Identification microflora related to growth performance in pigs based on 16s rRNA sequence analyses

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Abstract

Intestinal microorganisms have been shown to be important factors that affect the growth performance of pigs. Therefore, to investigate the effect of the intestinal microflora structure on the growth performance of pigs, samples from Duroc (n = 10), Landrace (n = 9) and Yorkshire (n = 21) pigs under the same diet and feeding conditions were collected. The fecal microbial composition was profiled via 16S ribosomal RNA (rRNA) gene sequencing. And we also analyzed their growth performance. We found that Duroc and Landrace had significant differences in average daily gain (ADG), feed efficiency ratio (FER), growth index (GI), and 100 kg daily age ($P < 0.05$). Moreover, through the analysis of the intestinal flora, we also identified that there were 18 species of intestinal flora with significant differences between Duroc and Landrace ($P < 0.05$). In order to eliminate the influence of genetic background, the intestinal differential flora of 21 Yorkshire pigs with different growth performance was analyzed. The results showed that there were significant correlations between *Barnesiella*, *Dorea*, *Clostridium* and *Lactobacillus* and pig growth performance. To explore the effect of intestinal flora on the growth performance of pigs at the molecular level, *Lactobacillus*, which has the highest content in the intestine, was selected for isolation and purification, and co-cultured with intestinal epithelial cells. The qPCR was used to determine the effect of *Lactobacillus* on MC4R gene expression in intestinal epithelial cells. The results showed that *Lactobacillus* inhibited MC4R gene expression in intestinal epithelial cells. The results provided useful reference for the further study on the relationship between intestinal flora and pig growth performance.

Introduction

There are a large number and a wide variety of symbiotic bacteria living in the intestines of animals. The number of microbes in the intestines of humans and animals is up to 10^{14}, nearly 10 times the number of animal body cells, and the mass is up to 1.2 kg, which is close to the mass of human liver. These microorganisms include bacteria, archaea, viruses and fungi, among which bacteria are the most numerou(Chen et al., 2015; Shimosato et al., 2015). Intestinal flora can provide nutrients and energy for the body, regulate immunity, antagonize pathogenic microorganisms, participate in metabolism, and even affect host behavior (Collins et al., 2012; Kim et al., 2016)

With the popularization of low-cost and large data "next-generation sequencing" technology, researchers have studied the microbial communities in soil, ocean, fresh water, air and other natural environments and discovered many unknown microorganisms, deepening their understanding of the microbial diversity in nature (Hyeun et al., 2015). Pig gut microbes are mainly distributed in the cecum, the number of microorganisms in intestinal contents per gram to 10^{12} ~ 10^{13} Colony-Forming Units (CFU), composed of 400 ~ 500 kinds of microbes, which mainly bacteroides (8.5% ~ 27.7%) and thick wall door of *clostridium* □□ group (10.8% ~ 29.0%), *clostridium* □ group (25.2%) for the advantage bacterium group (Leser et al., 2002).

The growth performance of animals is closely related to economic benefits, and improving the growth performance of animals is an important research direction in the breeding industry. Studies have shown

that gut microbes are also involved in regulating animal growth. Xin et al. found that *Lactobacillus johnsonii* BS15 could significantly improve the daily weight gain and diarrhea index of piglets, and improve the growth and development ability and disease resistance of piglets to a certain extent (Xin et al., 2020)., Yoshiaki et al found that adding *Enterobacter faecalis* to the diet of weaned piglets can effectively improve the growth performance of pigs, and adding *Enterococcus faecalis* and *Clostridium butyrate* to the diet may have certain effects on the change of intestinal flora structure (Yoshiaki et al., 2019). Niu et al. (2019) found that the bacterial abundance of *Clostridium* and *Turicibacter* in sow intestines was positively correlated with the apparent digestibility of ether extract, *Anaerofustis* and *Robinsoniella* were positively correlated with the apparent digestibility of crude fiber. *Collins bacterium* (collinsella) and *Sutterella* abundance and neutral detergent fiber (neutral detergent fiber) of apparent digestibility were positively correlated.(Yang et al., 2019; Yoshiaki et al., 2019). Li et al. (2019) used Yorkshire group to study the function of gut microbes found that gut microbes can improve nutrient digestibility of fattening pig growth and regulation of volatile fatty acids.

But different breeds in pig intestines are characteristic of the microbes, and as a pig widely used Duroc, Yorkshire and Landrace pigs, they have differences in growth traits. At present, the screening of growth-related microorganisms by comparing the differences of intestinal microorganisms among the three breeds has not been reported. Therefore, in order to analyze the intestinal flora differences of different breeds of pigs and screen out intestinal flora related to the growth performance of pigs, this study first to Duroc, Yorkshire and Landrace intestinal flora were analyzed, and the pig growth performance related to the intestinal flora has carried on the preliminary screening. And then to eliminate the influence of genetic background, this study used Yorkshire group of selected key flora and pig growth performance has carried on the correlation analysis, further from the pig growth performance against the screening of intestinal flora. Finally, the function of the selected key flora was verified at the cellular level. The purpose of this study is to screen out the key microflora related to the growth performance of pigs through the above studies, and to preliminarily explore their functions. It lays a foundation for improving the scientific theory of intestinal flora regulating pig economic characters.

Materials And Methods

Animals and growth performance measurements

First, we explored and identified the diversity of intestinal flora in pig intestines and the key intestinal flora related to pig growth performance forty breeding boars (Duroc, n =10; Landrace, n = 9; and Yorkshire, n = 21) with an average body weight (BW) of 97.97 ± 2.88 kg. Secondly, in order to eliminate the influence of genetic background, twenty-one Yorkshire pigs with an average BW of 96.62 ± 4.20 kg were collected. The three breeds were fed the same diet based on corn and soybean, grown in the same hog pen, and housed in comfortable temperature and humidity.

The BW of each animal was recorded after reaching approximately 20 kg and at the end of the experiment three months later. Body measurement traits, including body length (BL), body height (BH),

chest girth (CG), rump girth (RG), tube girth (TG) and backfat thickness (BT) were measured at the end of the experimental period. Collect and sort out the average daily gain (ADG), feed efficiency ratio (FER), growth index (GI), and 100kg daily age provided by the staff in the feedlot. All the measured data were corrected and analyzed by SPSS 22.0

Sample collection and 16S rRNA gene sequencing

Fecal samples were collected from all pigs via rectal massage at the end of the experimental period and stored in liquid nitrogen. Total genomic DNA was extracted using a QIAamp DNA Stool Mini kit (QIAGEN, CA, Hamburg, Germany) according to the manufacturer's instructions(Yang et al., 2014). The concentration and quantity of DNA was measured using a NanoDrop 1000 Spectrophotometer (NanoDrop, Germany), and the DNA concentration was diluted to 1 ng/μL using sterile water.

16S rRNA gene sequencing was performed by Shanghai Sangon Biotech on Illumina HiSeq 2500. The distinct V3-V4 regions of the 16S rRNA (ribosome ribonucleic acid) genes were amplified using specific primers (forward: GTGCCAGCMGCCGCGGTAA; and reverse: GGACTACHVGGGTWTCTAAT, with barcodes). Polymerase chain reactions (PCRs) were performed in triplicate in a total volume of 30 mL containing 4 μL of each primer, 30 ng of DNA template, 25 μL PCR Master Mix and molecular biology grade water as needed. The following PCR thermocycling conditions were used: an initial denaturation at 98°C for 3 min, followed by 30 cycles at 98°C for 45 s, 55°C for 45 s, and 72°C for 45 s, with a final extension at 72°C for 7 min. The PCR products were purified using Agencourt Ampure XP beads (Beckman Coulter, Inc.) and used to construct libraries. Finally, 24 samples were sequenced using a 250-bp paired-end Illumina HiSeq/MiSeq platform (Illumina, United States) at the Shanghai Sangon Biotech.

Sequence filtering and taxonomic assignments

The Illumina MiSeq™ raw image data were transformed by CASAVA base recognition analysis into the original sequences, known as raw data or raw reads, and the results were stored in FASTQ format. After removing the primer and adapter sequences, the paired reads were merged into single sequences according to the overlap between the paired-end reads. After that, the samples were identified and distinguished according to the barcode sequences to obtain the sample data. Finally, the data of each sample was quality-controlled and filtered to obtain valid data for each sample. USEARCH was used to remove the unamplified sequence regions of the pretreated sequence, after which sequencing errors were corrected (Edgar et al., 2010), and UCHIME was used to identify the chimeras (R. C et al., 2011). Subsequently, we performed BLASTn comparisons for the deleted chimeric sequences and representative database sequences, and the alignment results below a specific threshold were considered to be sequences outside the target region, and the partial sequences were removed.

Statistical analysis

Community diversity within and between groups was assessed using several indices, including the observed species, Chao1 estimator, abundance-based coverage estimation (ACE), Shannon and Simpson

indices, all of which were calculated using mothur (Schloss et al., 2009). The Wilcoxon rank-sum test was used to measure the differences in α-diversity values among the three groups, with $P < 0.05$ considered significant. Using the results of the taxonomic analysis, taxonomic comparisons between one or more samples at each classification level can be obtained. Correlation analysis is a classical method used to analyze the interactions between microorganisms. During the analysis, the species or Optical Transform Unit (OTU) with an abundance of more than 1% or with an abundance ranking in the top 100 were selected for bilateral test. SparCC (Friedman et al., 2012) was used to calculate the correlation coefficient and p value between each community/OTU, and the corrplot package (Wei et al., 2013) in R was used to plot the correlation matrix graph.

Phylogenetic measurements of β-diversity were also estimated using QIIME (Kuczynski et al., 2011). The unweighted UniFrac distance was used for principal coordinate analysis (PCoA) to compare the microbial communities from the three groups. Illustrations were generated using the vegan package in R. Linear discriminant analysis Effect Size (LEfSe*)* is used for the discovery and interpretation of biological markers and characteristics at multiple levels. This analysis uses statistical methods to assess different characteristics of the discovery and significance test, where the software first uses the non-parametric coefficient Kruskal-Wallis (KW) sum-rank test to detect the abundances and characteristics of the significant differences between groups, to identify any association between groups of subgroups. Subsequently, a Wilcoxon rank sum test (unpaired) is used to assess the differences in the feature of the group differences through a consistency check, after which an LDA discriminant analysis is performed to estimate the differences under the influence of the differences in the characteristics of group size. PICRUSt (Langille et al., 2013) was used to predict the functional enrichment from the 16S rRNA gene sequencing data with the Greengenes database. The significant differences between pairs of sample or multiple groups of Kyoto Encyclopedia of Genes and Genomes(KEGG) and Gene Ontology (GO) pathways were measured using STAMP(Parks et al., 2014).

Cell culture and isolation and purification of bacteria

Lactobacilli were isolated from fecal samples by reference to the test methods of Mirelahi M et al(Mirelahi et al., 2009). Then Lactic acid bacteria were cultured and the bacterial solution was prepared according to the concentration gradient of 10^6,$10^7$and 10^8, Store at 4°C, set aside. Subsequently, pig intestinal epithelial cells were isolated and cultured by referring to the method of Deguchi et al., (2006). After cell culture for 24h, the cells were treated with different concentrations of bacterial fluid prepared in the previous stage. After the cells were collected after the cells were co-cultured with the bacterial solution for 12h, RNA was extracted from the cells according to the instructions of the RNA extraction kit. The concentration and quantity of RNA was measured using a NanoDrop 1000 Spectrophotometer (NanoDrop, Germany), then the qualified RNA samples were then stored in a refrigerator at a low temperature for later use. A number of studies have shown that there is a significant correlation between the expression of MC4R gene and growth performance, so MC4R gene is considered as an important candidate gene for regulating growth performance(K. Kim et al., 2000). Based on the above research, MC4R gene was selected as the candidate gene in this study to study the influence of intestinal flora on

its expression. The Takara kit was used for reverse transcription and qPCR to detect the expression level of MC4R gene. CFX_3StepAmp+Melt was used for qPCR. The HMBS was selected as the internal reference gene.

Results

Growth performance analysis of three breeds of pigs

By comparing the growth performance of the three different pig breeds, the results showed that the GI (96.42), ADG (0.746 kg) and BL (101.1 cm) of Duroc pigs were significantly lower than those of the Landrace pigs (GI=118.17, P = 0.021; ADG=0.864 kg, P = 0.014; and BL =108.8 cm, P=0.027, respectively) (Figure 1). These three traits of Duroc pigs were also lower than those of Yorkshire, although the differences were not significant (P > 0.05). In contrast, the FER (2.489) and the days required to reach 100 kg (180.5 days) for the Duroc pigs were significantly higher than those observed for Landrace pigs, respectively (Fig.1). As for the other indicators, no significant differences among the three breeds were observed (Table S1).

Bacterial diversity and composition in three pig breeds

A total of 160,497 valid sequence reads were generated, after which OTUs with a 97% identity cut-off were then identified in the Duroc (1,222), Landrace (1,372), and Yorkshire (1,311) pigs (Table 1). Yorkshire pigs exhibited the highest Shannon diversity index (4.19) and had a more diverse bacterial community compared to the Landrace (4.13) and Duroc (3.75) pigs. For the ACE and Chao1, those observed for Duroc pigs (2,704.33 and 2,118.08, respectively) were lower than those observed for the Landrace (3,027.20 and 2,348.21, respectively) and Yorkshire pigs (2,725.23 and 2,170.14, respectively), showing that the richness of gut microbe species in Duroc pigs was lower than that of the other two breeds. In addition, the coverage for the three breeds was more than 98.8%, suggesting that most of the fecal bacterial diversity was captured (Table 1).

At the phylum level, *Firmicutes* and *Bacteroidetes* dominated the majority of the fecal microbiota regardless of breed, with other phyla including *Actinobacteria*, *Spirochetes*, *Proteobacteria*, *Planctomycetes*, *Fibrobacteres*, *Euryarchaeota*, *Verrucomicrobia*, *Chlamydiae* and *Tenericutes*. However, the bacterial community compositions of the breeds differed. For example, the proportion of the phylum *Firmicutes* was greater in the feces of Duroc pigs (89.72%), than in Landrace (81.13%) and Yorkshire pigs (82.6%) (P = 0.02 and 0.11, respectively). By contrast, the proportion of the phylum *Bacteroidetes* was higher in the feces of Landrace (11.03%) and Yorkshire pigs (7.36%) than in Duroc pigs (3.85%) (P = 0.03 and 0.15, respectively). Similarly, the proportion of the phylum *Proteobacteria* was higher in the feces of Landrace (2.06%) and Yorkshire pigs (2.25%) than in Duroc pigs (0.86%) (p = 0.01 and 0.007, respectively). Finally, the proportion of the phylum *Synergistetes* was greater in the feces of Landrace pigs than in Yorkshire pigs (P = 0.018) (Fig. 2 and Table S2).

At the genus level, *Lactobacillus*, *Streptococcus*, *Lachnospiracea* and *Barnestella* dominated the majority of the fecal microbiota regardless of breed, with other abundant genera including *Terrisporobacter*, *Anaerobacter*, *Treponema*, *Sporobacter*, *Oscillibacter*, *Gemmiger* and *Clostridium*. Although the composition of the intestinal microbiota of the three breeds of pigs was similar, the abundance and proportion of each taxon differed different to some extent, with Duroc and Landrace showing the greatest differences among the three breeds (Fig. 3).

Identification and functional prediction of key intestinal flora that affect pig growth performance

The PCoA results showed that the microbiomes of the Landrace were clearly separated from those of the Duroc pigs. Clustered samples indicate a high species composition similarity compared to separated samples. The Duroc samples were primarily concentrated in the yellow area at the top of the figure, whereas the Landrace samples were primarily concentrated in the light blue area at the bottom of the figure (Fig. 4). Most of the OTUs were shared among the three breeds (2,082), but 1,243, 1,455 and 730 OTUs were specifically observed in the Duroc, Landrace and Yorkshire breeds, respectively (Fig. S1).

Thirteen genera were shown to be significantly differentially represented between the three groups by LEfSe analysis, with 5 being more abundant in Yorkshire, 4 being more abundant in Landrace and 4 being more abundant in Duroc pigs. A cladogram showing the family and genus level abundance is shown in Fig. 5 B. *Coriobacteriaceae*, *Romoboutsia* and *Prevotella* were biomarkers in Yorkshire pigs, whereas *Lactobacillus* and *Dorea* were biomarkers in Duroc pigs, and *Enterobacteriaceae* and *Gammaproteobacterta* were biomarkers for Landrace pigs. Among them, the biomarkers for the Duroc and Landrace pigs differed significantly (Fig. 5 A).

The error chart used to compare differences clearly showed the different of intestinal microbiota between different groups. The following results were drawn from the comparison of intestinal microorganisms at the genus level. The Duroc and Landrace pigs had significant differences in 18 species of gut microbes (P <0.05)(Fig. 6A); the Duroc and Yorkshire pigs had significant differences in 8 species of gut microbes (P <0.05)(Fig. 6B); and the Landrace and Yorkshire pigs had significant differences in 5 species of gut microbes (P <0.05) (Fig. 6C). Thus, it was clear that the Duroc and Landrace pigs had the largest difference in intestinal microbiota composition, whereas the Landrace and Yorkshire pigs had the smallest difference in intestinal microbiota composition. The results showed that the contents of *Methanosphaera*, *Romboutsia*, *Cellulosibacter*, *Prevotella*, *Escherichia*, *Anaerobacterium*, *Parabacteroides*, *Megasphaera*, *Barnesiella* and *Acetanaerobacterium* in the intestinal tract of the Duroc pigs were all significantly lower than those observed in the Landrace pigs ($P < 0.05$), however, the contents of *Dorea*, *Salinispira*, *Clostridium*, *Lactobacillus*, *Bulleidia*, *Defluviitalea*, Pseudobutyrivibrio and *Anaeroplasma* of in the intestinal tract of the Duroc pigs were all significantly higher than those observed in the Landrace pigs ($P< 0.05$) (Fig. 6A). Similarly, the contents of *Allisonella*, *Acetanaerobacterium*, *Cellulosibacter*, *Prevotella*, *Hydrogenoanaerobacterium*, *Dialister* and *Terrimonas* in the intestinal tract of the Duroc pigs were all significantly lower than those observed in the Yorkshire pigs ($P < 0.05$), the content of *Salinispira* in the Duroc pigs was also significantly higher than that observed in the Yorkshire pigs ($P < 0.05$) (Fig. 6B).

Finally, the contents of *Cloacibacillus*, *Gallicola*, *Schwartzia* and *Enterococcus* in the intestinal tract of Landrace pigs were all significantly higher than that observed in the Yorkshire pigs (*P < 0.05*) (Fig. 6C and Table S3).

In this study, GO and KEGG were used to analyze the functions and pathways of intestinal microflora in pigs. The results showed thatRNA processing and modification, Inorganic ion transport and metabolism and Biosynthesis of Other Secondary Metabolites, Neurodegenerative Diseases, Digestive System, and Transport And Catabolism, Metabolism, Energy Metabolism, Glycan Biosynthesis and Metabolism. In all these aspects, Duroc pigs were significantly lower than Landrace pigs (*P < 0.05*). However, the functions and pathways of Duroc pig Replication, recombination and repair and Translation, Nervous System, Replication and repair, Cell Growth and Death, Xenobiotics Biodegradation and Metabolism were significantly higher than those of Landrace pigs (*P < 0.05*) (Fig. 7C and Fig. 8A). In addition, the functions and pathways of Energy production and conversion, Biosynthesis of Other Secondary Metabolites, Energy Metabolism and Metabolism of Duroc pigs were significantly lower than those of Yorkshire pigs (Fig. 7B and Fig. 8B). Finally, it is worth noting that the intestinal flora of Yorkshire and Landrace pigs showed no significant difference in function and pathway (*P > 0.05*) (Fig. 7C and Fig. 8C).

Analysis of intestinal flora diversity of Yorkshire pigs with different production performance.

In this project, the growth performance of Yorkshire pigs was first measured. After screening the individuals with significant differences in production performance, the intestinal flora diversity was measured and the differences were analyzed. The different flora was compared with the different flora screened out in the previous experiment. The key bacteria that may be related to the growth performance of pigs were further screened out. Firstly, intestinal flora of individuals with significant difference in ADG was analyzed. The results are shown in Figure 9A, there were 6 significantly different microflora in the intestines of the high ADG group and the low ADG group (*P < 0.05*), that *Dorea* and *Lactobacillus* in the high ADG group were significantly lower than those in the low ADG group(*P < 0.05*). But *Oscillibacter*, *Flavonifractor*, *Methanomassiliicoccus*, and *Unclassified* were significantly higher than the ADG low (*P < 0.05*). Then the intestinal flora of the significantly different individuals with FER was analyzed, a total of 12 different flora were found(*P < 0.05*)The results showed: the proportion of *Oscillibacter*, *Clostridium XlVb*, *Chlamydia*, *Methanomassiliicoccus*, *Treponema*, *Brevibacterium* and *Paraprevotella* in the intestinal tract of high FER group was significantly higher than that of low FER group (*P < 0.05*), However, *Slackia*, *Asteroleplasma*, *Bulleidia*, *Dorea* and *Parabacteroides* are significantly lower than those of the low FER group. Comparing with the results of previous trials, we identified five key species that may have regulatory effects on growth performance of pigs: *Clostridium*, bullet, *Dorea*, *Parabacteroides*, *Lactobacillus*.

In this research, SPSS was used to analyze the correlation between 5 candidate flora and FER, ADG, GI, 100kg daily age of Yorkshire pigs. The results showed that *Lactobacillus* was significantly negatively correlated with GI and ADG, with correlation coefficients of -0.514 and -0.499, respectively (*P < 0.05*). *Bulleidia* was significantly negatively correlated with ADG, FER and GI, with correlation coefficients of

0.556, -0.526, and 0.695, respectively (*P < 0.05*). *Dorea* was significantly negatively correlated with ADG and 100kg daily age, with correlation coefficients of -0.523 and -0.436, respectively(*P < 0.05*). *Clostridium* was significantly negatively correlated with GI, with correlation coefficients of -0.454 respectively (*P < 0.05*) (Table 2).

Cell level to verify the function of differential flora

Lactobacillus is a relatively common bacterium, and in this study, *Lactobacillus* was identified as a key bacterium that may affect the growth performance of pigs, *Lactobacillus* was selected in this study for functional exploration. After 12 hours of intestinal epithelial cell treatment with Lactobacillus, qPCR was used to identify MC4R gene expression in the experimental group and the control group. The results showed that the concentration of lactic acid bacteria in group 1×10^6, 1×10^7 and 1×10^8 inhibited the expression of MC4R gene, making the expression level of MC4R gene significantly lower than that of the control group (*P < 0.05*). And with the increase of lactobacillus concentration, its inhibitory effect on MC4R gene expression gradually increased (Fig. 10).

Discussion

There are a large number of intestinal microorganisms, and a large number of studies have shown that intestinal flora has a significant regulatory effect on the growth performance of animals. In this study, a similar phenomenon was found by analyzing the relationship between the growth performance of pigs and intestinal microorganisms.

The results of this study showed that regardless of the genetic background, the intestinal microbiota was composed of *Firmicutes* and *Bacteroidetes* at the phylum level, but the abundances and proportions of *Firmicutes* and *Bacteroidetes* in the intestinal tract of pigs of the different genetic backgrounds were different. Previous studies have suggested that genetic effects were significantly correlated with microbiome composition (Hildebrand et al., 2013). Furthermore, other studies have shown that pig breed affects the composition of *Firmicutes*, *Bacteroidetes*, and sulfate-reducing bacteria, which are higher in Chinese native pig breeds than in foreign breeds (L. Yang et al., 2014). *Firmicutes* and *Bacteroidetes* are also the two most abundant phyla in the healthy human gut microbiota, but the ratio of these two phyla varies among individuals (Zhang et al., 2015). A previous study reported similar results for the gut tract of other breeds of pig (Kim et al., 2011). Subsequently, in order to eliminate the influence of genetic background on the results, we studied the intestinal flora composition of Yorkshire pigs with different growth performance and obtained similar results. Another research examined the relationship between the composition of gut microbes and growth rates and fat accumulation and observed that *Sphingobacteria* in the phylum *Bacteroidetes* and *Deltaproteobacteria* in the phylum *Proteobacteria* were abundant in the gut, promoting fat production in some animals (Yang et al., 2016; Zhao et al., 2015). *Bacteroidetes* and *Proteobacteria* also play an important role in the growth performance of pigs(Yang et al., 2016), similarly, we also found that *Bacteroidetes* and *Proteobacteria* were significantly different in the intestines of pigs with different growth performance. In addition, genus level results observed in this

study indicated that Lactobacillus, Streptococcus, *Lachnospiracea* and *Barnesiella* are the core bacterial genera in pig intestines. Previous studies reported that *Prevotella* and *Streptococcus* are the most abundant bacterial genera in the intestines of pigs(Ramayocaldas et al., 2016), which was somewhat different from the results of this study. Furthermore, another study showed that *Prevotella* decreased from 30–4.0% of all bacteria in the guts of pigs the aged, which is related to the gradual increase in the intestinal digestion and absorption capacity of pigs(Kim et al., 2011). Therefore, it was speculated that the increasing intestinal digestive capacity of pigs led to a gradual decrease in the proportion of *Prevotella* in the intestines of pigs, while *Lactobacillus* contributed to the increasing intestinal digestive capacity, leading to the large accumulation of *Lactobacillus* in the intestines of pigs.

We found that the intestinal microbiota compositions of the Duroc and Landrace pigs were significantly different, primarily with respect to *Anaeroplasma*, *Acetanaerobacterium*, *Pseudobutyrivibrio*, *Defluvitalea*, *Barnesiella*, *Megasphaera*, *Bulleidia*, *Clostridium*, *Salinispira*, *Parabacteroides*, *Dorea*, *Anaerobacterium*, *Escherichia*, *Prevotella*, *Cellulosibacter*, *Romboutsia*, *Lactobacillus* and *Methanosphaera*. Similarly, our functional predictions have shown that differences in intestinal flora can lead to differences in function related to the growth performance of pigs. Subsequently, we analyzed the intestinal flora of Yorkshire with different growth properties, and the results showed that *Dorea*, *Lactobacillus*, *Bulleidia*, *Clostridium*, and *Parabacteroides* were again identified with significant differences. Therefore, the above bacteria groups were preliminarily considered to be related to the growth performance of pigs. The subsequent correlation analysis between the growth performance of pigs and the key flora showed that the above flora was indeed the key flora to regulate the growth performance of pigs. Some studies have come to similar conclusions that *Methanosphaera*, *Prevotella* and *Romboutsia* are linked to fat accumulation (Guo et al., 2018), it has been shown that *Escherichia/ Shigella*, *Parabacteroides* and *Megasphaera* have a specific correlation with the growth performance of pigs (Yin et al., 2018). This suggests that intestinal flora does have an effect on pig performance, but the exact mechanism is unclear. There is another point worth noting, *Salinispira*, a bacterium unique to the Duroc gut, was identified in this study. However, because there are currently no reports on the function of *Salinispira* in the intestinal tracts of pigs, this bacterium requires further research.

Previous studies revealed that many species of *Escherichia-Shigella* and *Romboutsia*, which are most abundant in the intestinal tract, contribute to the degradation of glucose and fructo-oligosaccharides(Delgado-Andrade et al., 2017; Gerritsen et al., 2017). In this study, these two bacteria groups were also found to be significantly different in the intestines of Duroc and Landrace pigs, but there was no significant difference in the contents of *Escherichia-shigella* and *Romboutsia* in the intestines of Yorkshire pigs with different growth performance. This suggests that the levels of these two types of gut flora associated with fat accumulation in pigs may be influenced by the genetic background. Many species of Lactobacillus and Streptococcus (the prevalent genera in the colon) contribute to lactic acid production(Ruas-Madiedo et al., 2017).

Another study suggested that the enzymatic digestion and absorption of starch constitutes the predominant functions of the small intestine, while the large intestine primarily functions to ferment non-

starch polysaccharides via bacteria and produces SCFAs, which serve as important nutrients for the epithelium and body tissues (Serena et al., 2008). Lactobacillus plays a key role in this process, indicating that Lactobacillus has an impact on the growth performance of pigs. In this study, it was found that at the genus level, Lactobacillus is the species with the largest proportion in the intestinal tract, and the content of Lactobacillus in the intestinal tract of pigs with different growth performance is significantly different. In addition, there is a negative correlation between the growth performance of pigs and the content of Lactobacillus in the intestines. The higher the content of Lactobacillus, the lower the growth performance of pigs. This conclusion is consistent in the two experiments of this study. The OTUs associated with Streptococcus were associated with lactic acid-producing bacteria, and *Escherichia-Shigella* and *Romboutsia* are associated with glucose degradation and absorption (Delgado-Andrade et al., 2017; Gerritsen et al., 2017). This conclusion is similar to the results of this study and to some extent supports the results of this study. In addition, this study also found that the greater the difference in pig growth performance, the greater the number of significantly different flora in their intestines. But not all differences in gut flora affect function. Many members of these families show a high potential for fermenting various polysaccharides and dietary proteins (Meehan et al., 2014; Su et al., 2014).

In summary, the intestinal flora of Duroc, Landrace and Yorkshire pigs were determined via 16S rRNA sequencing in this study. Through functional prediction of intestinal flora of different pig breeds, the differences in intestinal flora among the different pig breeds was shown to potentially lead to the differences in the growth performance of the pigs, and these results were also verified to some extent in the phenotypic determination results at the beginning of the study. Moreover, we analyzed the correlation between the flora and growth performance in Yorkshire populations, and finally screened out *Lactobacillus*, *Barnesiella*, *Clostridium* and *Dorea* which were significantly related to the growth performance of pigs. On the other hand, this study elucidated the effect of *Lactob acillus* on MC4R gene expression in pig intestinal epithelial cells, providing some references for studying the influence of flora on host phenotypes. These findings can enhance our understanding of the relationship between intestinal flora and the growth performance of pigs and provide a theoretical basis for subsequent studies on the regulation of host growth performance by intestinal microflora on.

Abbreviations

rRNA: ribosomal RNA; ADG: Average daily gain; FER: Feed efficiency ratio; GI: Growth index; BW: Average body weight; BL: Body length; BH: Body height; CG: Chest girth; RG: Rump girth; TG: Tube girth; BT: Backfat thickness; PCRs: Polymerase chain reactions; ACE: Abundance-based coverage estimation; PCoA: Principal coordinate analysis; LEfSe: Linear discriminant analysis Effect Size; KW: Kruskal-Wallis; KEGG : Kyoto Encyclopedia of Genes and Genomes; GO: Gene Ontology.

Declarations

Acknowledgments

None.

Authors' Contributions

XLL and XJL conceived and designed the experiments; YX, XLL and RQ contributed analytical tools; KW and MW analyzed the data; XJL, MW and XLL wrote the manuscript; DD, XH and CL provided the test sample; XJL funded the project. All authors read and approved the final manuscript.

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Availability of data and materials

The sequencing raw data in this study was deposited in NCBI Sequence Read Archive (SRA) under accession number SUB6206792.

Ethics approval and consent to participate

All animal experiments were approved by the Institutional Animal Care and Use Committee of Henan Agricultural University (Permit Number: 11-0085).

Consent for publication

No applicable.

Competing Interests

We certify that there are no conflicts of interest with any financial organization regarding the materials discussed in the manuscript.

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Tables

Table 1. Diversity indices and summary of the 16S rRNA gene pyrosequencing data

Measurement	Yorkshire(n=5)	Duroc(n=10)	Landrace(n=9)
Valid reads	55000	52551	52946
OTUs	1311	1222	1372
Shannon diversity index	4.19	3.75	4.13
ACE estimator of species richness	2725.23	2704.33	3027.2
Chao 1 estimator of species richness	2170.14	2118.08	2348.21
Coverage/%	99	99	98.86
Simpson	0.08	0.12	0.07

OTUs: operational taxonomic units; ACE: abundance-based coverage estimator. Calculations were performed based on the OTU definition at > 97% sequence identity. The numbers shown in the table are the average value for each breed. There was no significant difference in all indicators among the three groups (P >0.05)

Table 2 Correlation analysis between key flora and pig growth performance

Item	Lactobacillus	Parabacteroides	Dorea	Bulleidia	Clostridium
ADG	-0.499*	0.114	-0.523*	0.556*	-0.220
FER	0.159	-0.221	0.038	-0.526*	0.271
GI	-0.514*	0.216	-0.350	0.695*	-0.454*
100kg daily age	0.378	-0.091	0.436*	-0.420	0.089

* indicates significant correlation($P<0.05$), while non-superscript indicates insignificant correlation (P>0.05).

Figures

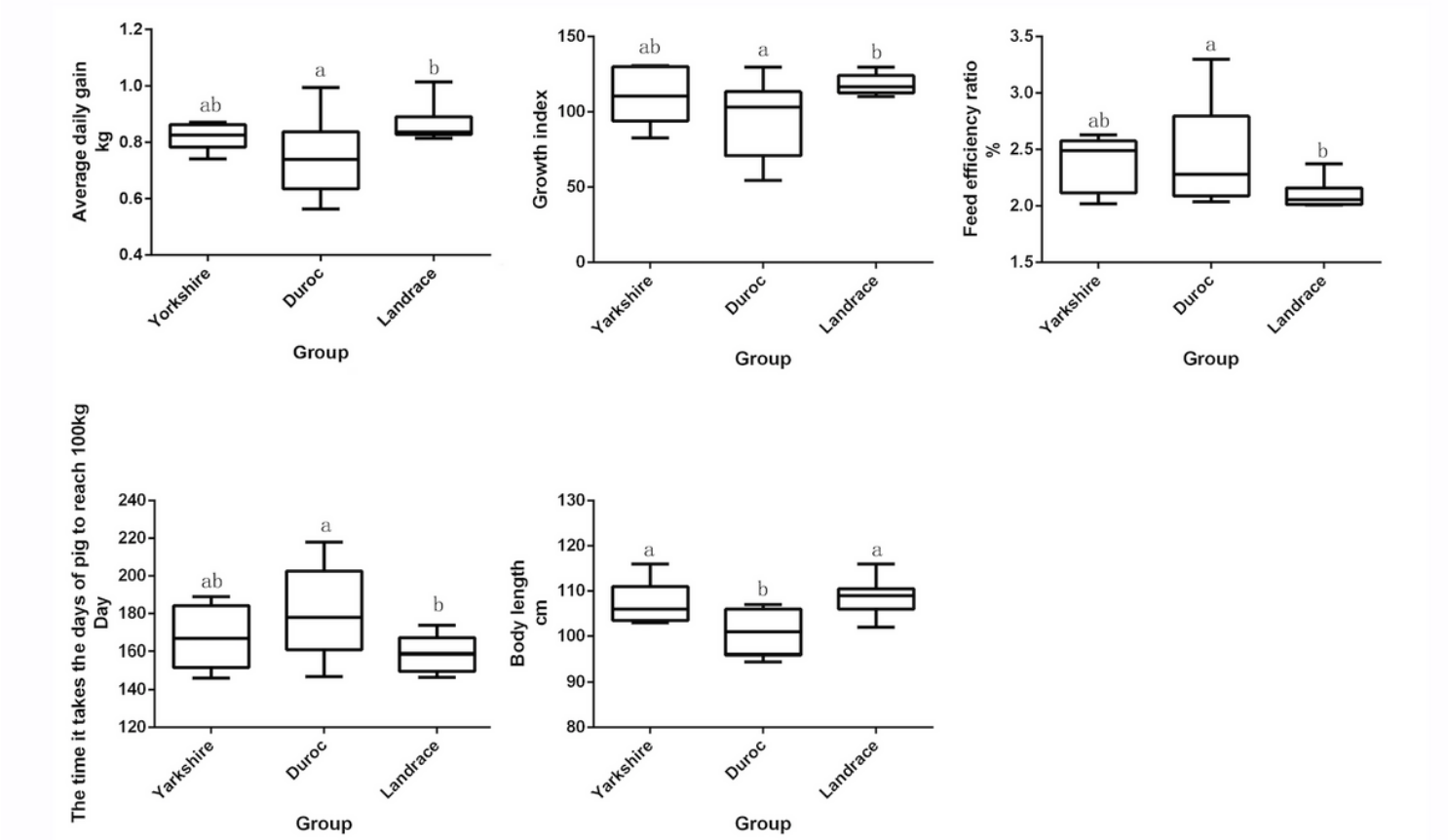

Figure 1

Distribution of growth traits of the three male pig breeds. The error bars represent standard deviations. The middle line in the bar represent the median. ab Means in the same set of data with different superscript letters differ significantly ($P < 0.05$).

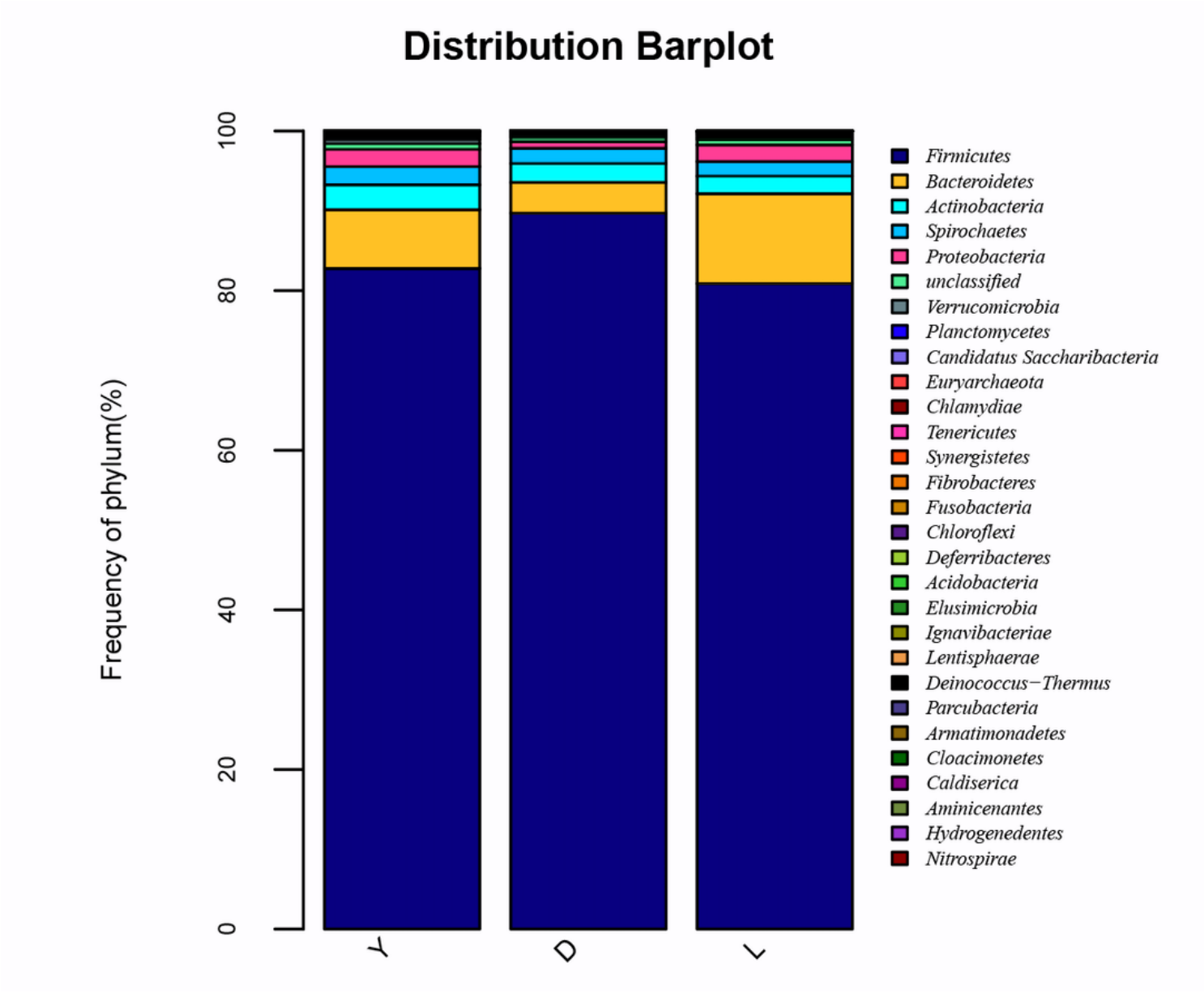

Figure 2

Distribution of bacterial phyla and their abundances in the fecal microbiota of the boars of the three assayed pig breeds. D: Duroc; L: Landrace; Y: Yorkshire.

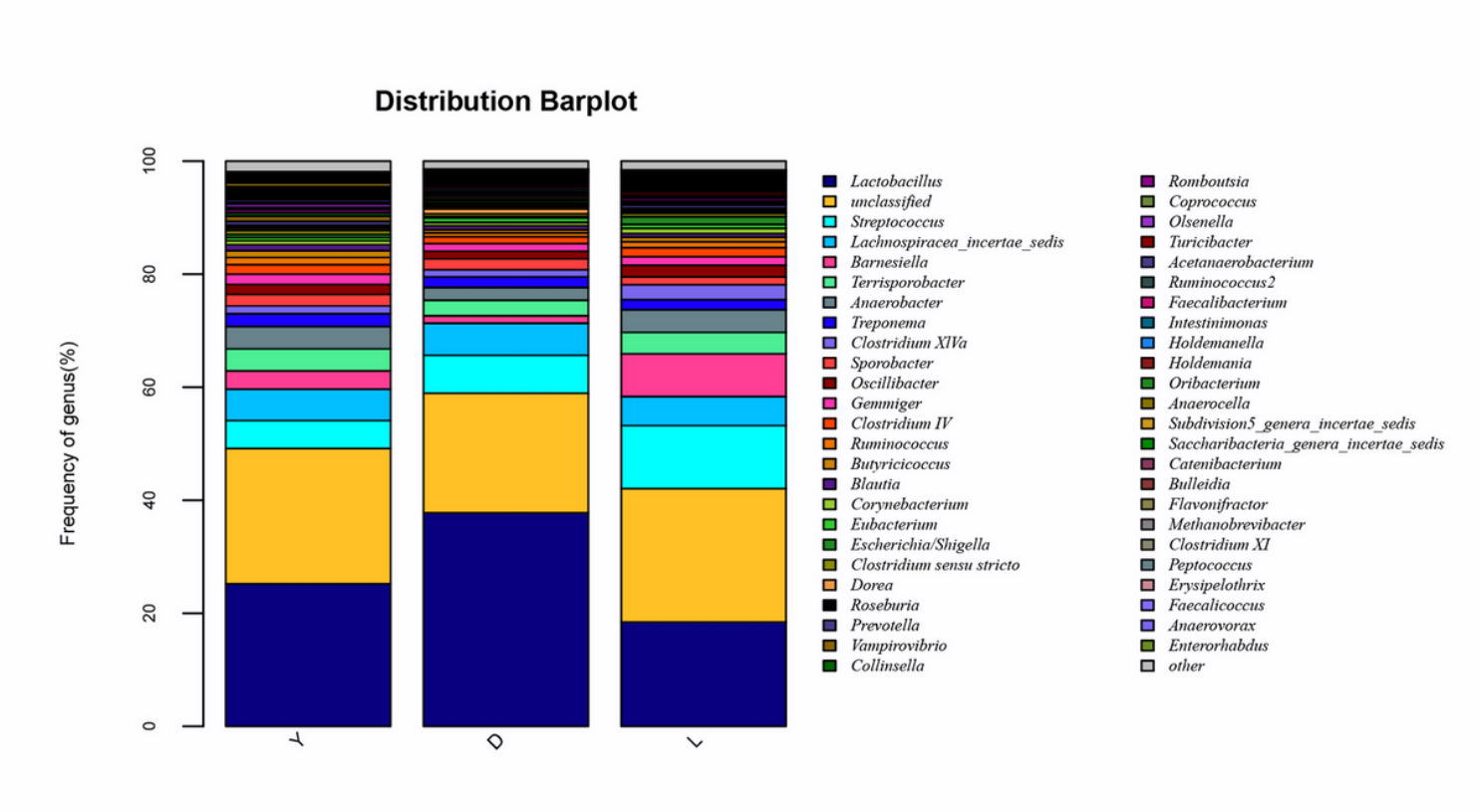

Figure 3

Distribution of bacterial genera and their abundances in the fecal microbiota of the three boar pig breeds.

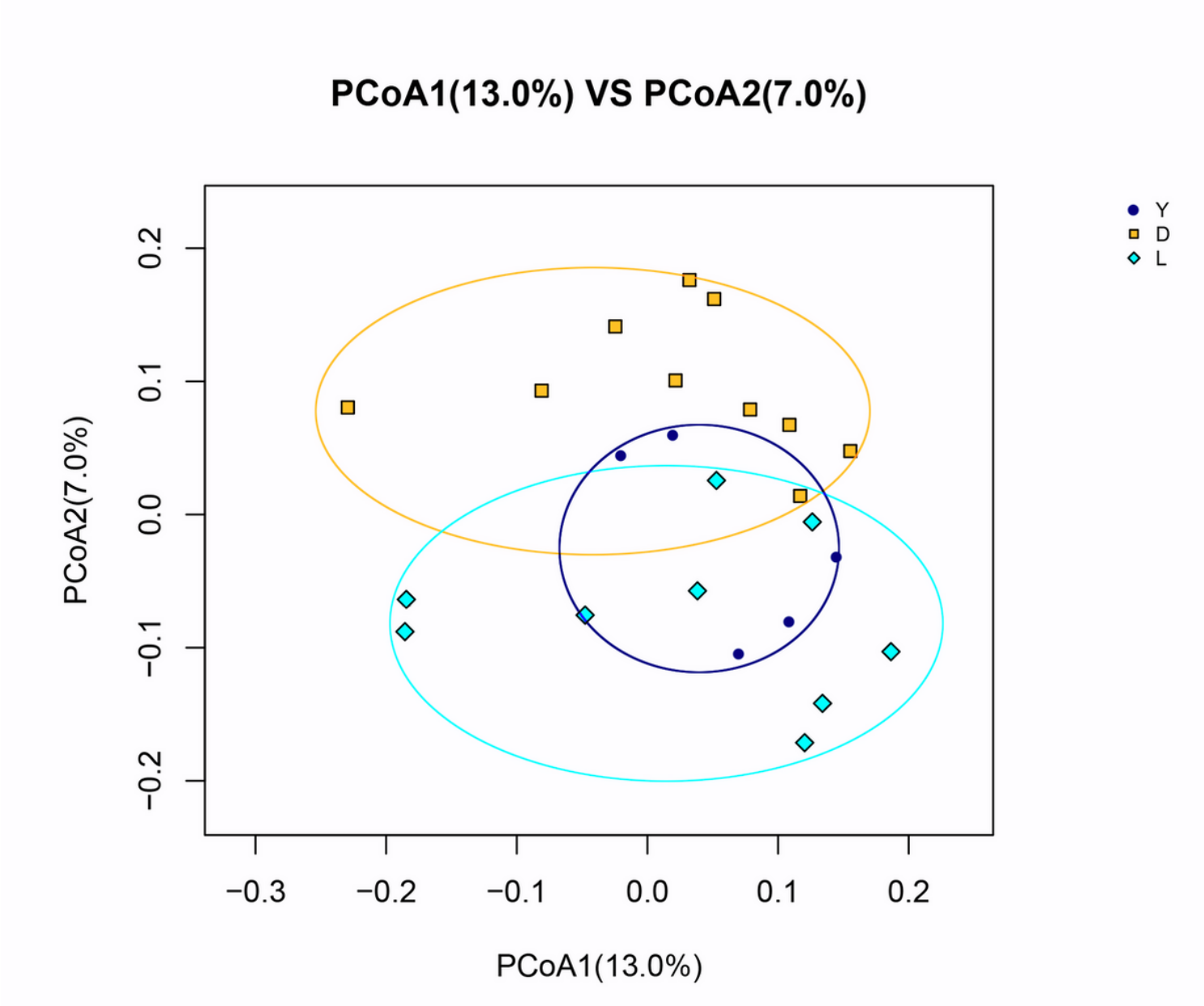

Figure 4

The PCoA analysis based on unweighted UniFrac distances. Each point represents a sample. The first principal component is plotted on the X-axis, and the second principal component is plotted on the Y-axis. The colors indicate different breeds. The percentages shown on each axis indicate the contribution to the discrepancy among the samples.

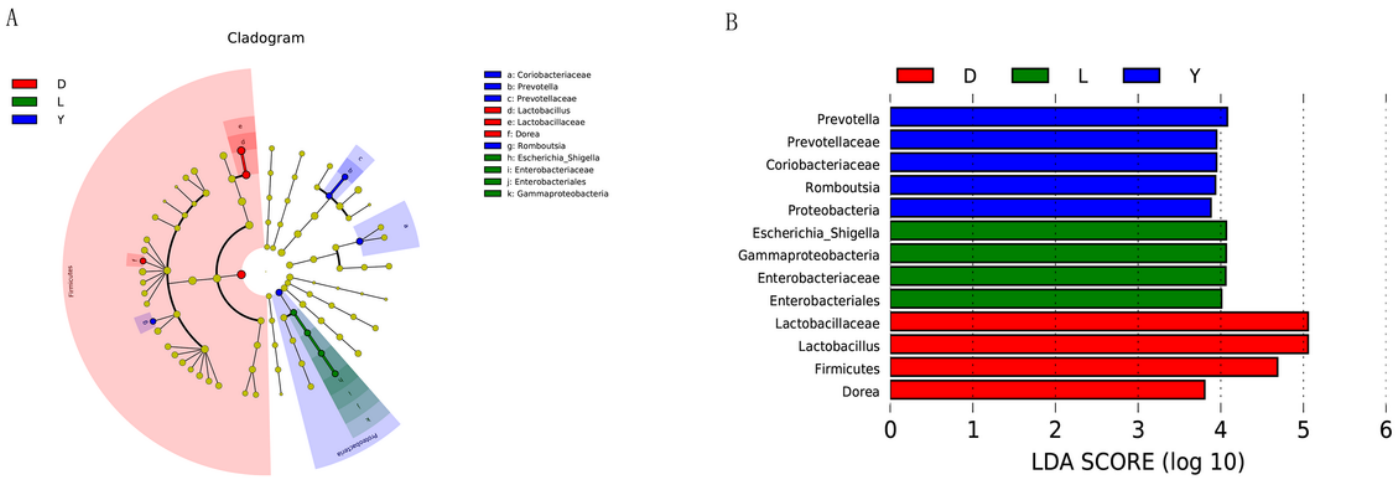

Figure 5

Differential analysis of the bacterial taxa in the intestinal microbiomes of the three groups. (A) Phylogenetic tree of the microbial communities in the three groups. The phylogenetic tree with taxonomic nodes, where the diameter of the nodes indicates the relative abundance, shows the intestinal micromicrobiota of the Landrace, Duroc and Yorkshire pigs. Different groups are labeled with different colors. The red areas indicate that the species of bacteria were more abundant in the Duroc pigs, the blue areas indicate that the species of bacteria were more abundant in the Yorkshire pigs, and the green areas indicate that the bacteria are more abundant in the Landrace pigs. (B) OTUs differentially represented at the genus level in Landrace, Duroc, and Yorkshire pigs, as identified by LEfSe. Histogram showing OTUs that were more abundant in Landrace (green color), Yorkshire (blue color) or Duroc (red color) pigs, ranked by effect size.

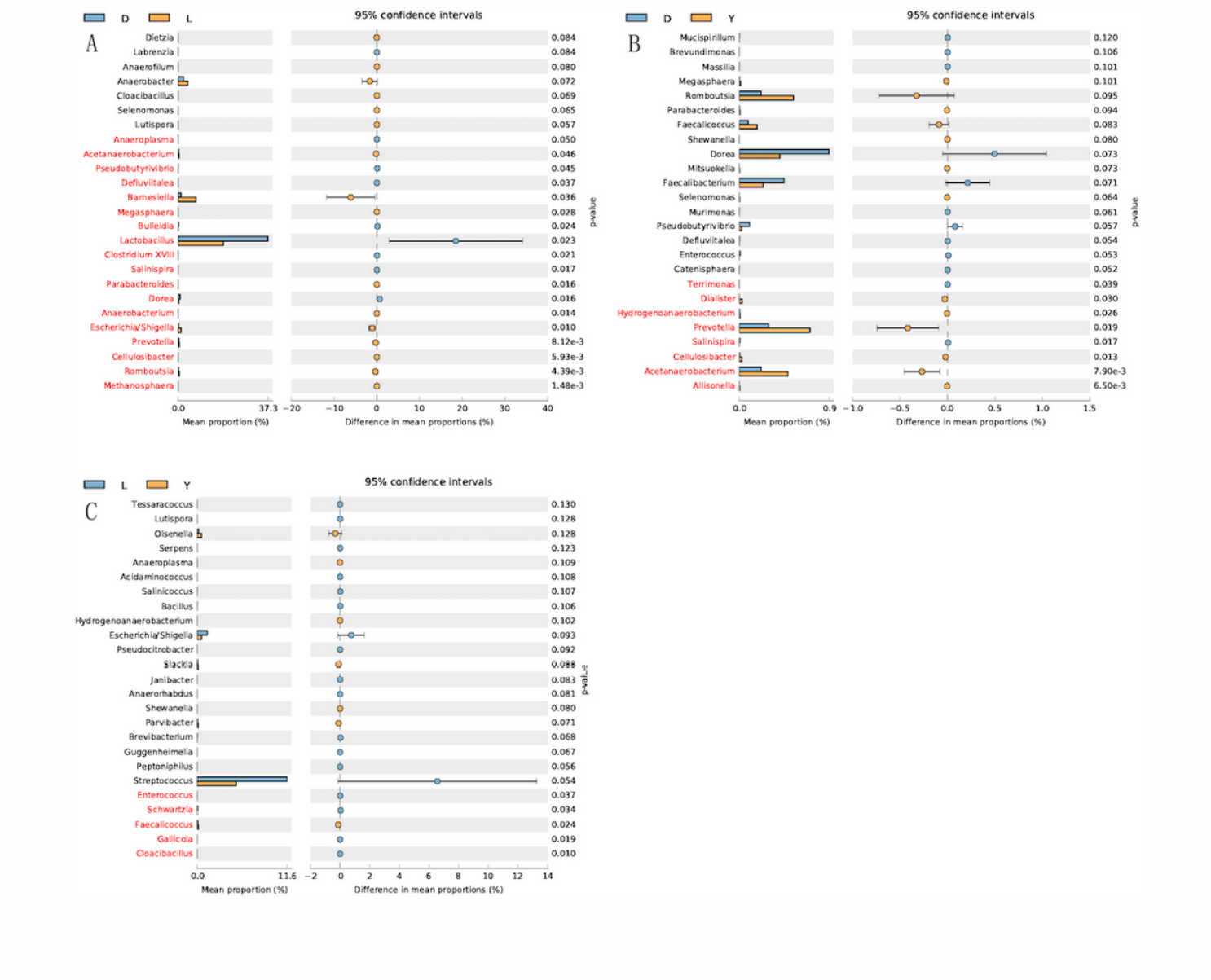

Figure 6

Error chart for the comparison of differences. The left part of the figure shows the abundance ratio of different microorganisms in the two groups, while the middle part shows the difference in the proportion of species classification abundance at the 95% confidence interval. The value on the far right is the p value, where a p value of < 0.05 indicates a significant difference, and species classification is marked in red. Only the 25 with the lowest p values are listed. (A) Intestinal microbial difference analysis for Landrace and Duroc pigs. (B)Intestinal microbial difference analysis of Yorkshire and Duroc pigs. (C) Intestinal microbial difference analysis of Yorkshire and Landrace pigs.

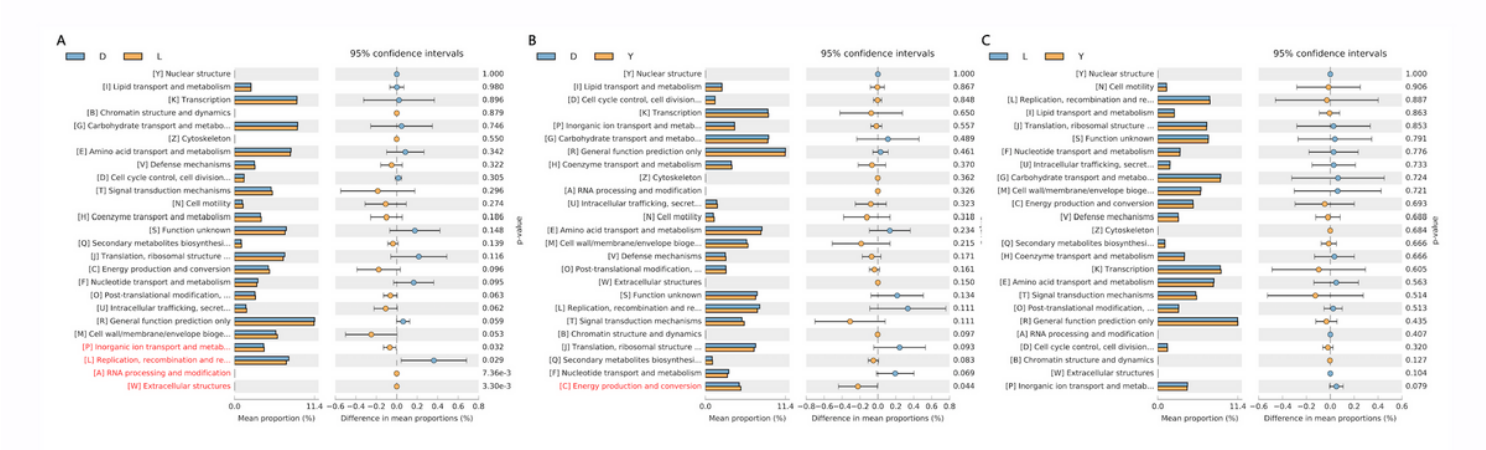

Figure 7

Functional prediction of differential intestinal flora in three group using GO. (A) Analysis of functional differences between intestinal flora of Landrace and Duroc. (B) Analysis of functional differences between intestinal flora of Yorkshire and Duroc. (C) Analysis of functional differences between intestinal flora of Yorkshire and Landrace.

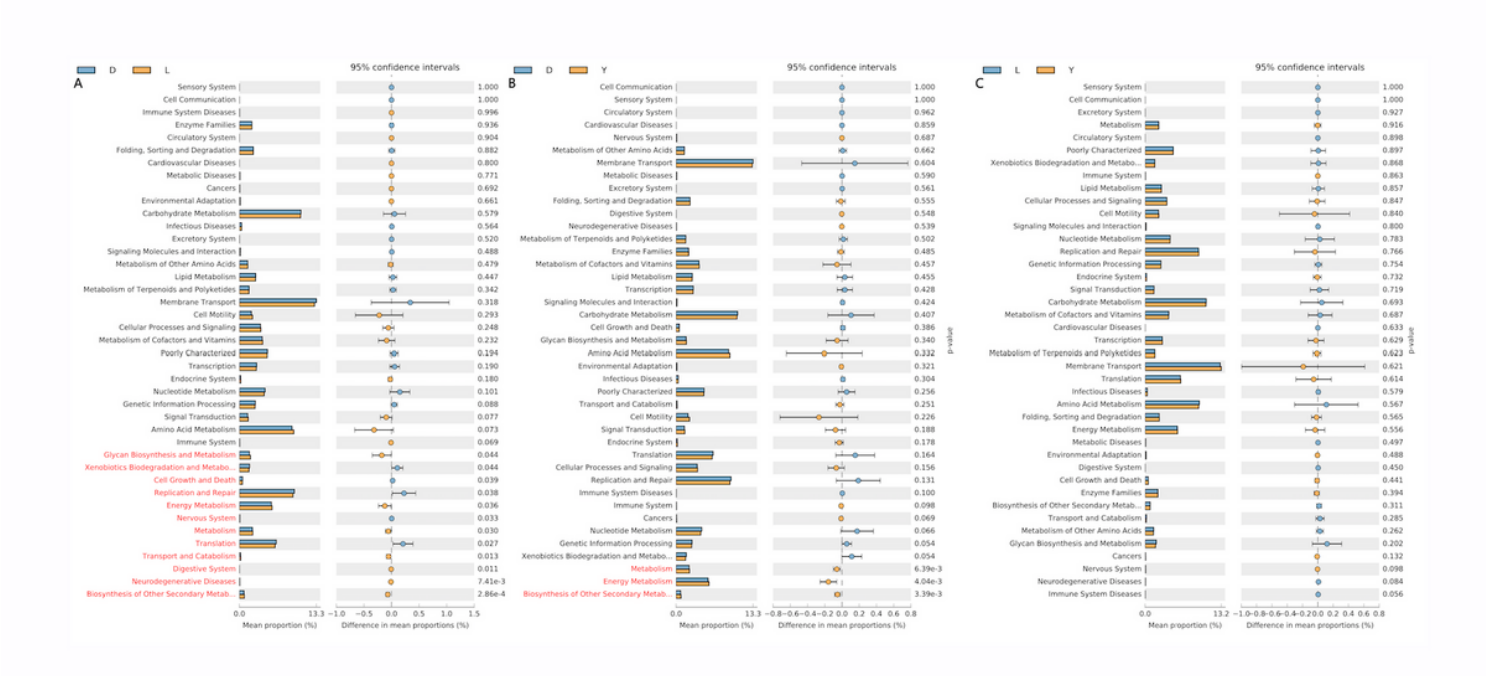

Figure 8

Predicted function of the microbial flora of the three groups using KEGG. The third level KEGG pathways are shown in the post-hoc plot. (A) Analysis of KEGG pathways differences between intestinal flora of Landrace and Duroc. (B) Analysis of KEGG pathways differences between intestinal flora of Yorkshire and Duroc. (C) Analysis of KEGG pathways differences between intestinal flora of Yorkshire and Landrace. The significance of the gene distribution between groups was determined by ANOVA with a $P < 0.05$.

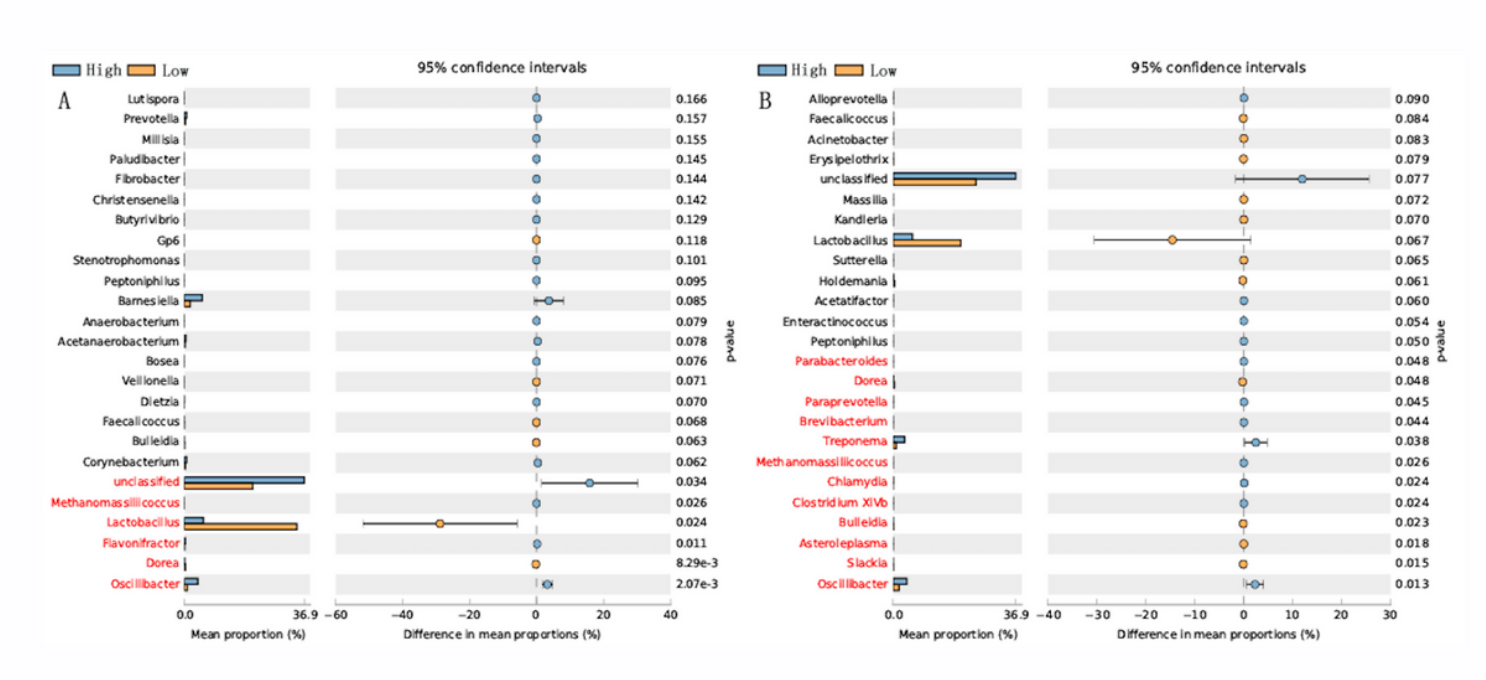

Figure 9

Analysis chart of Intestinal flora diversity of Yorkshire pigs with significant difference in growth performance. (A) Analysis chart of intestinal flora diversity of Yorkshire pigs with significant difference in ADG. (B)Analysis chart of intestinal flora diversity of Yorkshire pigs with significant difference in FER. The left part of the figure shows the abundance ratio of different microorganisms in the two groups, and the middle part shows the difference proportion of species classification abundance within the 95% confidence interval. The value on the far right is P value, $P < 0.05$ indicates significant difference, and species classification is marked in red.

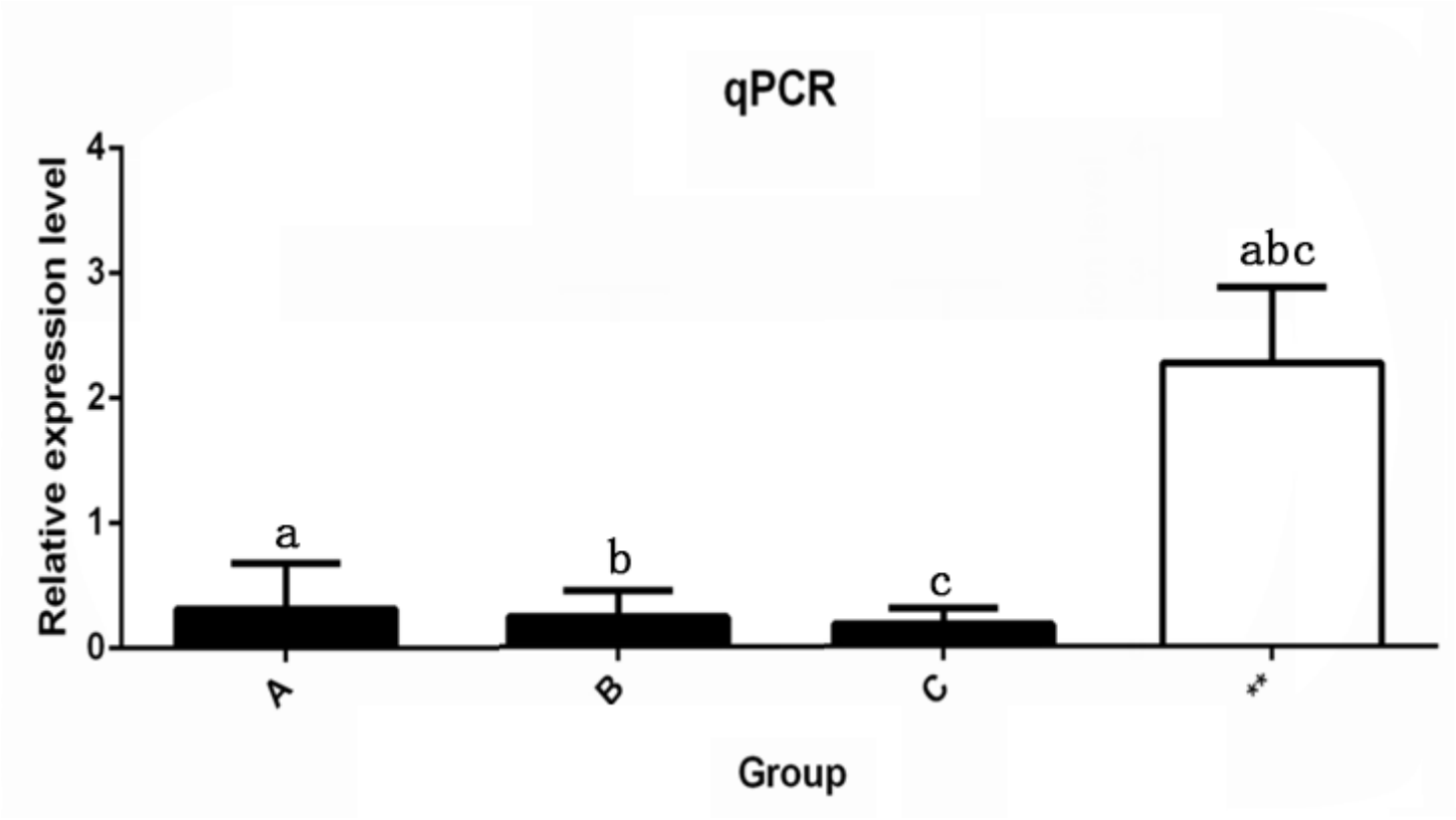

Figure 10

Bar chart of qPCR results. (A) Lactobacillus was treated with A concentration of 1×106, (B) Lactobacillus was treated with A concentration of 1×107, (C) Lactobacillus was treated with A concentration of 1×107, ** represents blank treatment control group.

Supplementary Files

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