis of nutrient transporters mRNA abundances in the duodenal, jejunal, and ileum tissue homogenates from weaning piglets of two groups of pigs from parents with different EBV_FCR is presented in Figure 1. Results were normalized using GAPDH expression as a housekeeping control gene in each real-time PCR. Data are presented as means $\pm$ SE (n = 8) in arbitrary units. The Ct values for amplifying GAPDH cDNA were similar between the two pig groups (data not shown). Also, no statistical differences were observed between the two pig groups in the gene expression of the nutrient transporters studied.

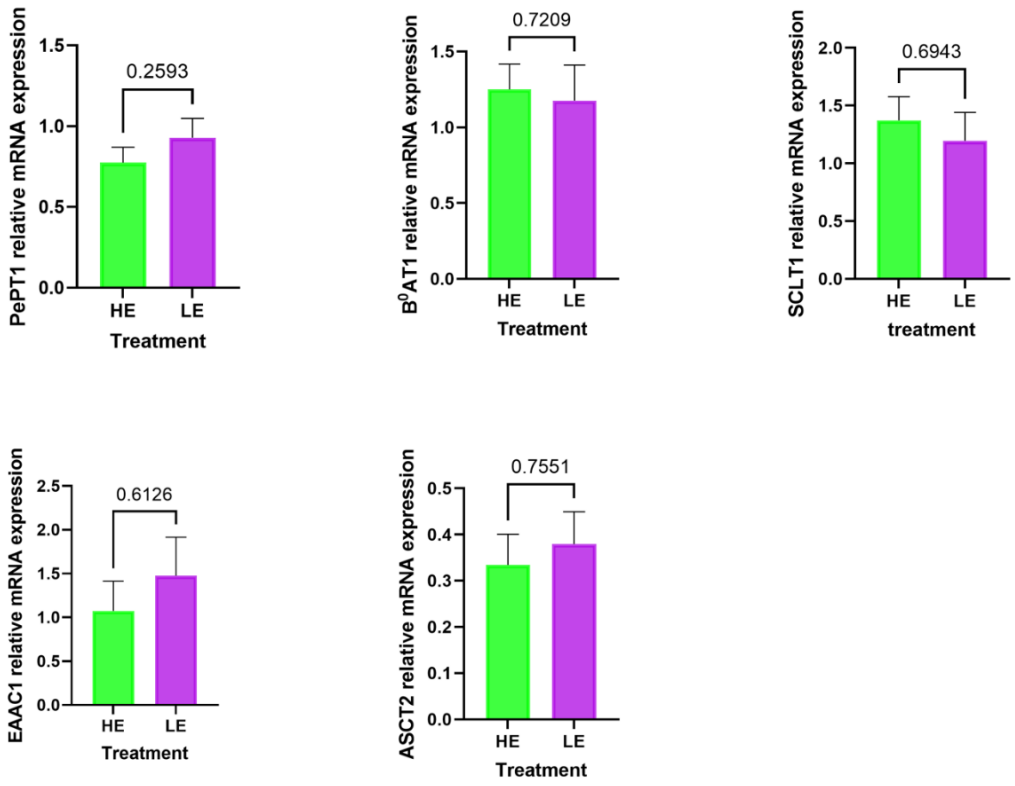
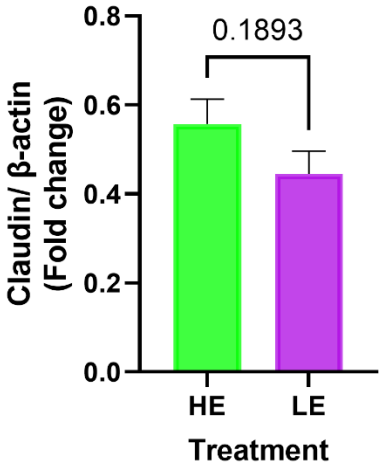


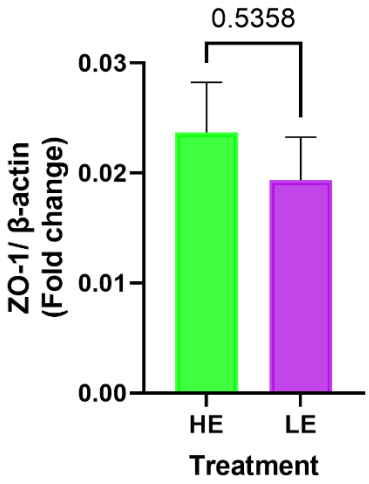
Figure 5.1 Relative mRNA expression of nutrient transporters in jejunum (SEM bars, n = 8). Glyceraldehyde 3-phosphate dehydrogenase (GAPDH) was used as the reference gene. SGLT1: sodium/glucose cotransporter 1; EAAC1: excitatory amino acid transporter 1; ASCT2: glutamine transporter; B^0AT1: neutral amino acid transporter; PepT1: peptide transporter 1.

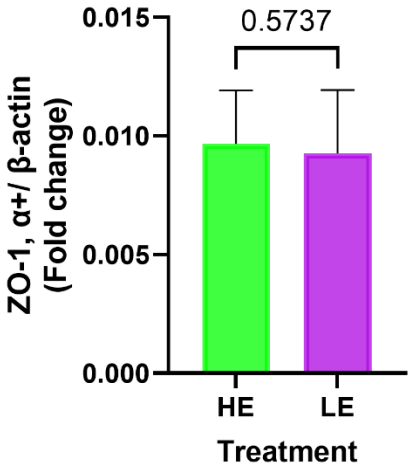
5.4.3 Tight junction protein expression

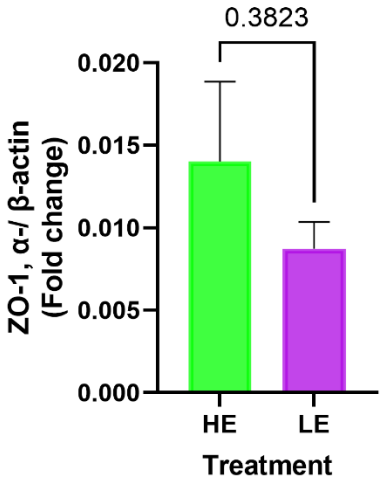
Western blot analyses showed the presence of two ~223 and ~214 kDa for ZO-1 protein bands in total jejunal tissue homogenate (Fig. 5.2A). A protein band ~22 kDa was found for Claudin-1. There were no significant differences in ZO-1 and Claudin-1 protein abundances in the jejunal tissue homogenates (Fig. 5.2B).

(A)


0.1893
0.8
0.6
0.4
0.2
0.0
Claudin/ β-actin
(Fold change)
HE
LE
Treatment


0.5358
0.03
0.02
0.01
0.00
ZO-1/ β-actin
(Fold change)
HE
LE
Treatment


0.5737
0.015
0.010
0.005
0.000
ZO-1, α+/ β-actin
(Fold change)
HE
LE
Treatment


0.3823
0.020
0.015
0.010
0.005
0.000
ZO-1, α-/ β-actin
(Fold change)
HE
LE
Treatment

(B)

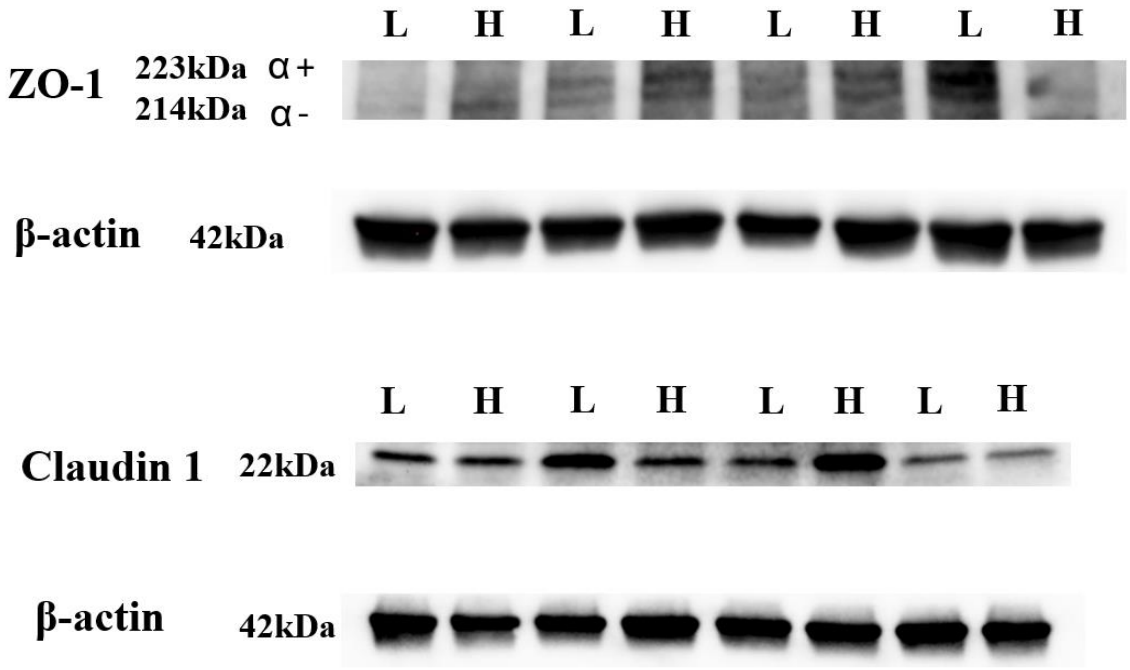


Figure 5.2 Tight junctions proteins expression in jejunum determined by Western Blotting (n=8). Protein bands for ZO-1: zonula occludens-1 and claudin-1 (A); Protein expression is represented as a fold change from the reference β-actin (B)

5.4.4 Digesta microbiota across the GIT

After quality control of the sequencing data with DADA2, there were, on average, 39506 reads per sample (ranging from 20716 to 65309) for a total of 57 samples resulting in 953 ASVs with 2 or more counts. The relative abundances of major phyla differed ($P < 0.05$) among sites, with cecum being similar to colon and different from jejunum and ileum, regardless of pig group (Figure 5.3, Appendix 5 and 6). At the phylum level, the cecum and colon were dominated by *Firmicutes* and *Bacteroidetes*, whereas the jejunum and ileum were dominated by *Firmicutes* ($P > 98\%$) and to a very small extent by *Proteobacteria* ($P < 1\%$). Bacteroidetes were almost nonexistent in the small intestine, whereas this phylum had the highest relative abundance in the cecum of LE pigs accounting for more than 34% of the microbiota in these pigs. Bacteroidetes were significantly higher in the LE pigs compared to HE pigs in the cecum ($P < 0.05$), and they tended to be higher in the colon of LE pigs compared to HE pigs ($P = 0.076$).

Actinobacteria accounted for less than 0.2% regardless of site and pig group, except for a higher relative abundance of this phylum in the cecum of LE pigs compared to HE pigs (0.14 vs. 0.05%, $P < 0.05$). At the genus level, the relative abundances of significant genera differed among sites and between genotypes (Figure 5.3 and Figure 5.4, Appendix 7 and 8). At all the sites besides jejunum with no statistical difference between pig groups, *Lactobacillus* was significantly higher in HE pigs compared to LE pigs. The relative abundance of this genus varied from more than 90% (jejunum of HE pigs) to 37% in the colon of LE pigs. In the large intestine, *Prevotella*, *Blautia*, and *Faecalibacterium* were significantly higher in LE pigs compared to HE pigs. *Prevotella*, for example, was twice more abundant in the cecum of LE pigs (32%) than in the cecum of HE pigs (16%). The same was true for the other two genera, with relative abundances twice higher in LE than HE pigs. *Streptococcus* relative abundances varied from more than 6% to more than 35%, but there were no statistical differences between pig groups or GIT sites. *Roseburia* was present exclusively in the

large intestine, and there were no statistical differences between pig groups or between cecum and colon, which accounted for less than 5% of the total microbiota found in these two GIT sites.

The alpha diversity varied among sites of the digestive tract, with the cecum and colon having the highest richness and diversity than the small intestine, regardless of pig group (Figure 5.5). Bray-Curtis Non-metric multidimensional scaling (nMDS) plot showed a significant difference in bacterial community structure in different locations of GIT of pigs of HE and LE. In this plot, the Bray-Curtis dissimilarity matrix was used, and two clear clusters for the small and large intestine are seen (ANOSIM: R = 0.3476, $P < 0.001$ for all 8 groups, Figure 5.6). PCoA analysis showed that genotype affected the composition of microbiota in the ileum and cecum ($P < 0.05$) and showed a tendency ($P = 0.051$) to affect the composition of the microbiota in the colon between the two pig groups (Figure 5.7).

In order to identify specific bacterial genera characteristics of the two pig groups within each GIT location, we performed an LDA analysis coupled with LDA Effect Size (LEfSe). Figure 5.8 (Appendix 9) shows the genera differentially represented between the two pig groups and GIT sites. *Lactobacillus* (jejunum_HE) and *Prevotella* (cecum_LE) had very high LDA scores. *Blautia* was also identified as an enterotype of the cecum in LE pigs. In addition, V25 and SMB53 were enterotypes of the ileum of LE pigs, whereas genera such as *Megasphaera*, *Ruminococcus*, and *Dorea* were enterotypes of LE pigs' colon.

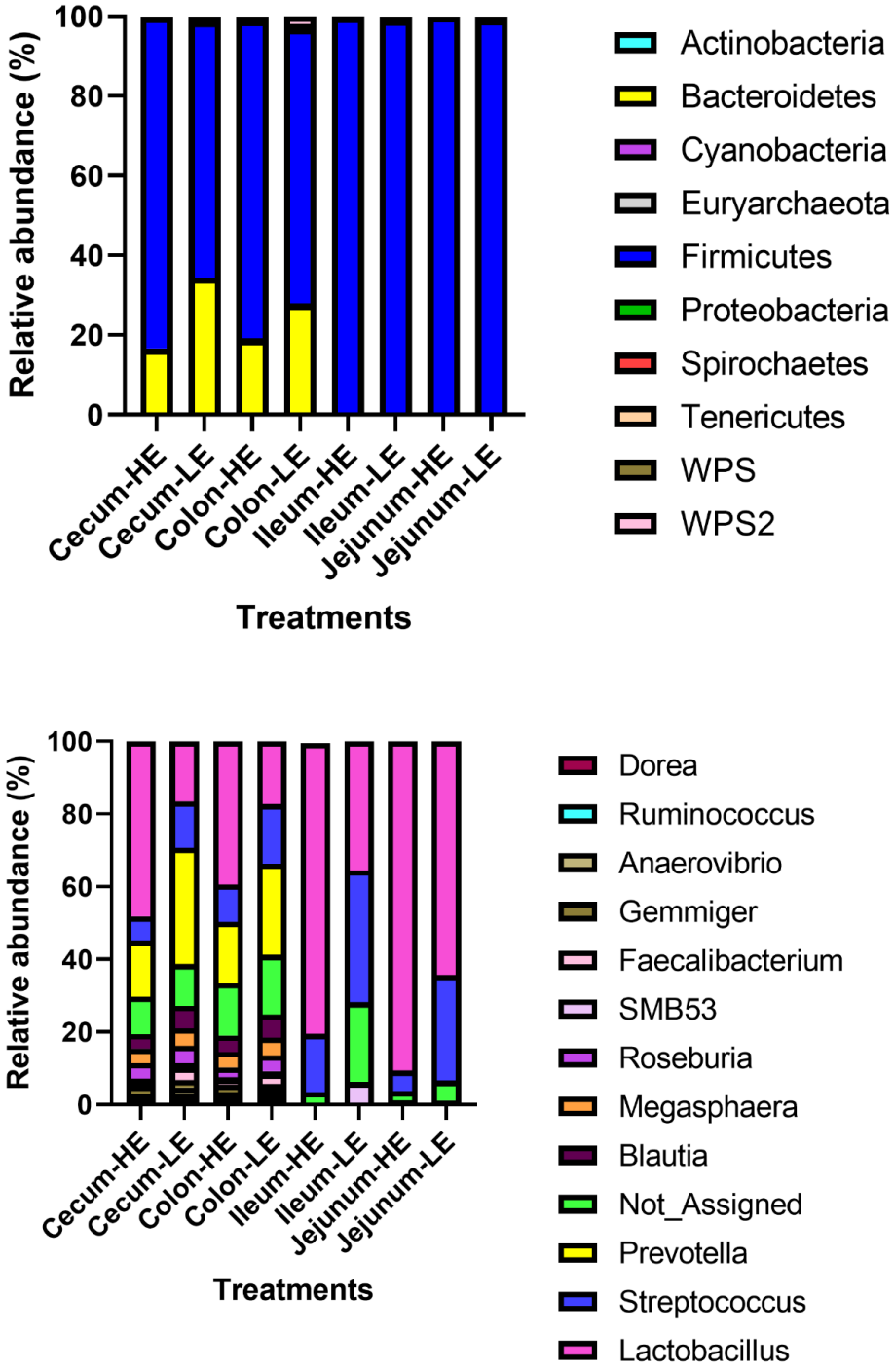


Figure 5.3 Relative abundances (%) of abundant phyla (Top graph) and genera (bottom graph), assessed by treatment (HE vs. LE), for jejunum, ileum, cecum, and colon.

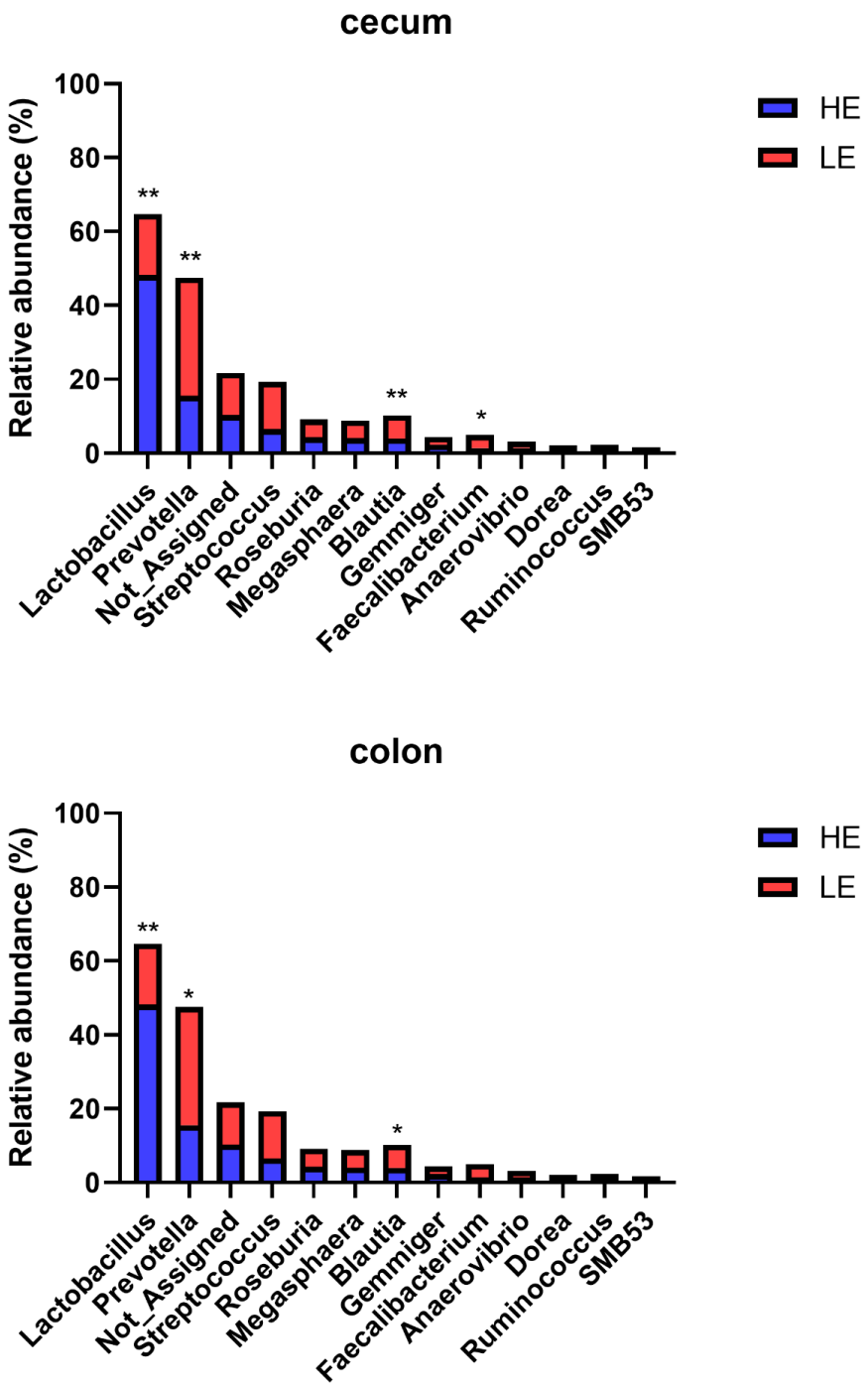


Figure 5.4 Effects of treatment (HE vs. LE) on the most abundant genera of cecum and colon *Relative abundance of genera differed significantly ($P < 0.05$) between treatments, ** relative abundances of genera differ significantly ($P < 0.001$) between treatments.

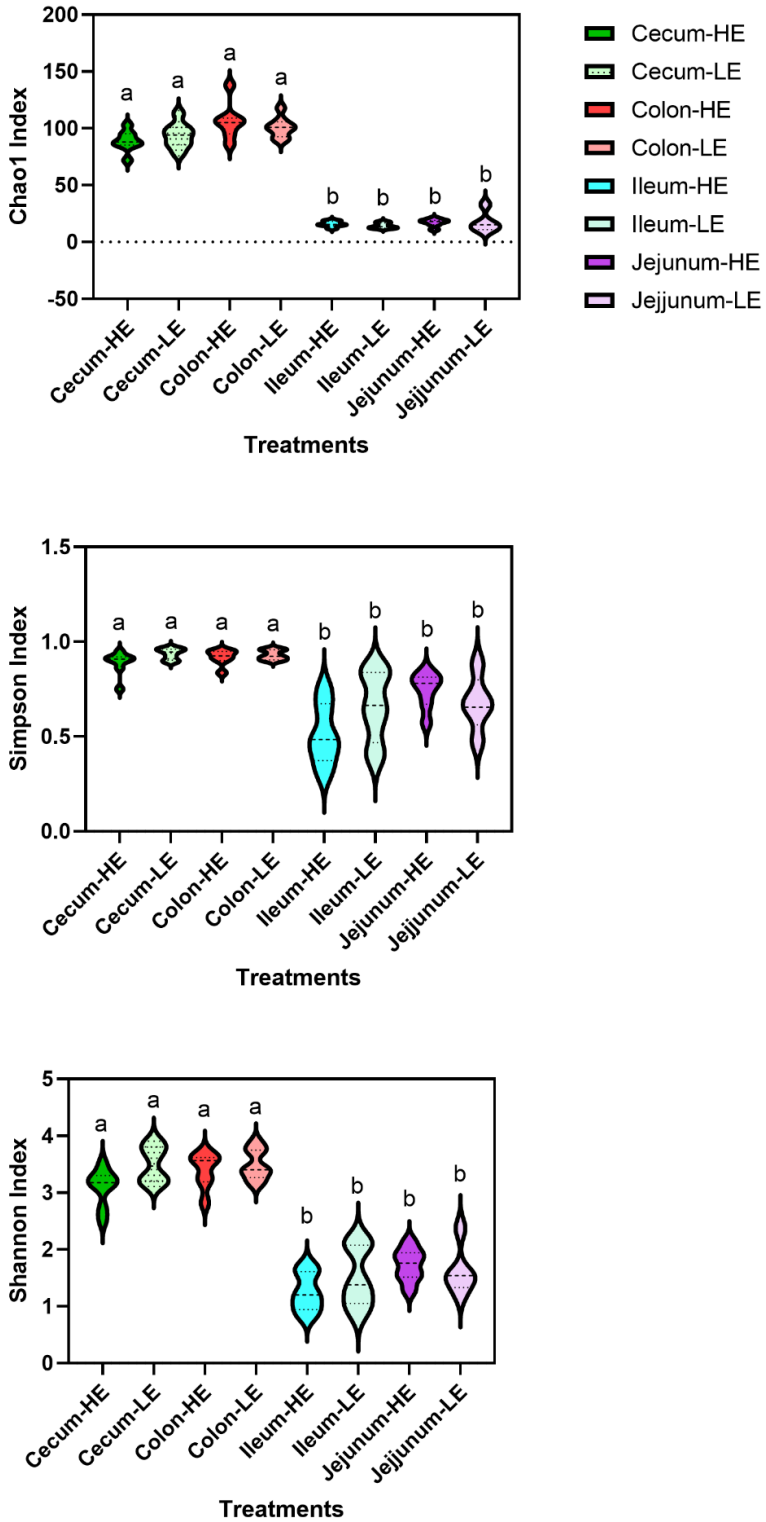


Figure 5.5 Effects of genotype (HE vs. LE) on bacterial richness and diversity indices throughout the digestive tracts of pigs.

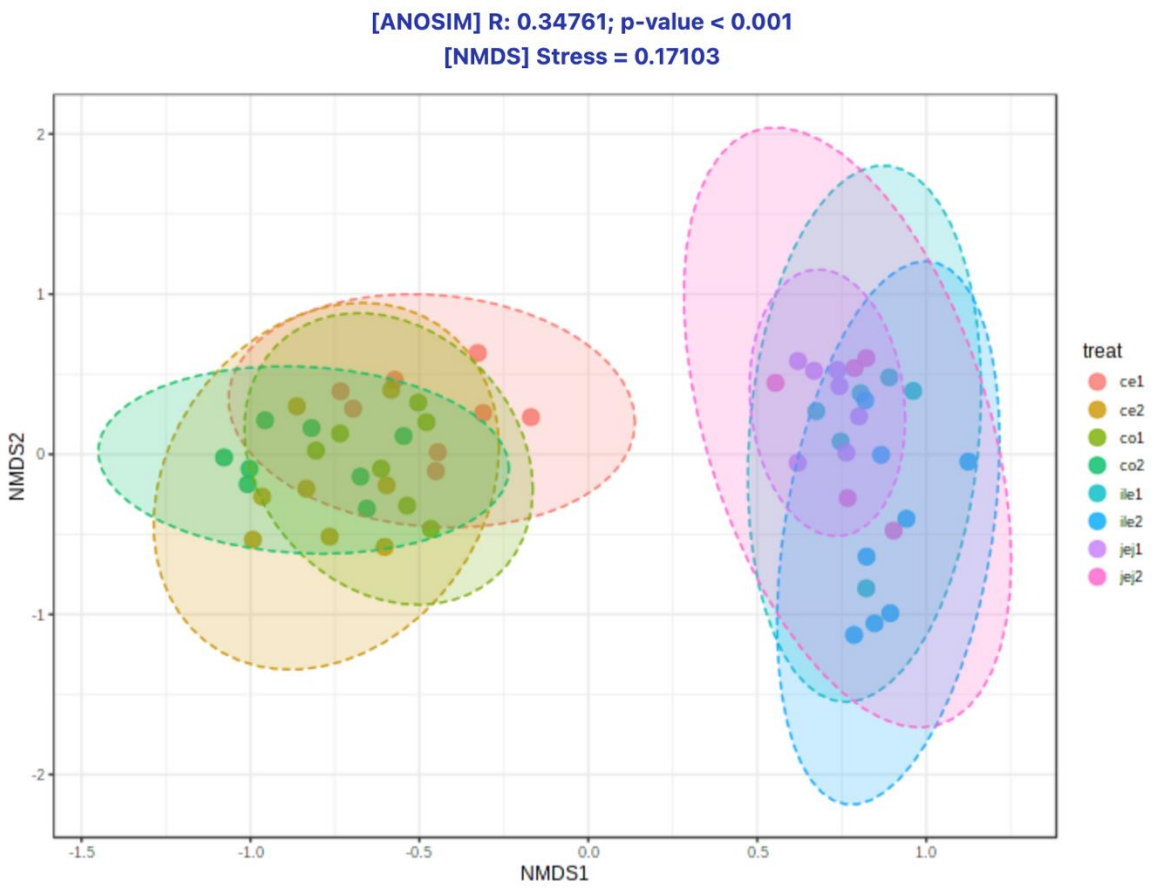


Figure 5.6 Non-metric multidimensional scaling (nMDS) plot showing significant difference in bacterial community structure in different locations of GIT of pigs of HE and LE. Bray-Curtis dissimilarity matrix was used to create the nMDS plot. (ANOSIM: R = 0.3476, $P < 0.001$ for all 8 groups).

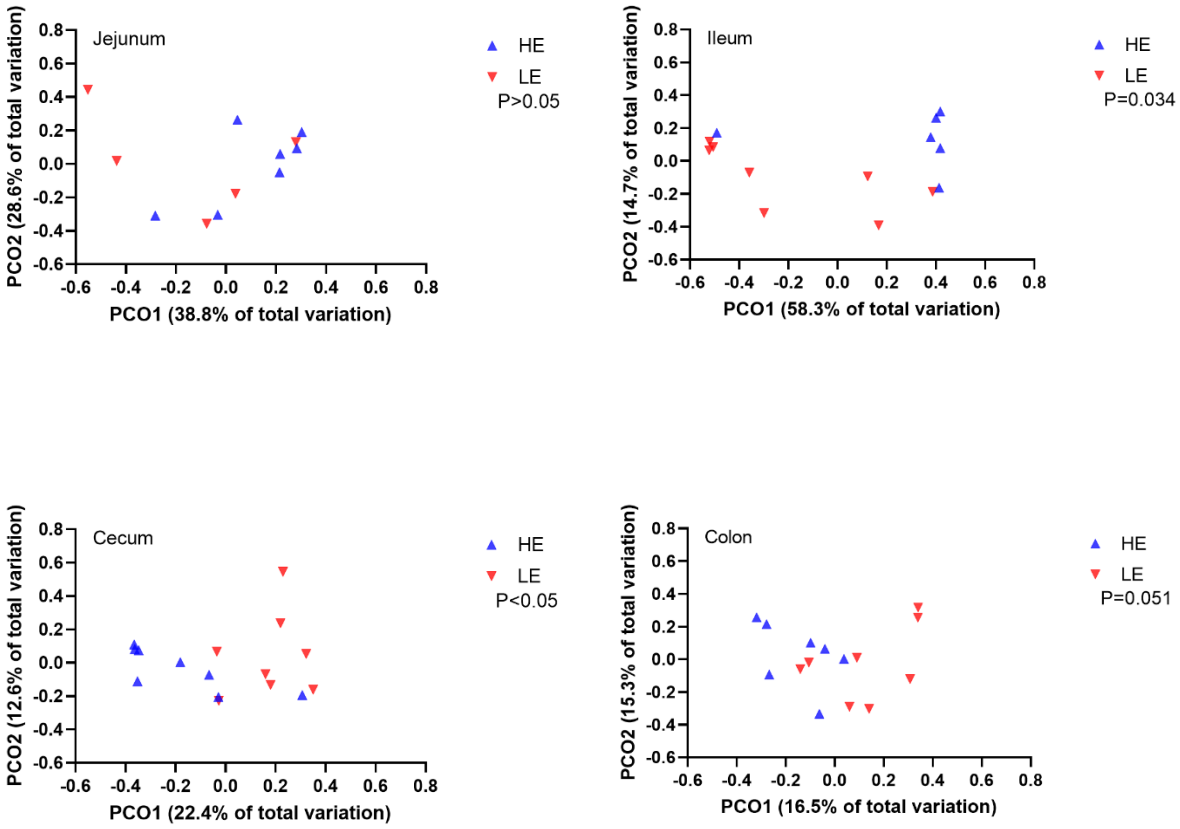


Figure 5.7 Principal coordinate analysis (PCoA) of Bray-Curtis dissimilarities of treatments (HE, LE) by jejunum, ileum, cecum and colon of nursery pigs.

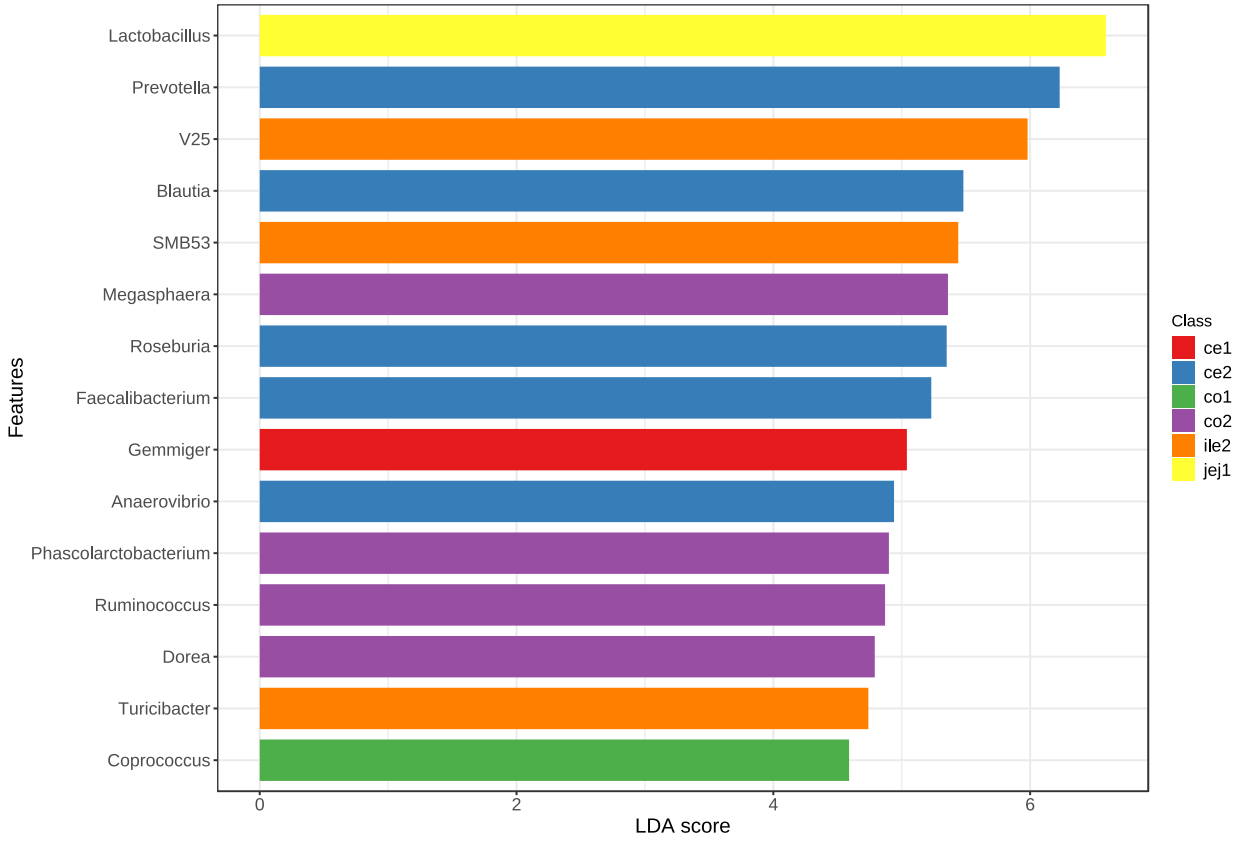


Figure 5.8 LDA analysis of gut microbiota data collected from 2 pig groups selected for feed efficiency and at various GIT locations. Color corresponds to the enterotype in which genus was found to be most abundant. Ce1 = cecum_HE; Ce2 = cecum_LE; Co1 = colon_HE; Co2 = colon_LE; ile2 = ileum_LE; Jej1 = jejunum_HE.

5.5 Discussion

5.5.1 Enzyme activities, nutrient transporters gene expression and tight junction protein expression

Several hydrolases and nutrient transporters are located at the enterocyte brush border and play significant roles in digesting and absorbing nutrients (Wang et al., 2020). In the present study, it was investigated if any differences in the digestive and absorption capacities of the small intestine exist between two pig groups selected for feed efficiency. In a companion manuscript by Wu et al. (2022, in press) from the same study, we found no differences in either feed efficiency or the apparent total tract nutrient and energy digestibility between two pig groups selected for feed efficiency. To gain further insight into the digestive capacities, several hydrolyses' maximal specific activity (V_{max}) throughout the small intestine were determined. Furthermore, the small intestine hydrolysis activities are locally specific, generally decreasing from the jejunum to the ileum (Henning et al., 1994; Fan et al., 1999). It was found that no pig group effects existed, but V_{max} differed among the small intestine segments. For alkaline phosphatase, V_{max} was higher in the duodenum than in the jejunum and ileum. Fan et al. (1999) also found V_{max} to be higher in the duodenal, intermediate in the jejunal, and lowest in the distal ileal brush border membrane. There was a decrease in maximal specific activity of this enzyme as we moved across the small intestine from the duodenum to the ileum.

However, the absolute values in the present study were quite different from ALP values reported by Fan et al. (1999). According to this study, V_{max} varied from 3.5 to 7.7 μmol/mg/min, more than 100 times higher than the ALP results obtained in the present study. On the other hand, ALP activities in the present study were more than 20 times higher than reported by Wellington et al. (2021). It is unclear why these considerable differences among studies, but the pH used in the

activity measurements differed. Fan et al. (1999) used the optimal pH for the enzyme, i.e., pH of 10.5, as opposed to the physiological pH of 7.4 used in our and Wellington et al. (2021) studies. Also, numeric values of kinetic parameters such as V_{max} are specific to the substrate's concentrations used, and these were different among the three studies.

Moreover, the enzyme activity is dependent on the pig's physiological stage. Fan et al. (2002) showed that ALP maximal specific activity was lowest in the adult pig, intermediate in suckling pig, and highest at the weaning and post-weaning stages. Wellington et al. (2021) and the present study were conducted with post-weaning pigs. Lackeyram et al. (2010) concluded that 98% of jejunal mucosa ALP maximal activity was associated with the apical membrane and the remaining 2% existed as intracellular soluble ALP. Therefore, depending on the basis where the activity is measured could explain differences. However, in both studies, ALP was measured on tissue homogenate. Therefore, these differences are not apparent and need further investigation. This enzyme is responsible for hydrolyzing phosphoric ester bonds of organic compounds and playing a major role in fat absorption, among other functions (Zhang et al., 1996). Based on V_{max}, there seem to be no differences in the above functions of ALP between the two pig groups. Nevertheless, the enzyme affinity K_m comparison between the two pig groups and across the small intestine segments needs to be investigated in the future. By measuring the K_m, more insight into the differences across the GIT segments would be gained to know if differences would be due to higher affinities or a higher expression of the enzyme due to more ALP enzyme molecules.

The pig groups did not differ between sucrase, maltase, and maltase-glucoamylase specific activities. However, sucrase maximal specific activity increased as it moved across the small intestine from the duodenum to the jejunum and ileum. These results agree with sucrase activity measured by Fan et al. (1999) in gut luminal homogenates. Furthermore, sucrase and maltase are

capable of hydrolyzing disaccharides (maltose) and trisaccharides (maltotriose), which are two major intermediate digestive carbohydrate products after salivary and pancreatic α-amylase digestion (Van Beers et al., 1995). Therefore, our results suggest no differences in sucrase, or maltase were observed between the two pig groups. Also, the maltase-glucoamylase (MGAM), a carbohydrate enzyme involved in starch digestion and the production of α-dextrins, besides disaccharides and trisaccharides (Semenza, 1986), was not affected by the pig group.

In our study, the specific enzyme activity values were measured at the V_{max} level and not below V_{max} as in this case, the activity would be affected by the substrate concentrations used and cannot reflect changes in the enzyme digestive capacity nor the enzyme protein abundances. Lackeyram et al. (2010) concluded that weaning decreased the V_{max} at the transcriptional level by reducing the steady-state ALP gene mRNA abundance. The sucrase, maltase, and MGA activity measured in jejunum tissue homogenates were much higher in Lackyram et al. (2012) study than in our study, which is surprising as these enzymes usually increase as the pig grows, and the previous authors used neonatal pigs as opposed to post-weaning piglets in our study. Fan et al. (2002) reported a gradual increase in V_{max} of sucrase from suckling through adult stages. Jejunal maltase activity was 2 times higher in our study than in Wellington et al. 2021.

On the other hand, sucrase activity was 2 times lower in our study. These variations in activity response are partly explained by the same factors discussed for the ALP. In addition, it could be due to feed and nutrient intake by the pigs. Wellington et al. (2021) concluded that at 56d, the nutrient level significantly contributed to intestinal function and enzyme activity compared to birth weight when compared to restricted-fed pigs with no restricted-fed ones.

Different nutrient transporters were considered in the present study as a measure of the absorptive capacity of the small intestine, mainly for glucose and amino acids between the two pig

groups. SGLT1 is believed to be the major route for the absorption of dietary glucose across the luminal membrane of pig enterocytes (Moran et al., 2010). Although numerically higher in the HE group, the relative mRNA expression of this transporter was not statistically different between the two pig groups. However, in a study by Vigors et al. (2016) with pigs divergent in feed efficiency based on RFI, it was found that the low-RFI pigs had higher relative gene expression levels of the sodium/glucose co-transporter 1 (SGLT1) in the jejunum.

The relative expression of several amino acid transporters was investigated. Di- and tri-peptides are transported across the apical membrane via the H+-dependent peptide transporter system (PepT1) (Leilach and Ganapathy, 1996) and further hydrolyzed to free AA by enterocytic intracellular peptidases (Fan et al., 2004). EAAC1 is the primary Na+-dependent Glu transporter expressed in enterocytes (Fan et al., 2004). ASCT2 mediates Na+-dependent transport of small neutral AA (Kanai and Hediger, 2004). The preferred substrates of the B^0AT1 are large aliphatic AA, aromatic amino acids, and small neutral amino acids. Results were not statistically different between the two pig groups and were similar to Wellington et al. (2021). Except for this last transporter, all the other amino acid transporters analyzed were numerically higher for the LE group than the HE group. These results suggest that LE pigs required higher gene expression of amino acid transporters to keep similar growth performances as the HE pigs.

Wellington et al. (2021) reported that the sodium-dependent neutral amino acid transporter (B^0AT1) and ASCT2 (glutamine transporter) were downregulated in restricted pigs compared to normally fed pigs. The absence of differences in the relative expression of nutrient transporters between the two pigs agrees with the fact that there were no differences in digestibility and growth performance, and this is most likely due to the diet composition. Changes in nutrient transporters'

duodenal, jejunal, and ileum expression may be observed when less easily digestible ingredients and diets are fed instead of the premium diets used.

In multicellular organisms, such as pigs, absorption and transport across the epithelium of water, ions, and organic molecules is tightly regulated by the intestinal epithelial barrier, which consists of the apical plasma membrane of the enterocytes and the intercellular tight junctions (TJs). TJs are the most apical multiprotein complexes that regulate epithelial permeability and paracellular diffusion (Wang et al., 2014). In the present study, we targeted the TJs' paracellular transport, which forms a continuous, embracing belt between adjacent epithelial cells. The most critical transmembrane proteins are the members of the claudin family, which determine junctional permeability (Zhao et al., 2021). In our study, the protein expression of claudin1 was not different between the two pig groups selected for FE, suggesting that junctional permeability was not affected by the FE genotype group.

Peripheral membrane proteins, such as zonula occludens1 (ZO-1), did not differ between the two pig groups. ZO-1 proteins are crucial for tight junction assembly and maintenance, and our results suggest that these two functions were not different between the two pig groups selected for FE based on EBV_FCR of parent lines. TJs proteins limit intestinal epithelial cell permeability and protect mucosal cells from being exposed to bacteria and toxins (Zhao et al., 2021). Any disruption of these TJs would result in impaired intestinal function, leading to obstructed nutritional absorption and reduced growth performance of pigs. From our study, we can conclude that pigs selected for lower FE did not differ from pigs selected for higher FE in terms of intestinal epithelial cell permeability. This result, together with the lack of differences in digestive enzyme activity and mRNA expression of nutrient transporters, supports the similar growth performance

between the two pig groups reported by Wu et al. (2022, in press), suggesting neither digestion nor absorption was affected by FE selection.

5.5.2 Digesta microbiota across the GIT

In the present study, we investigated the changes in microbiota composition and diversity of digesta samples from different locations of GIT of the two pig groups selected for FE. In a companion study, fecal microbiota was not different between the two pig groups (Wu et al., 2022, in press). Nevertheless, the effect of the pig group on microbiota composition at the various sites of the GIT of these two groups was not known, and it was investigated in this study.

Firmicutes was the dominant phylum all along the GIT sites studied at the taxonomic level, which agrees with other studies (e.g., Gresse et al., 2019; Adhikari et al., 2019; Gardiner et al., 2020; Crespo-Piazuelo et al., 2019; Quan et al., 2018). Despite that, the relative abundances of major phyla differed among sites, with cecum being similar to colon and different from jejunum and ileum, regardless of pig group. Greese et al. (2019), using PcoA analysis, also reported distinct clusters of microbial numbers and diversity between the large and small intestines. At the phylum level, the cecum and colon were dominated by *Firmicutes* and *Bacteroidetes*, whereas the jejunum and ileum were dominated by Firmicutes ($P > 98\%$) and to a very small extent by *Proteobacteria* ($P < 1\%$). The reason for the very small abundance of *Proteobacteria* in the small intestine of pigs in the present study is unclear.

Crespo-Piazuelo et al. (2019) reported much higher abundances of *Proteobacteria* but did not find any *Proteobacteria* in jejunum and ileum. There seems to be a much higher variability among samples in the study of Crespo-Piazuelo et al. (2019), which could explain differences in the *Proteobacteria* between the two studies. *Bacteroidetes* were almost nonexistent in the small intestine, whereas this phylum had the highest relative abundance in the cecum of LE pigs

accounting for more than 34% of the microbiota in these pigs. Crespo-Piazuelo et al. (2019) also reported tiny percentages of *Bacteroidetes* in the jejunum and ileum. *However, Bacteroides* were significantly more abundant in the LE pigs than in HE pigs in the cecum and tended to be higher in the colon than in HE pigs ($P = 0.076$). *Actinobacteria* accounted for less than 0.2% regardless of site and pig group, except for a higher relative abundance of this phylum on the cecum of LE pigs compared to HE pigs (0.14 vs. 0.05%, $P < 0.05$).

At the genus level, the relative abundances of significant genera differed among sites and between genotypes. At all the sites besides jejunum with no statistical difference between pig groups, *Lactobacillus* was significantly higher in HE pigs compared to LE pigs. The relative abundance of this genus varied from more than 90% (jejunum of HE pigs) to 37% in the colon of LE pigs. In the large intestine, *Prevotella*, *Blautia*, and *Faecalibacterium* were significantly higher in LE pigs compared to HE pigs. *Prevotella*, for example, was twice more abundant in the cecum of LE pigs (32%) than in the cecum of HE pigs (16%). The same was true for the other two genera, with relative abundances twice higher in LE than HE pigs. In the studies by Adhikari et al. (2019) and Crespo-Piazuelo et al. (2018), *Lactobacillus* and *Prevotella* were the two major genera reported in the digesta of various GIT sites with *Lactobacillus* positively associated with high feed efficiency and *Prevotella* associated with low feed efficiency. Similarly, Bergamasschi et al. (2020) and Verschuren et al. (2018) also reported a positive association between *Lactobacillus* and feed efficiency, and Gardiner et al. (2020) also reported a positive association between *Prevotella* and low feed efficiency.

Streptococcus relative abundances varied from more than 6% to more than 35%, but there were no statistical differences between pig groups or GIT sites. Aliakbari et al. (2021) and Quan et al. (2018) reported a higher abundance of *Streptococcus* in the high-RFI pigs (low FE) pigs

compared to the high-efficiency pigs. Similarly, in the present study, this genus was numerically higher in the LE pig group, but there were no statistical differences. *Roseburia* was present exclusively in the large intestine, and there were no statistical differences between pig groups or between cecum and colon, accounting for less than 5% of the total microbiota found in these two GIT sites. He et al. (2019) reported a higher abundance of this *Roseburia* in low-RFI pigs (High FE). The reason for these differences is unclear but could be due to the pigs' breeds, diet, and age (Luo et al., 2022) used in both studies. *Prevotella, Blautia,* and *Dorea* were positively associated with LE pigs at different GIT locations. Although *Prevotella* is associated with low feed efficiency pigs, *Blautia* and *Dorea,* on the other hand, have been reported to be associated with high feed efficiency pigs (e.g., Bergamaschi et al., 2020). Differences in the pig breeds, sampling ages, and or diets could be behind the differences between our study and the one by the previous studies. Furthermore, the previous studies sampled feces rather than digesta along the GIT.

Higher microbial diversity is often considered an attribute of gut health (Aliakbari et al., 2021). Aliakbari et al. (2021) reported negative correlations between alpha-diversity metrics and five growth traits, including RFI and FCR. These negative correlations imply that selecting animals for improved FE (lower RFI or FCR) will increase intestinal microbial community diversity. In the present study, the alpha diversity metric varied among sites of the digestive tract, with the cecum and colon having the highest richness and diversity than the small intestine, regardless of pig group, and similar results were reported by other studies (e.g., Gardiner et al., 2020; Crespo-Piazuelo et al., 2019; Quan et al., 2018). In addition, some studies (e.g., Aliakbari et al., 2021; Gardiner et al., 2020) have reported higher richness and diversity metrics in high feed efficiency pigs compared to low feed efficiency pigs. Although this was also true for the present study

numerically, there were no statistical differences in the alpha or beta diversity metrics between the two pig groups.

It is believed that higher diversity in more feed-efficient pigs might be related to better gut health and resilience to feed changes. However, the premium diet used in the present study may have masked some potential statistical effects of the pig group. Bray-Curtis Non-metric multidimensional scaling (nMDS) plot showed a significant difference in bacterial community structure in different locations of GIT of pigs of HE and LE. The Bray-Curtis dissimilarity matrix was used in this plot, and two clear clusters for the small and large intestine are seen (ANOSIM: R=0.3476, $P < 0.001$ for all 8 groups). In addition, PCoA analysis showed that genotype affected the composition of microbiota in the ileum and cecum ($P < 0.05$) and showed a tendency ($P = 0.051$) to affect the composition of the microbiota in the colon between the two pig groups.

The segregation between the small and large intestine compartments was evident from the microbial numbers and diversity indices. This separation between the small and large intestine could be due to differences in the physicochemical conditions and or differences in substrate availability between the foregut and hindgut (Gresse et al., 2019; Kelly et al., 2017). The microbiota composition and diversity differences found between pig groups and GIT sites suggest that functionality differences in the microbiota may exist, which needs further investigation.

5.6 Conclusion

In this study, we found that two pig groups selected for feed efficiency did not differ in terms of carbohydrate and lipid hydrolases activities nor the nutrient absorption capacities based on gene expression results of nutrient transporters. Also, gut barrier integrity does not seem to be a factor behind feed efficiency differences between the two pig genotypes. However, microbiota composition and diversity varied along the GIT, and some differences within each site between

the two genotypes were present, suggesting different microbiota functionalities that need to be further investigated.

6.0 CHAPTER 6 GENERAL DISCUSSION AND CONCLUSION

6.1 General discussion

Feed efficiency could be impacted by many factors, including genetic and environmental factors, such as diet, management, and the animals' physiological and health state (Patience et al. 2015). In this study, there was no significant difference in nutrient digestibility and energy metabolism of nursery pigs selected for feed efficiency. However, the low-efficiency pig group was found to have lower urine energy excretion, which is unclear. Low-efficiency pigs could need more nitrogen in the large intestine for fermentation, therefore maintaining more urea and energy and excreting less urea in the urine. The FHP was higher in the low FE group suggesting a higher maintenance energy requirement by this group compared to the high FE group. NE was also numerically 17% lower for the low FE group but statistically not different from the high FE group. Although these results suggest that high FE pigs had lower maintenance energy requirements and HP and, therefore, a higher NE than the other pig group, these results need to be seen with caution as our RQ values were unrealistically high and, therefore, HP and FHP are not reliable. Future indirect calorimetry with pigs from these genotypes needs to be considered to clarify possible maintenance energy requirements and the NE value of the diet. As HP results are questionable in our study, we could not calculate retained energy (RE) nor RE as protein or fat. Therefore, in the future, carcass composition in terms of protein and lipids analysis should be considered.

There was a trend that high-efficiency pigs had larger hearts and lungs. However, ADFI, ADG, and FCR were similar between the two pig groups at this stage. Therefore, the genetic difference might not be enough for the two groups to have significant differences regarding the growth performance until a later stage, for example, at the grower-finisher or finisher stages.

Another reason could be the premium diet used in this study, which could increase nutrient absorption and digestibility and mask the differences between the two pig groups.

The gut microbiota is critical in pig physiology and homeostasis, particularly in energy harvest, nutrient digestion, and intestinal health, and it is expected to be associated with FE (McCormack et al., 2017). Factors including breed, age, sex, growth traits, and diet could affect the gut microbiota composition of pigs (Wang et al., 2020; Homan et al., 2017). Therefore, the gut microbiota compositions are different in various studies. In our study, there was no significant difference in terms of fecal microbiota composition, and *Firmicutes* and *Bacteroidetes* were found as the most abundant phyla in feces at the nursery stage, which agrees with the other studies (e.g., Crespo-Piazuelo et al., 2019; Quan et al., 2018); *Prevotella* and *Roseburia* were the two major genera, which is similar with the findings in the study of Homan et al. (2017) and Ke et al. (2019). The corn-soybean meal-based diet might be one of the reasons that led to the similar microbiota composition of the two pig groups, as Verschuren et al. (2018) observed no significant correlations between FE and gut microbiota composition in a corn-soybean meal-based diet.

According to the other studies, high-efficiency pigs had higher bacterial diversities (Wang et al., 2021; Gardiner et al., 2020; Si et al., 2020). This study found no significant difference in bacterial diversity at the feature level (ASV level). However, high-efficiency pigs were found to have higher alpha diversity at the genus level in the Shannon index, and there was a trend of beta diversity difference when the phylogenetic distances and the abundance of ASVs were considered. Alpha diversity includes richness and evenness. Although richness was not different between the two pig groups at the feature or genus level, the measure of evenness at the genus level was higher for the high-efficiency group based on the Shannon index.

Prevotella was reported as a potential biomarker, and it is associated with the fermentation of plant polysaccharides (Wang et al., 2021; Ivarsson et al., 2014; Tan et al., 2017). Similarly, we found that the low-efficiency pigs had more *Prevotella* in the feces, and *Chlamydia, Lachnosphira*, *Streptococcus*, and *V4*, which were more abundant in high-efficiency pigs, could also be linked to FE. Furthermore, nitrogen metabolism, amino acid metabolism, and transport system were positively connected to FE of pig's efficiency (Yang et al., 2017; Quan et al., 2020; Jiang et al., 2021), but our present study found similar predicted functionalities, which were dominated by amino acid metabolism and carbohydrate metabolism, among the high and low FE pig groups. In this situation, body composition, growth traits, and RFI should be further investigated as they could affect the microbiome composition and predicted functionalities.

Enzymes in the enterocyte brush border play an important role in nutrient digestion and absorption (Wang et al., 2020). For example, ALP plays an essential role in fat absorption, and sucrase, maltase, and maltase-glucoamylase (MGAM) are essential to carbohydrate degradation (Zhang et al., 1996; Van Beers et al., 1995). In this study, V_{max} of ALP, sucrase, maltase, and MGAM of duodenum, jejunum, and ileum was measured, and no significant difference was found between the high and low-efficiency pig groups. To further understand the digestive capacities, the enzyme affinity K_m should be further investigated in the future.

Gene expression of nutrient transporters is a measurement of the absorption capacity of the small intestine. SGLT1 (nutrient transporter mainly for dietary glucose) (Moran et al., 2010), EAAC1 (primary Na^+-dependent Glu transporter), PepT1 (H^+-dependent peptide transporter) (Leilach and Ganapathy, 1996), B^0AT1 (neutral amino acid transporter); and ASCT2 (neutral amino acid exchanger) were measured in this study, but their relative mRNA expression levels

were found not significantly different between the two pig groups, which indicates that the pigs selected for FE had similar nutrient absorption capacities.

Tight junctions could modulate the epithelial permeability and paracellular diffusion and tight junction proteins could protect the mucosal cells from bacteria and toxins by reducing the permeability of intestinal epithelial (Wang et al., 2014; Zhao et al., 2021). Tight junction Claudin 1 and ZO-1 are essential in controlling junctional permeability and tight junction assembly and maintenance (Zhao et al., 2021). Moreover, no significant difference was found between the two pig groups regarding the tight junction protein Claudin-1 and ZO-1 expression level in this study, which suggests that there was no significant difference in intestinal epithelial cell permeability between the pigs selected for efficiency in this study. The protein expression of other tight junction proteins such as Claudin-3, Occludin, and others such as ZO-2 and ZO-3 should be compared between the two pig groups. Furthermore, transepithelial Electric Resistance (TEER) and cell permeability measurements (e.g., FD4) would help to gain further insight into any potential differences in the gut barrier function of the two pig groups.

This study also determined the gut microbiota composition of jejunum, ileum, cecum, and colon of pigs selected for high and low FE. In both pig groups, cecum and colon were found to have similar microbiome taxa, which agrees with the results of Quan et al. (2020). *Firmicutes* were the two pig groups' major phylum among jejunum and ileum, and *Firmicute* and *Bacteroidetes* were the two most abundant phyla in the cecum and colon. *Bacteroidetes* and *Actinobacteria* were observed to have higher relative abundance in the cecum of the HE pig group at the phylum level. At the genus level, the HE pig group contained more *Lactobacillus* in the jejunum, and the LE pig groups had a higher relative abundance of *Prevotella*, *Blautia,* and *Faecalibacterium* in the cecum and colon. Likewise, *Prevotella* was a potential biomarker for low FE pigs (Gardiner et al., 2020)

and *Lactobacillus* was observed to positively correlate with high FE pigs (Verschuren et al.,2018; Bergamasschi et al., 2020). The alpha diversity of different gut regions tested in this study was significantly different, and the cecum and colon had the highest alpha diversity.

The previous studies found that high FE pigs had higher microbiota diversity than low FE pigs (Aliakabari et al., 2021, Gardiner et al., 2020). However, the HE and LE groups did not have a significant difference in alpha or beta diversity, which could be due to the premium diet ingredients. The PCoA results showed a correlation between genotype and the microbiota composition of ileum and cecum digesta. The link between gut microbiota and genotype could be more precise if the functionality analysis is determined in the future.

6.1 General conclusion

In conclusion, this study indicates that, at the nursery stage, pigs with predicted low and high feed efficiency did not differ in growth performance, nutrient absorption capacity, and gut barrier integrity. The composition of fecal microbiota the predicted functionality of fecal microbiota were similar between the two pig groups; however, the gut microbiome composition varies in cecum and ileum between the high and low efficiency groups. The high-quality diet, age, and growth trait could be the reasons that obscured the genetic effect on the growth performance of nursery pigs. The growth performance of growing and finishing pigs from parents with high and low EBV_FE and the functionalities of gut microbiota in different gut sections need further investigation.

7.0 CHAPTER 7 FUTURE DIRECTIONS

Future directions include:

1) To evaluate the growth performance of growing and finishing pigs from parents with high and low EBV_FCR;
2) To investigate the fecal microbiome composition and predicted functionalities of growing and finishing pigs from parents with high and low EBV_FCR;
3) To explore the microbiome composition and predicted functionalities of different gut regions of pigs from parents with high and low EBV_FCR;
4) To test the body composition, growth traits, and RFI of nursery pigs from parents with high and low EBV_FCR; and
5) To determine the functionality of the gut microbiome in different gut sections.

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Appendix 1. Statistics, *P* values and FDR adjusted *P* values for genotype effects on microbiota at the phylum level.

	P values	FDR	Statistics
Chlamydiae	0.009218	0.1567	197.5
Spirochaetes	0.10164	0.4522	172
Firmicutes	0.10997	0.4522	171
Lentisphaerae	0.12684	0.4522	169
Tenericutes	0.13808	0.4522	168
Bacteroidetes	0.15961	0.4522	90
Deferribacteres	0.19449	0.4723	162
Actinobacteria	0.41755	0.7887	106
Verrucomicrobia	0.47243	0.8031	147.5
Proteobacteria	0.5641	0.8718	144
Synergistetes	0.6166	0.8735	141.5
Fibrobacteres	0.79124	0.9605	135.5
Elusimicrobia	0.82213	0.9605	122
Euryarchaeota	0.88016	0.9605	123.5
Cyanobacteria	0.926	0.9605	125
TM7	0.96047	0.9605	129.5

Appendix 2. Statistics, *P* values and FDR adjusted *P* values for genotype effects on microbiota at the genus level.

	P values	FDR	Statistics
Chlamydia	0.009218	0.5807	197.5
Prevotella	0.056216	0.9564	77
Streptococcus	0.079568	0.9564	175
Lachnospira	0.079654	0.9564	175
Treponema	0.10164	0.9564	172
SMB53	0.1053	0.9564	171
L7A	0.12548	0.9564	169
Mogibacterium	0.1929	0.9564	163
Mucispirillum	0.19449	0.9564	162
CF231	0.22401	0.9564	161
Peptococcus	0.23438	0.9564	160
Acidaminococcus	0.2401	0.9564	159.5
Catenibacterium	0.2503	0.9564	97
Campylobacter	0.25423	0.9564	159
Turicibacter	0.27651	0.9564	155.5
Butyricicoccus	0.29153	0.9564	104.5
Megasphaera	0.30452	0.9564	156
p_75_a5	0.30869	0.9564	155.5
Lachnobacterium	0.31033	0.9564	154.5
Collinsella	0.34067	0.9564	102.5
Dialister	0.34472	0.9564	152.5
Helicobacter	0.36178	0.9564	107
Dorea	0.36567	0.9564	103.5
Victivallis	0.38627	0.9564	150.5
Oscillospira	0.42302	0.9564	150
Coprococcus	0.44504	0.9564	149
Methanosphaera	0.45269	0.9564	148
Gemmiger	0.46767	0.9564	148

02d06	0.4739	0.9564	147.5
Mitsuokella	0.49748	0.9564	146.5
Methanobrevibacter	0.51009	0.9564	145.5
Faecalibacterium	0.51475	0.9564	146
Sutterella	0.53395	0.9564	111
Oxalobacter	0.55751	0.9564	144
Lactobacillus	0.5641	0.9564	144
Eubacterium	0.59765	0.9564	113.5
Asteroleplasma	0.6441	0.9564	115.5
Clostridium	0.65105	0.9564	115.5
Slackia	0.69354	0.9564	138.5
vadinCA11	0.70397	0.9564	117.5
Sphaerochaeta	0.72747	0.9564	118.5
Anaerovibrio	0.75205	0.9564	137
Butyricimonas	0.75458	0.9564	134.5
Succinivibrio	0.78044	0.9564	136
YRC22	0.78061	0.9564	120.5
Fibrobacter	0.79124	0.9564	135.5
Subdoligranulum	0.79379	0.9564	122
Blautia	0.80913	0.9564	135
RFN20	0.80913	0.9564	121
Roseburia	0.80913	0.9564	121
Pseudobutyrivibrio	0.83491	0.9564	132.5
Butyrivibrio	0.85047	0.9564	133.5
Anaerostipes	0.86063	0.9564	123
Defluviitalea	0.86249	0.9564	133
Desulfovibrio	0.86462	0.9564	133
Parabacteroides	0.86529	0.9564	123
Bacteroides	0.91372	0.9876	125
Bulleidia	0.92493	0.9876	131
Ruminococcus	0.95556	1	130

rc4_4	0.9827	1	129
Phascolarctobacterium	0.98518	1	129
Oribacterium	1	1	128

Appendix 3. Statistics, LDA scores, *P* values and FDR adjusted *P* values for genotype effects on microbiota at the genus level

	P values	FDR	H	L	LDA score
Chlamydia	0.009301	0.6604	8716.4	2489	-3.49
Prevotella	0.026172	0.7752	3177000	4107700	5.67
Lachnospira	0.038182	0.7752	45593	28636	-3.93
Streptococcus	0.054589	0.7752	191330	111050	-4.6
V4	0.054589	0.7752	3528400	2931200	-5.48
L7A	0.10807	0.9136	2277.7	1664.3	-2.49
SMB53	0.1372	0.9136	15324	4245.4	-3.74
Peptococcus	0.16889	0.9136	3326.2	2414.2	-2.66
Acidaminococcus	0.17262	0.9136	18478	11871	-3.52
Mucispirillum	0.17538	0.9136	2691.4	737.06	-2.99
Dorea	0.20005	0.9136	19109	26449	3.56
Catenibacterium	0.2278	0.9136	14066	23028	3.65
Treponema	0.2278	0.9136	469320	199980	-5.13
Turicibacter	0.2347	0.9136	850.78	542.85	-2.19
Victivallis	0.25361	0.9136	1369.6	1040.2	-2.22
Desulfovibrio	0.2582	0.9136	5426.8	4894.3	-2.43
Mogibacterium	0.26617	0.9136	4463.1	2925.7	-2.89
p_75_a5	0.2744	0.9136	7733	5648.7	-3.02
Dehalobacterium	0.27922	0.9136	115.21	21.963	-1.68
Butyricicoccus	0.28117	0.9136	1130.7	909.61	-2.05
Lachnobacterium	0.29218	0.9136	7685.4	1705	-3.48
Megasphaera	0.30887	0.9136	106380	82302	-4.08
Oscillospira	0.30887	0.9136	157840	137070	-4.02
CF231	0.30887	0.9136	168150	125850	-4.33
02d06	0.34608	0.9136	15980	16675	2.54

Anaerofustis	0.34686	0.9136	154.93	75.293	-1.61
Coprococcus	0.36571	0.9136	77206	73712	-3.24
Coprobacillus	0.37596	0.9136	256.29	114.48	-1.86
Gemmiger	0.38603	0.9136	51785	41477	-3.71
Campylobacter	0.38603	0.9136	81517	34281	-4.37
Oxalobacter	0.41626	0.9492	1812.9	1445.3	-2.27
Fibrobacter	0.42782	0.9492	33774	38656	3.39
Collinsella	0.44594	0.9583	1572.7	1876.7	2.18
vadinCA11	0.4589	0.9583	3168.5	5175.6	3
Helicobacter	0.47382	0.9612	2812.3	2693.3	-1.78
Lactobacillus	0.49752	0.9634	364760	366330	2.9
Eubacterium	0.52171	0.9634	22164	25964	3.28
Sutterella	0.52171	0.9634	27767	28877	2.74
Slackia	0.55842	0.9634	498.68	384.88	-1.76
Dialister	0.57452	0.9634	10165	10562	2.3
Methanobrevibacter	0.58181	0.9634	4269.1	1755	-3.1
Bacteroides	0.59521	0.9634	1135	1007.8	-1.81
Faecalibacterium	0.59775	0.9634	155240	141840	-3.83
Subdoligranulum	0.60117	0.9634	1246.1	2313.2	2.73
Methanosphaera	0.61697	0.9634	919.55	839.44	-1.61
Clostridium	0.62417	0.9634	13649	19590	3.47
RFN20	0.67845	0.9779	106610	121520	3.87
Anaerovibrio	0.70626	0.9779	58719	52375	-3.5
Parabacteroides	0.70626	0.9779	9316.8	10002	2.54
Roseburia	0.70626	0.9779	382150	419080	4.27
YRC22	0.75027	0.9779	1411.8	1842.8	2.34
Pseudobutyrivibrio	0.75462	0.9779	1181.9	1262.7	1.62
Mitsuokella	0.76302	0.9779	24509	21001	-3.24

Ruminococcus	0.76302	0.9779	180480	164210	-3.91
Defluviitalea	0.78769	0.9779	1145	857.04	-2.16
Oribacterium	0.79192	0.9779	7357.2	7222.4	-1.84
Bulleidia	0.79192	0.9779	38516	36668	-2.97
Butyricimonas	0.80629	0.9779	510.68	589.18	1.6
Succinivibrio	0.8211	0.9779	262020	440370	4.95
Phascolarctobacterium	0.85053	0.9779	31413	28340	-3.19
Sphaerochaeta	0.87692	0.9779	1655.2	1539.5	-1.77
Anaerostipes	0.90898	0.9779	2696.3	2456.5	-2.08
Asteroleplasma	0.92435	0.9779	1249.8	1323.6	1.58
Blautia	0.93991	0.9779	50096	51916	2.96
Butyrivibrio	0.93991	0.9779	7216.9	4605.4	-3.12
Bilophila	0.96419	0.9779	15.857	18.957	0.407
Enterococcus	0.96419	0.9779	88.864	93.556	0.525
Shuttleworthia	0.96419	0.9779	592.26	546.95	-1.37
rc4_4	0.96543	0.9779	339.38	601.77	2.12
Anaerotruncus	0.97793	0.9779	1781.1	1284.8	-2.4
Anaerobiospirillum	0.97793	0.9779	325.44	253.09	-1.57

Appendix 4. Statistics, *P* values and FDR adjusted *P* values for genotypes effects on functionality at the genus level.

	P values	FDR	Statistics
Translation ribosomal structure and biogenesis	0.03874	0.4492	183
RNA processing and modification	0.04084	0.4492	73.5
Inorganic ion transport and metabolism	0.19637	0.5915	163
Nucleotide transport and metabolism	0.20987	0.5915	162
Carbohydrate transport and metabolism	0.25423	0.5915	97
Defense mechanisms	0.27033	0.5915	158
Chromatin structure and dynamics	0.27365	0.5915	157.5
Cell cycle control cell division chromosome partitioning	0.28267	0.5915	157
Secondary metabolites biosynthesis transport and catabolism	0.28709	0.5915	157
Signal transduction mechanisms	0.28709	0.5915	99
Cell wall membrane envelope biogenesis	0.30452	0.5915	156
Replication recombination and repair	0.32262	0.5915	155
General function prediction only	0.53915	0.9124	111
Energy production and conversion	0.64202	0.9392	141
Amino acid transport and metabolism	0.72398	0.9392	118
Posttranslational modification protein turnover chaperones	0.72398	0.9392	118
Coenzyme transport and metabolism	0.78044	0.9392	120
Intracellular trafficking secretion and vesicular transport	0.80913	0.9392	135
Cell motility	0.83577	0.9392	122
Lipid transport and metabolism	0.89653	0.9392	132
Transcription	0.89653	0.9392	132
Function unknown	0.98518	0.9852	129

Appendix 5. Statistics, *P* values and FDR adjusted *P* values for treatment (genotype x GIT section) effects on microbiota at the phylum level.

Phylum	*P* values	FDR	Statistics
Bacteroidetes	3.9899E-08	3.9899E-07	47.747
Actinobacteria	4.4721E-05	0.0001191	31.774
Euryarchaeota	4.707E-05	0.0001191	31.654
Firmicutes	4.7627E-05	0.0001191	31.627
Spirochaetes	0.027169	0.054338	15.784
Proteobacteria	0.05822	0.097034	13.627
Tenericutes	0.08616	0.12309	12.469
WPS2	0.21939	0.27424	9.4896
Cyanobacteria	0.27124	0.30138	8.7482
WPS	0.70176	0.70176	4.6568

Appendix 6. Statistics, *P* values and FDR adjusted *P* values for treatment (genotype x GIT section) effects on microbiota at the genus level.

Genus	*P* values	FDR	Statistics
Ruminococcus	3.7116E-08	5.8443E-07	47.908
Prevotella	4.1516E-08	5.8443E-07	47.659
Blautia	5.1066E-08	5.8443E-07	47.198
Dorea	5.7131E-08	5.8443E-07	46.948
Roseburia	6.9575E-08	5.8443E-07	46.509
Oscillospira	9.6269E-08	5.9425E-07	45.785
Anaerovibrio	9.9041E-08	5.9425E-07	45.721
Faecalibacterium	1.1888E-07	6.2413E-07	45.313
Phascolarctobacterium	1.4828E-07	6.9195E-07	44.819
Gemmiger	2.8396E-07	1.1926E-06	43.362
Coprococcus	3.9044E-07	1.4525E-06	42.646
CF231	4.1501E-07	1.4525E-06	42.508
Megasphaera	4.7151E-07	1.5233E-06	42.221
Peptococcus	5.4244E-07	1.6273E-06	41.905
Collinsella	9.1822E-07	2.571E-06	40.715
Eubacterium	2.8866E-06	7.5774E-06	38.111
Mogibacterium	3.5897E-06	8.8686E-06	37.613
Oribacterium	1.2491E-05	2.9145E-05	34.745
Lachnospira	2.3026E-05	5.0899E-05	33.325
Lactobacillus	0.0001284	0.0002696	29.284
Clostridium	0.0001568	0.0003137	28.808
Acidaminococcus	0.0002012	0.0003841	28.213
Catenibacterium	0.0007325	0.0012819	25.086
Methanobrevibacter	0.0008409	0.0014127	24.748
Methanosphaera	0.0009184	0.0014835	24.532
Succinivibrio	0.0012562	0.0019541	23.759
Rothia	0.0035817	0.0053725	21.131
Turicibacter	0.0042799	0.0061985	20.676
Parabacteroides	0.004555	0.0063014	20.517
Desulfovibrio	0.004651	0.0063014	20.463
Mitsuokella	0.016512	0.021672	17.141
Treponema	0.027169	0.034579	15.784
SMB53	0.060613	0.074875	13.51
Campylobacter	0.08616	0.10339	12.469
Streptococcus	0.10009	0.11677	12.014
Butyrivibrio	0.13383	0.15191	11.112
Bulleidia	0.20588	0.22755	9.7056
Dialister	0.23281	0.25071	9.2855
Corynebacterium	0.33512	0.35188	7.9714
rc4	0.63329	0.64873	5.2188
YRC22	0.70481	0.70481	4.6317

Appendix 7. *P* values genotype effects for each of the GIT sections studied on microbiota at the phylum level.

Phylum	Cecum	Colon	Ileum	Jejunum
Actinobacteria	0.016	ns		ns
Bacteroidetes	0.0036	0.0755		ns
Cyanobacteria	ns	ns		
Euryarchaeota	ns	ns		
Firmicutes	0.0023	0.0569	ns	ns
Proteobacteria	ns	ns	ns	ns
Spirochaetes	ns	ns		
Tenericutes	ns	ns		
WPS	ns	ns		
WPS2	ns	ns		

ns - not significant ($P > 0.05$).

Appendix 8. *P* values genotype effects for each of the GIT sections studied on microbiota at the genus level.

Genus	Cecum	Colon	Ileum	Jejunum
Dorea	ns	ns		
Ruminococcus	0.0675	ns		
Anaerovibrio	ns	ns		
Gemmiger	ns	ns		
Faecalibacterium	0.0151	0.0527		
SMB53	ns	ns	ns	ns
Roseburia	ns	ns		
Megasphaera	ns	ns		
Blautia	0.0019	0.0316		
Not_Assigned	ns	ns	0.0249	ns
Prevotella	0.0048	0.0864		
Streptococcus	ns	ns	ns	ns
Lactobacillus	0.0018	0.0059	0.0449	ns

ns - not significant ($P > 0.05$).

Appendix 9. Statistics, LDA scores, *P* values and FDR adjusted *P* values for genotype and GIT sections effects on microbiota at the genus level

Genus	*P* values	FDR	ce1	ce2	co1	co2	ile1	ile2	jej1	jej2	LDAscore
Lactobacillus	0.0001284	0.0002696	16887	5035.5	13764	4894.1	32849	10238	33019	30927	4.15
Streptococcus	0.10009	0.11677	2350.5	4546.9	3564.6	5681.4	6748	18589	1777.7	16115	3.92
Prevotella	4.1516E-08	5.8443E-07	6085.8	13069	7172.7	9393.5	0	2	5	2	3.82
V23	0.000636	0.0011614	3743.6	3997.8	5362.9	5218.6	1248	10307	784.29	2400.2	3.68
SMB53	0.060613	0.074875	205.1	382.8	194.7	215.9	205.2	2837.5	353.43	451.2	3.12
Blautia	5.1066E-08	5.8443E-07	1423.8	2278.2	1677.6	2112.9	0	1.125	4.5714	27.8	3.06
Megasphaera	4.7151E-07	1.5233E-06	1503.6	1788.8	1520.6	1702.4	1.333	0	3.7143	0	2.95
Roseburia	6.9575E-08	5.8443E-07	1557	1581	1004.4	1364.6	0	0	0	11	2.9
Faecalibacterium	1.1888E-07	6.2413E-07	489.6	1309.8	578	927.3	0	0	0	0	2.82
Gemmiger	2.8396E-07	1.1926E-06	828	794.3	739.7	474	0	0	3.4286	18.4	2.62
Anaerovibrio	9.9041E-08	5.9425E-07	470.6	704.5	523	499.4	0	0	1.5714	0	2.55
Phascolarctobacterium	1.4828E-07	6.9195E-07	316.5	490.1	535	570	0	0	0.2857	0	2.46
Turicibacter	0.0042799	0.0061985	78.5	124.9	56.43	30.63	95.5	556.3	3	22.4	2.44
Ruminococcus	3.7116E-08	5.8443E-07	281.8	525	352.7	515.4	0	0	0	0	2.42
Dorea	5.7131E-08	5.8443E-07	288.9	458.1	409	437.6	0	0	0	2.2	2.36
Coprococcus	3.9044E-07	1.4525E-06	235.5	206.4	306.7	267.8	0	0	0	0	2.19
Succinivibrio	0.0012562	0.0019541	13.25	189.6	27.71	289.5	0	0	0	0	2.16
Oscillospira	9.6269E-08	5.9425E-07	66	39	161	92.63	0	0	0	1.4	1.91
Clostridium	0.0001568	0.0003137	107.9	151.6	82.29	136.9	0	74.75	0	0	1.89

Ce = cecum; co = colon; ile = ileum; jej = jejunum;

1= high efficiency pig group; 2= low efficiency pig group.