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Characteristics of faecal bacterial flora and volatile fatty acids in Min pig, Landrace pig, and Yorkshire pig

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ABSTRACT

Background: In order to study the influence of long-term growth process and evolution environment on intestinal bacteria of different breeds, the intestinal bacteria and volatile fatty acids among the faeces of Min, Landrace and Yorkshire pigs were analysed by Illumina high-throughput sequencing of the 16S-rDNA and gas chromatography.

Results: The shared core microbiota of Landrace, Yorkshire and Min pig were 1273, accounting for 69.56% of total abundance of organisms. The proportion of *Firmicutes* in Min pig faeces (57.89%) was significantly higher than that in Landrace and Yorkshire pig faeces (47.01% and 46.40%, respectively) ($P < 0.05$), but that of *Bacteroidetes* was exactly opposite. Moreover, Min pig presented more highly efficient membrane transport, environmental adaptation, carbohydrate transport, and metabolism than Yorkshire pig ($P < 0.05$). The acetic acid/total volatile fatty acid ratio in Min pig was significantly higher than that in Landrace pig ($P < 0.05$), and the isobutyric acid/ total volatile fatty acid ratio in Min pig was significantly larger than that in Yorkshire pig ($P < 0.05$). Furthermore, the content of branched chain volatile fatty acids in Min pig was significantly higher than that in Yorkshire pig ($P < 0.05$).

Conclusions: This study demonstrated that Min pig, as an excellent breed in the cold area of China, possessed special intestinal floral structure compared to the imported pigs in order to satisfy their physiological and metabolic demands, which may influence their characteristics such as resistance to cold, diseases, and crude feeding, and the ability to deposit intramuscular fat.

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1. Introduction

Numerous microbial species are distributed worldwide and possess specific characteristics. Even the same species may present distinct character based on the regional factors. For example, the Min pig is one of the eight indigenous pig breeds of China. It has numerous extraordinary properties, such as good meat quality, high litter size, cold resistance, disease resistance, strong adaptation to roughage and significant hybridisation effect [1,2]. In recent decades, to explore the excellent traits of different pig breeds, China has introduced several pig breeds from other countries; of these, the most widely used are Landrace pigs, Yorkshire pigs, and Duroc pigs. All imported pig breeds represent their specific apparent characters, which are different from the Min pig; however, people ignore the advantages of Min pig as an indigenous and excellent pig breed in China. Thus, it is necessary to explore the factors responsible for these excellent traits in fast and efficient excavated pigs.

Feed utilisation, disease resistance and energy metabolism are directly related to different pig varieties, despite these being local or imported pig breeds; herein, another crucial factor is the composition and diversity of intestinal microflora [3,4,5]. The animal's apparent properties and the intestinal microflora have an inseparable relationship, which constitutes a complex and huge ecosystem in the host body. The number of intestinal microflora genes in pigs is approximately 150 times higher than that in humans, and most of these genes are unknown [6,7]. In general, the host intestinal flora can be divided into three types, namely beneficial, harmful, and neutral bacteria. First, beneficial bacteria, also defined as probiotics, such as *Bifidobacteria* and *Lactobacillus*, can produce numerous metabolites to accelerate food digestion, curb the growth of pathogenic microorganisms and control toxin synthesis; however,

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harmful bacteria are able to manufacture various harmful substances in the intestine. Under normal circumstances, these substances can be cleaned by the body. Once the harmful bacteria grow rapidly, they can damage the host immune system, leading to various diseases and even death. Moreover, neutral bacteria possess dual functions, such as *Escherichia coli* and *Enterococcus*, which are beneficial to the body under normal conditions. Once the growth is excessive, they become pathogenic and injure the body. Several researchers have found that the structure and diversity of the intestinal microbial community are closely related to species [8] animal age [9] and growth environment [10]. Meng found that weaning transition can change the proportion and structure of microbiota [11]. Thus, the intestinal microflora can achieve a dynamic balance, co-exist mutually with the host and maintain health and normal physiological function of the host, which is crucial for the healthy and normal growth of animals [12,13].

Some studies have reported that the intestinal flora could stimulate the development of immune system to enhance the host immune function [14,15] and prevent the infestation of pathogenic microorganisms [16]. Moreover, Lievin found that certain *Bifidobacteria* could inhibit the growth of pathogenic bacteria by producing bacteriocin [17]. Furthermore, harmful bacteria and substances can enter the body by increasing intestinal cell permeability, whereas the intestinal microflora can prevent pathogen invasion by reducing intestinal epithelial cell permeability [18,19]. Additionally, the intestinal flora may also affect the host's cellular processes and metabolic pathways of the tissue system [20]; however, this flora is directly related to the host in numerous ways, including the fibre type involved in their diet [21,22] composition and concentration of fibre in the diet [23,24,25] length of time to feed fibre [9] and supplements in diets [26,27,28].

Each species has a unique structure of intestinal microflora. For example, *Proteobacteria* account for majority of flora in the intestine of fish, and the abundance of *Firmicutes* is twice that of *Bacteroidetes*; whereas, in humans, *Firmicutes* and *Bacteroidetes* are highly abundant, with almost similar proportions [29,30]. Even in the same species, the composition of intestinal flora differs among different breeds. One study reported that the diversity of intestinal microflora in obese twins was low [31] which is in accordance with Zupancic's finding [32]. Guo found that high abundance of *Pachytene* and low abundance of *Bacteroides* were observed in the intestine of obese pigs [33]. In addition, Yang reported that significant differences were observed in the intestinal microflora in different pig breeds, by studying the intestinal microflora composition in eight pig breeds [5]. High similarity was observed in the intestinal microflora of Yorkshire pigs and Duroc pigs and also in those of Bama pig, Erhualian pig, and Xiaomeishan pig from China [5]. Su confirmed that breeds and growth stages influence the intestinal microflora of pigs [4].

The animal metabolism mainly involves two pathways: host metabolism and intestinal microflora metabolism, which form a co-metabolic relationship to control the entire metabolism process [34]. Volatile fatty acids (VFAs) are volatile short-chain fatty acids that are mainly produced from undigested carbohydrates in food by anaerobic bacterial fermentation *in vivo*, and generally comprise 1–6 carbon chains. They mainly include acetic acid, propionic acid, butyric acid, isobutyric acid, valeric acid, and isovaleric acid, and are the main products of dietary fibre fermentation by intestinal bacteria [35]. Several studies have reported that acetic acid is produced by the colonic anaerobes fermenting undigested and unabsorbed carbohydrates in the foregut, propionic acid is the dominant product of *Bacteroides* fermentation and butyric acid is principally produced by *Firmicutes* metabolism [36,37,38]. Moreover, acetic acid, and propionic acid are involved in the energy metabolism process of the liver, and butyric acid can provide energy for digestion and absorption process of intestinal epithelial cells [39,40]. Furthermore, butyric acid protects the host from pathogens [41,42] and reduces the flow rate of chyme in the intestine to facilitate nutrient absorption by intestinal epithelial cells [43,44]. Therefore, VFAs produced by intestinal bacteria can participate in numerous body activities, such as immunity, obesity, and blood sugar regulation [45].

The intestinal microflora is crucial for the growth and development of the body; however, the relationship among intestine, metabolites, and host remains unclear. At present, most studies focus on the effect of intestinal flora on the host, but the mechanism between pig breeds and intestinal flora remains unclear. The studies on Min pig are limited. We propose two scientific hypotheses: (1) the structure of intestinal flora in Min markedly differs from that of the other pig breeds, and (2) the growth performance and health of Min pig are controlled by their own intestinal flora. Our objectives are to detect the differences in the intestinal flora of various pig breeds, that is Landrace pig (SLA), Yorkshire pig (SLW), and Min pig (SMIN), on faecal flora structure and metabolites, clarifying the effects of intestinal flora on growth performance and metabolism, and exploring the unique metabolic model of Min pig. This experiment provides a theoretical basis for studying the intestinal flora of pigs and provides a preliminary basis for further exploring the mechanism of differential pathways. Furthermore, it is of great theoretical significance to understand and explore this excellent resource of Min pig, clarifying the growth performance and metabolism of pigs and promoting the development of the pig industry.

2. Materials and methods

2.1. Ethics statement

This study was approved by the Animal Care and Use Committee of the Northeast Agricultural University. The collection of captive pig stool samples was approved by following the Guide for the Care and Use of Laboratory Animals of Northeast Agricultural University (NEAU-[2011]-9).

2.2. Animal management, sampling and DNA extraction

A single-factor experiment was designed. Three treatment groups were divided into SLA, SLW, and SMIN. Each group had six replications. All pigs were fed the same feeding diet and were provided similar lifestyle in Lanxi Breeding Pig Farm of Heilongjiang province. The diets were formulated to meet or exceed all nutrient requirements of the sows recommended by NRC [46] (3.10 Mcal ME/kg, 178.5 g/kg crude protein, 9.9 g/kg Ca, 5.0 g/kg available P, 28.8 g/kg crude fat, 31.4 g/kg crude fibre). All pigs were placed on a small ground and subjected to exercise from 5:30 am to 6:00 am, and were then brought back to their pigsties for feeding at 6:00 am every morning. Similarly, this process was repeated at 3:00–3:30 pm at the same ground and they were brought back to their pigsties at 3:30 pm every afternoon. The faecal samples of SLA, SLW, and SMIN were collected in the morning. All fresh faecal samples were stored in liquid nitrogen tank with full liquid nitrogen and immediately transported to the laboratory. Thereafter, the DNA of the samples was extracted using a stool DNA kit (OMGEA, kit 50, Shanghai, China).

Next, 1 g faeces and 10 ml Buffer SP1 were added into 15 ml centrifuge tube. The tube was centrifuged at 4000× *g* for 15 min. All supernatants and their 1/3 volume of Buffer SP2 were added into a new 15 ml centrifuge tube. The tube was shaken well by vortexing (Qilinbeier, VORTEX-5, Jiangsu, China) for 20 s and then placed on ice for 5 min, followed by centrifugation at 3000× *g* for 10 min. Subsequently, all supernatants and the same volume

of isopropanol were added into a new 15 ml centrifuge tube, after inversing 10 times, at 3000× *g*, and centrifuged for 10 min. Then, all the supernatants were discarded and 250 μl Elution Buffer was added into the centrifuge tube. The tube was shaken well and placed in a water bath at 70℃ for 5 min. All liquid and 200 μl HTR were added to a new 1.5 ml centrifuge tube. Next, the tube was shaken well by vortexing for 10 s and kept at room temperature for 2 min, followed by centrifugation at 13,000× *g* for 2 min with a centrifuge. Thereafter, 250 μl supernatant was transferred to a new 1.5 ml centrifuge tube, and 10 μl protease K and 250 μl BL Buffer were added. The tube was shaken by a vortex oscillator for 10 s and then placed in a water bath at 70℃ for 5 min. Next, 250 μl absolute ethanol was added into the centrifuge tube and shaken by vortex oscillator for 10 s. The whole sample (including precipitation) was placed in the column, and the collecting tube was centrifuged at 13,000× *g* for 1 min. Furthermore, the solution and collecting tube were discarded, and a new collecting tube was replaced. Next, 500 μl HB Buffer was added into the column and centrifuged at 13,000× *g* for 1 min, and thereafter the solution and collecting tube were discarded. Later, 750 μl DNA Wash Buffer was added into the column and centrifuged at 13,000× *g* for 1 min, and thereafter the solution and collecting tube were discarded. Eventually, 200 μl Elution Buffer (preheated at 70℃) was added to the column and centrifuged at 13,000× *g* for 1 min.

2.3. PCR amplification and sequencing

To amplify the V3–V4 region of the 16S-rRNA gene for Illumina deep sequencing, universal primers, 338F: 5′-ACTCCTACGGGAGG CAGCA-3′ and 806R: 5′-GGACTACHVGGGTWTCTAAT-3′) were used. PCR was performed in a total reaction volume of 20 μl: H_2O 13.25 μl, 10 × PCR ExTaq Buffer 2.0 μl, DNA template (100 ng/ml) 0.5 μl, prime1 (10 mmol/l) 1.0 μl, prime2 (10 mmol/l) 1.0 μl, dNTP 2.0 μl and ExTaq (5 U/ml) 0.25 μl. After an initial denaturation at 95℃ for 5 min, amplification was performed by 30 cycles of incubation for 30 s at 95℃, 20 s at 58℃ and 6 s at 72℃, followed by a final extension at 72℃ for 7 min. The amplified products were purified and recovered using 1.0% agarose gel electrophoresis. Finally, library construction and sequencing steps were performed by Beijing Biomarker Technologies Co. Ltd (Beijing, China).

2.4. Bioinformatics analysis

The bioinformatics analysis in this study was completed on the Biomarker biocloud platform (www.biocloud.org). To obtain the raw tags, paired-end reads were merged using FLASH (v1.2.7, http://ccb.jhu.edu/software/FLASH/) [47]. Raw tags were then filtered and clustered in the next steps. The merged tags were compared to the primers, and the tags with more than six mismatches were discarded using the FASTX-Toolkit [48]. Tags with an average quality score < 20 in a 50 bp sliding window were truncated using Trimmomatic (http://www.usadellab.org/cms/?page=trimmomatic) [49] and tags shorter than 350 bp were removed. We identified possible chimaeras by employing UCHIME [50,51] a tool included in mothur software (http://drive5.com/uchime) [52]. The denoised sequences were clustered using USEARCH (version 10.0), and tags with similarity s97% were regarded as an operational taxonomic unit (OTU). Taxonomy was assigned to all OTUs by searching against the Silva databases (Release128, http://www.arb-silva.de) using the uclust within QIIME [53,54,55,56].

2.5. VFA analysis

The method of VFA analysis was based on the study of Morlein and Tholen [57]. About 2 g faecal sample was added into a plastic bottle containing 400 μl of 25% metaphosphoric acid and crotonic acid (internal standard), centrifuged at 13,000× *g* for 10 min and filtered by 0.22 nm water filter membrane twice, then stored at 4℃ for 48 h for later analysis of VFA using gas chromatography. Gas chromatography (6890N; Agilent Technologies, Avondale, PA, USA) was equipped with a 30 m HP-INNOWax 19091N-213 (Agilent) capillary column (0.32 mm i.d. and 0.50 mm film thickness). The chromatograph oven was programmed as follows: 120°C for 3 min, 10℃/min increment to 180℃, and then held for 1 min. The injector and detector were maintained at 220℃ and 250℃, respectively. Nitrogen was used as the carrier gas (flow rate 2.0 mL/min). The following formula was used to calculate the value of VFA content in the samples:

$$Yi = (Fi \times Wsj \times Aij)/Asj \times 1000/Wj,$$

where Yi = VFA content (mg/g), Fi = correction factor, Wsj = weight of internal standard mixed with the sample, Aij = peak area of fatty acid, Asj = peak area of internal standard, Wj = sample weight, and (Fi × Wsj × Aij)/Asj = VFA weight in the sample. In total, six mixed external standards contained the internal standard. Fi was calculated using an external standard curve. The calculation formula for Fi is as follows:

$$Fi = (Wi/Ws)/(Ai/As),$$

where Wi = weight of VFA in external standards, Ws = weight of internal standard in external standards, Ai = peak area of VSA in the external standard curve and As = the peak area of internal standard in the external standard curve.

2.6. Statistical analysis

Total trait values were analysed by ANOVA of SPSS (20.0) for a randomised complete block with a factorial arrangement of treatments. The factorial treatment arrangement comprised three different pig breeds. If differences in treatment means were detected by ANOVA, Duncan's multiple range test was applied to separate means. A significance level of $P \leq 0.05$ was considered as statistically significant.

3. Results

3.1. Analysis of 16S-rDNA gene sequencing

In total, 1,232,234 effective tags were acquired from six SLA, six SLW and six SMIN faecal samples. Next, 66,880.5–69,606.2 (mean: 68,457.4 ± 815.4) effective tags (mean length: 416.3 bp) were obtained from per sample (Table 1). In total, 1830 OTUs were obtained at a sequence similarity level of 97%, and 1486–1593 (mean: 1552.7 ± 33.6) OTUs were obtained from each sample (Fig. 1a). The rarefaction curves of OTUs in 18 independent samples are illustrated in Fig. 1b. With an increase in the number of sequences, the OTU number initially revealed an upward trend, and then became smooth and steady. This indicates that most of the bacteria types in 18 independent samples had been detected. According to the ribosome database, 13 phyla, 22 classes, 25 orders, 39 families and 82 genera were detected in the bacteria.

Table 1
Sequence information.

Items	SLA	SLW	SMIN
Total effective sequences	413,314.0	401,283.0	417,637.0
Mean of effective sequences	68,885.7	66,880.5	69,606.2
Average length of effective sequences (bp)	417.3	417.0	414.5

SLA: Landrace pig; SLW: Yorkshire pig; SMIN: Min pig.

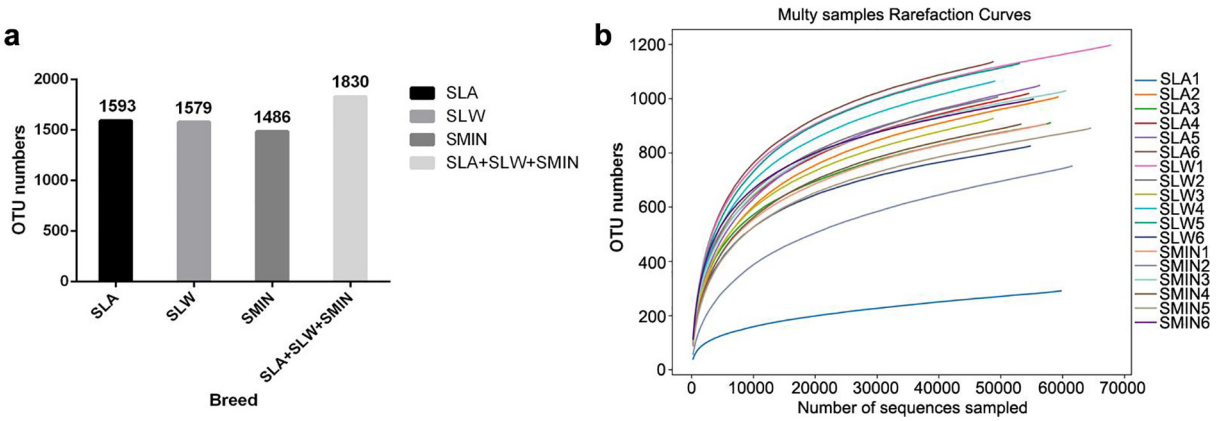

Fig. 1. Histogram and rarefaction curves of operational taxonomic units (OTUs). (a) Histogram of OTU number in three groups. The *x*-axis presents different groups, and the *y*-axis displays the OTU number. The OTU similarity threshold of 97% was considered. $^{*}P < 0.05$ (Student's t-test). (b) The rarefaction curves of OTUs. The *x*-axis reveals the number of effective sequences in each sample, and the *y*-axis displays the OTU number. Each curve of different colour indicates a different sample. Increase in extracted sequences decreased the OTU number.

3.2. Diversity analysis of faecal flora

Alpha-diversity indexes of the three different groups are illustrated in Fig. 2. Alpha-diversity indices reflected the abundance and consistency, mainly comprising Shannon index, Simpson index, ACE index and Chao1 index. No significant difference was observed in SLA, SLW, and SMIN ($P > 0.05$). In terms of the diversity of OTUs, the Shannon index in SLA (4.71 ± 0.32) was nearly identical to that of SMIN (4.70 ± 0.21). Although the Shannon index in SLW (5.10 ± 0.08) was slightly higher than that in the other two groups, SLW in Simpson (0.02 ± 0.00) was the least, which indicated that the diversity of OTUs in SLW was lower than that in SLA (0.03 ± 0.01) and SMIN (0.03 ± 0.01). Moreover, according to the OTU abundance, the amounts of ACE and Chao1 in SLW (1170.31 ± 59.86 and 1177.54 ± 63.08, respectively) were the highest, and these amounts in SLA (1052.53 ± 135.62 and 1055.14 ± 13 6.76, respectively) were lower than those in SMIN (1072.97 ± 26.25 and 1080.76 ± 25.86, respectively) and SLW.

Beta-diversity indexes could reflect species composition through space. In terms of non-metric multi-dimensional scaling (NMDS; the information of PC1 and PC2 are summarised in Table S1), the dissimilarity of the microbial community and

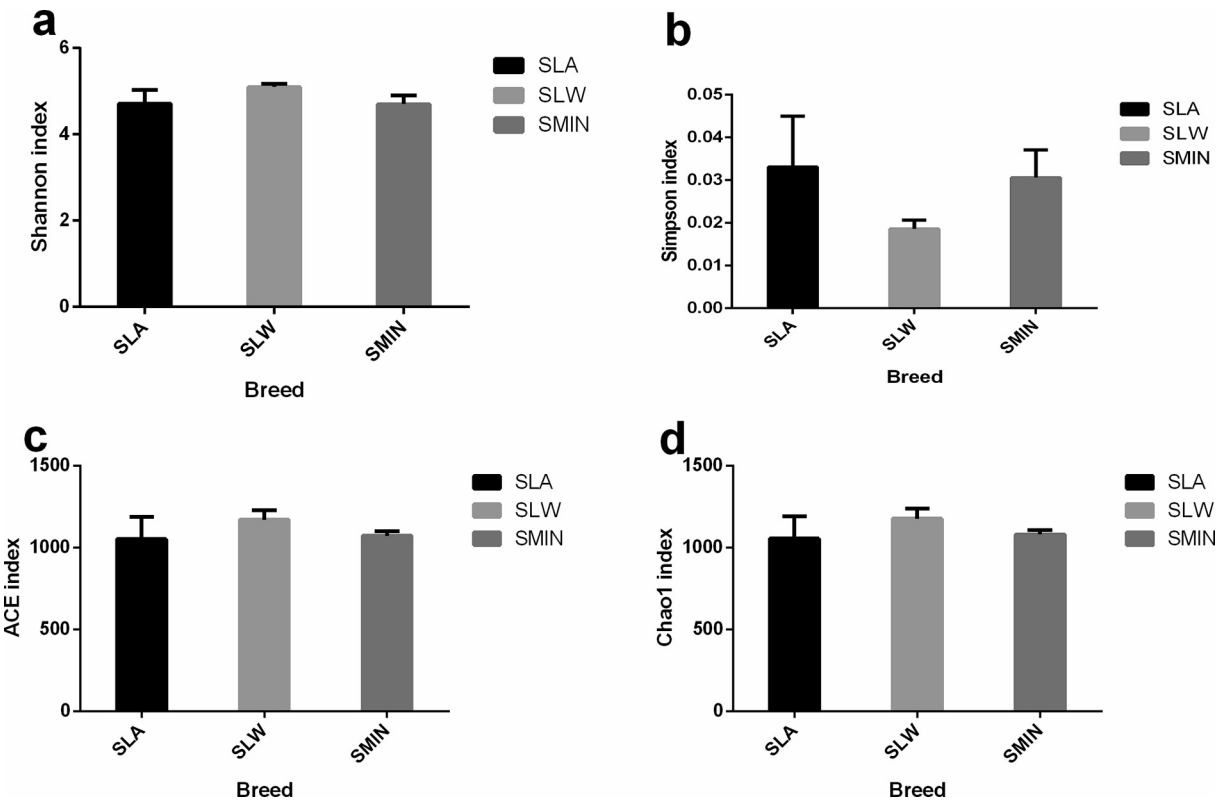

Fig. 2. Histogram of Alpha-diversity indexes among three groups. (a) Shannon and (b) Simpson indexes reflect the diversity of OTU. (c) ACE and (d) Chao1 indexes reflect the OTU abundance. Smaller Simpson and greater Shannon index indicate higher diversity of the microbiota; greater ACE or Chao1 index indicates the higher the expected species richness of the microbiota.

distinct structure among SLA, SLW, and SMIN (Fig. 3) are revealed. Different groups' Beta distances are illustrated in Fig. 4, and the related data are summarised in Tables S2, S3, and S4. Beta distance of SLA and SLW is illustrated in Fig. 4a ($P > 0.05$). The beta distances of the SLA and SMIN are presented in Fig. 4b ($P < 0.05$), and those of SLW and SMIN are illustrated in Fig. 4c ($P < 0.01$). In general, the data in Fig. 3 reveal that the results of this experiment were available and reliable, and those in Fig. 4 indicate that significant differences were observed in the species composition between SMIN and the other two groups ($P < 0.05$).

The species diversity of SLA, SLW, and SMIN is illustrated in Fig. 5. The share core microbiota of SLA, SLW, and SMIN were 1273. In addition, 139 core microbiota of SLA and SLW, 73 core microbiota of SLW and SMIN and 70 core microbiota of SLA and SLW were found. The results indicated that the similarity between SLA and SLW was higher than that between SMIN and the other two groups.

3.3. Abundance analysis of faecal bacteria

According to the relative abundance, the top 10 phyla and the top 10 genera of the faecal flora in SLA, SLW, and SMIN are illustrated in Fig. 6 as two histograms. In terms of the top 10 phyla of faecal bacteria, the dominant bacteria of SLA, SLW, and SMIN were Firmicutes (47.01%, 46.40% and 57.89%, respectively) and Bacteroidetes (35.08%, 34.16% and 28.29%, respectively), followed by Spirochaetae (7.67%, 8.19% and 5.57%, respectively), Proteobacteria (6.18%, 3.46% and 2.74%, respectively), Verrucomicrobia (1.10%, 2.74% and 3.01%, respectively), Tenericutes (1.24%, 2.29% and 1.05%, respectively) *and* Lentisphaerae (0.96%, 1.27% and 0.54%, respectively) (Fig. 6a).

For the top 10 genera of the faecal bacteria, the dominant bacteria of SLA, SLW, and SMIN were *Christensenellaceae_R-7_group* (8.02%, 10.15% and 19.34%; respectively), *Rikenellaceae_RC9_gut_group* (5.01%, 6.26% and 9.85%; respectively), *Treponema_2* (7.29%, 7.80% and 4.87%; respectively), *Uncultured_bacterium_f_Bacteridales_S24-7_group* (4.94%, 7.40% and 2.89%; respectively), *Ruminococcaceae_UCG-005* (5.23%, 4.78% and 4.25%; respectively), *Prevotellaceae_UCG-001* (5.67%, 5.43% and 1.59%; respectively), followed by *Ruminococcaceae_NK4A214_group* (3.03%, 3.36% and 3.35%; respectively), *Ruminococcaceae_UCG-014* (2.12%, 3.31% and 2.46%; respectively), *Prevotellaceae_NK3B31_group* (3.26%, 3.06% and 0.40%; respectively) and *Ruminococcaceae_UCG-010* (1.90%, 2.31% and 2.36%; respectively) (Fig. 6b).

3.4. Significant difference analysis of faecal flora among SLA, SLW, and SMIN

The cladogram and LDA scores of faecal bacterial differences by LefSe analysis in the three different groups are illustrated in Fig. 7a and 7b, respectively. The abundance of Clostridiales and Firmicutes in SMIN was significantly higher than that in SLA and SLW ($P < 0.05$). Moreover, the abundance of bacteria in *the bacteroidales_S24_group* in SLW was significantly higher than that in SMIN and SLA ($P < 0.05$). Furthermore, according to the *Prevotellaceae*, *Prevotellaceae_UCG_001*, *Prevotellaceae_NK3B31_group*, *Prevotellaceae_9* and *Rumen_bacterium*, the abundance of these bacteria in SLA was significantly larger than that in SMIN and SLW ($P < 0.05$).

3.5. Functional gene prediction of faecal flora among SLA, SLW, and SMIN

The changes and differences in the metabolic pathways of functional genes in faecal bacteria among SLA, SLW, and SMIN in the KEGG database and COG database are presented in Fig. 8. For metabolic pathways in the KEGG database (Fig. 8a), significant differences were observed in the metabolic pathways between SLW and SMIN on membrane transport and environmental adaptation ($P < 0.05$). In addition, for metabolic pathways in the COG database (Fig. 8b), significant differences were observed in the metabolic pathways between SLW and SMIN on translation, ribosomal structure and biogenesis, signal transduction mechanisms and

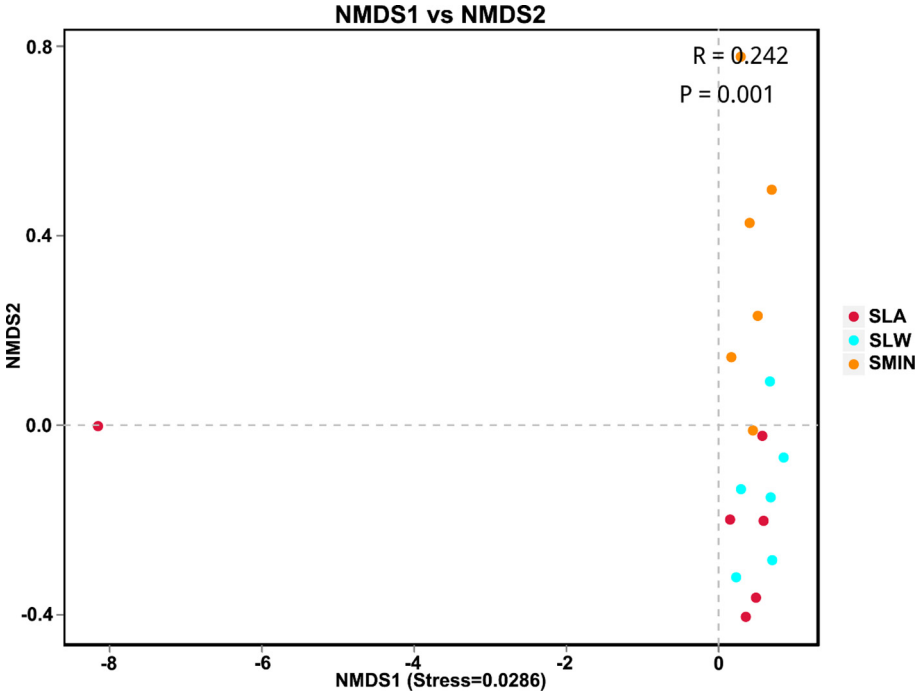

Fig. 3. Non-metric multi-dimensional scaling (NMDS) analysis among three groups. Each point represents one sample and each colour indicates a different group. The distance between points indicates the difference level. Stress lower than 0.2 indicates that the result of NMDS analysis is accurate and correct. A closer sample indicates higher similarity.

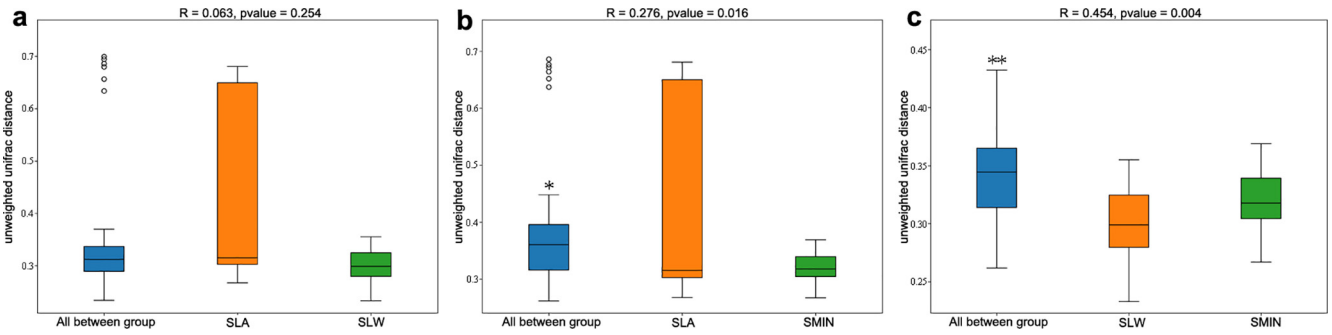

Fig. 4. Box plot of different groups Beta distance (ANOSIM Analysis). (a) Beta distance of SLA and SLW. (b) Beta distance of SLA and SMIN. (c) Beta distance of SLW and SMIN. The *x*-axis represents the grouping and the *y*-axis represents the distance calculated by Unweighted_unifrac. *R*-value range is between −1 and 1. The R-value ≤0 represents no significant inter-group and intra-group differences, and R-value >0 indicates that inter-group differences are greater than the intra-group differences. The *P*-value represents the confidence level of the statistical analysis; $^{*}P < 0.05$ reflects significant inter-group and intra-group differences. $^{**}P < 0.01$ reflects extremely significant inter-group and intra-group differences.

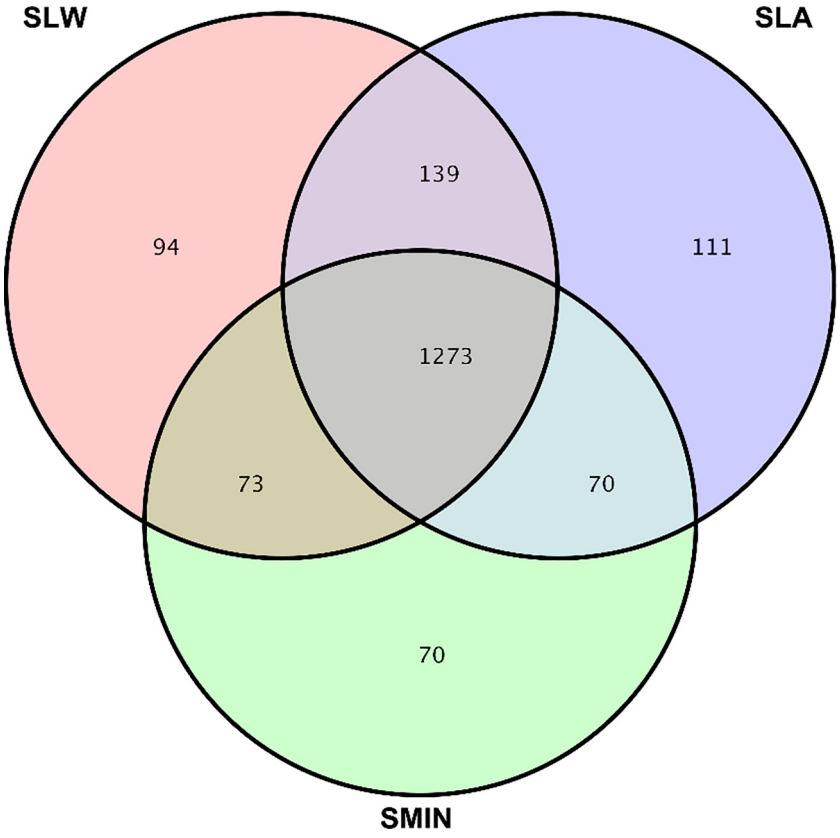

Fig. 5. Venn of microbial diversity (OTUs). The Venn diagram displays the number of OTUs (97% sequence identity) shared or specific among SLA, SLW, and SMIN.

carbohydrate transport and metabolism ($P < 0.05$). Furthermore, regardless of the KEGG and COG databases, no significant difference was observed between SLA and SMIN in the metabolic pathway ($P > 0.05$).

The content and proportion of VFAs in the faeces of pigs in different pig breeds are summarised in Table 2. According to the six VFAs and total volatile fatty acids (TVFA), the content of VFA in SLA was the highest, whereas that in SLW was the lowest. Significant differences were observed in the content of VFAs among the SLA, SLW, and SMIN, except for propionic acid and butyric acid ($P < 0.05$). Moreover, the contents of six VFAs and TVFA were significantly different between SLA and two other groups ($P < 0.05$), after which the contents of acetic acid, isobutyric acid, isovaleric acid, pentanoic acid and TVFA significantly differed between SMIN and SLW ($P < 0.05$); however, no significant differences were observed in the proportion of isovaleric acid/TVFA, pentanoic acid/TVFA and branched chain volatile fatty acids (BVFA)/TVFA among SMIN, SLA and SLW ($P < 0.05$), which was followed by a significant difference in isobutyric acid/TVFA, butyric acid/TVFA, isovaleric acid/TVFA, pentanoic acid/TVFA and BVFA BVF/TVFA between the SLW and two other groups ($P < 0.05$). Furthermore, the percentages of acetic acid/TVFA, isobutyric acid/TVFA, pentanoic acid/TVFA and BVFA BVF/TVFA were significantly different between SMIN and SLA ($P < 0.05$).

4. Discussion

The host and intestinal flora have a strong connection, which is influenced by physiological characteristics and the internal envi-

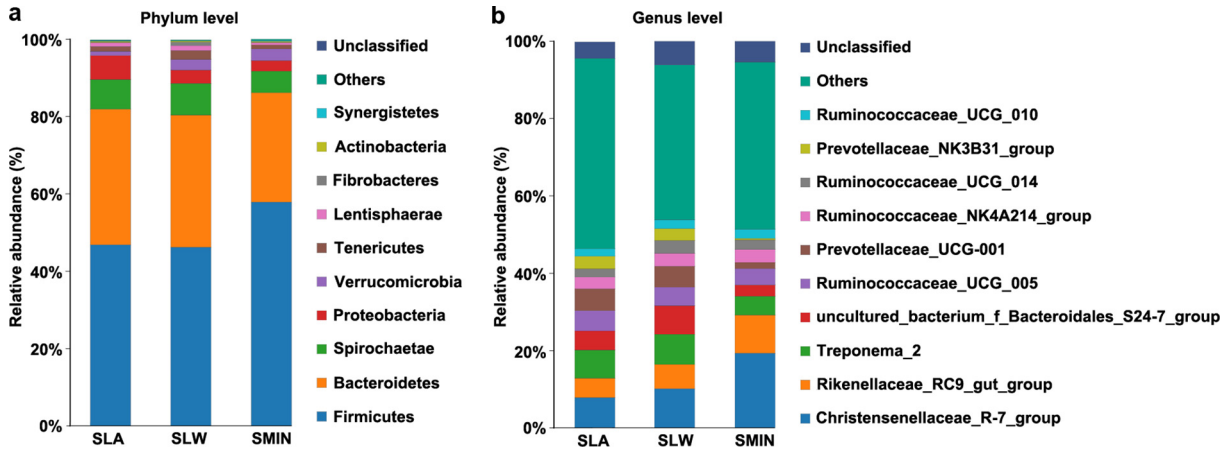

Fig. 6. Histogram of relative abundance among SLA, SLW, and SMIN. The *x*-axis indicates different groups and *y*-axis represents relative abundance shown as percentage. (a) Relative abundance of the top 10 phyla. (b) Relative abundance of the top 10 genera. Other species were presented as 'Others'.

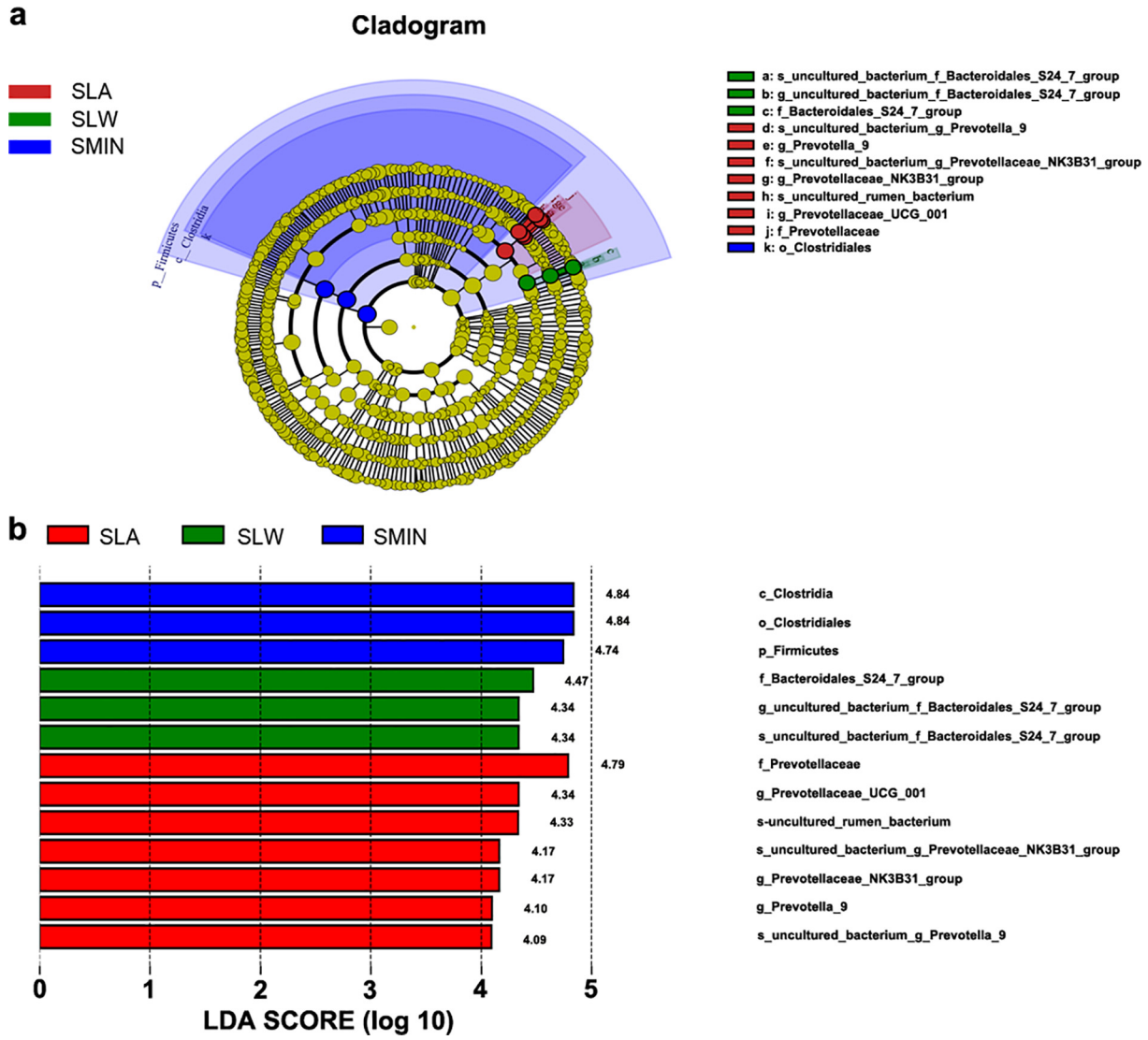

Fig. 7. LefSe analysis. (a) The cladogram diagram represents the significant different microbial species in SLA, SLW, and SMIN. Three different colours (red, green and blue) represent three different groups, with the species classification at the level of phylum, class, order, family and genus from the inside to the outside. The red, green and blue nodes in the phylogenetic tree indicate numerous significant differences among SLA, SLW, and SMIN in microbial species. Yellow nodes represent species with no significant difference among three groups. (b) Species with significant difference having an LDA score greater than the estimated value. The default score is 4.0. The length of the histogram indicates the LDA score, which is the degree of influence of species with significant difference among three groups.

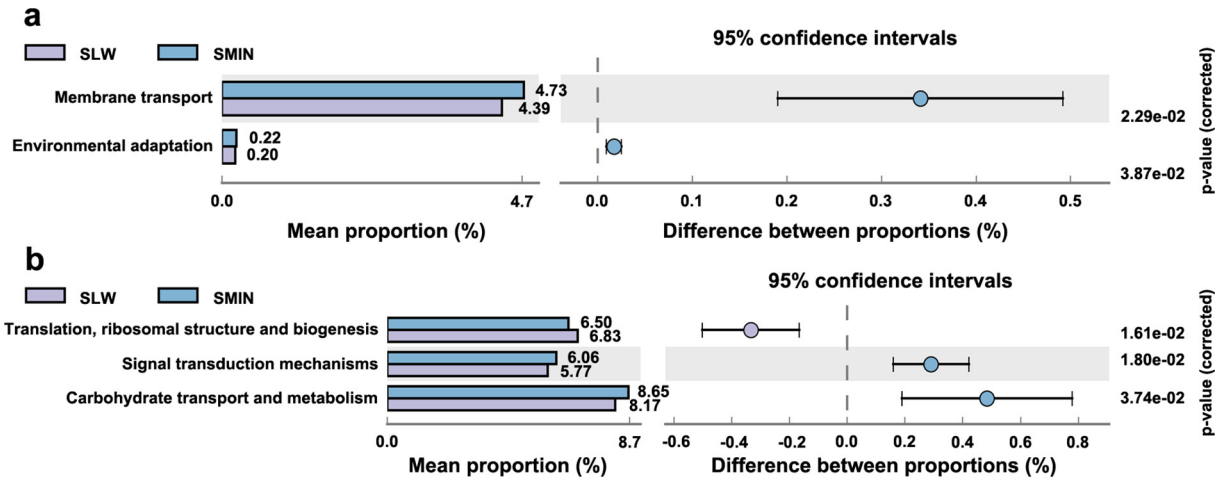

Fig. 8. Abundance radio of different function between SLW and SMIN. (a) The abundance ratio of different function between SLW and SMIN in KEGG database. (b) The abundance ratio of different function between SLW and SMIN in COG database. PICRUSt analysis. The graphs present the abundance ratio of different functions in two groups of samples. The middle value indicates the difference between proportions of functional abundance in the 95% confidence interval, and the rightmost value is the *P*-value. $P < 0.05$ represents significant difference.

Table 2
Content and percentage of VFA in faeces of different pig breeds.

Items	SLA	SLW	SMIN	*P* value
Acetic acid (mg/g)	51.51 ± 2.96^{a}	16.45 ± 1.35^{c}	31.75 ± 1.95^{b}	<0.001
Propionic acid (mg/g)	44.09 ± 3.54^{a}	13.14 ± 1.26^{b}	20.30 ± 2.30^{b}	<0.001
Isobutyric acid (mg/g)	4.00 ± 0.32^{a}	0.78 ± 0.08^{c}	2.05 ± 0.20^{b}	<0.001
Butyric acid (mg/g)	7.03 ± 1.04^{a}	2.79 ± 0.41^{b}	3.11 ± 0.36^{b}	<0.001
Isovaleric acid (mg/g)	5.94 ± 0.40^{a}	1.03 ± 0.04^{c}	2.61 ± 0.24^{b}	<0.001
Pentanoic acid (mg/g)	2.11 ± 0.23^{a}	0.29 ± 0.02^{c}	0.75 ± 0.12^{b}	<0.001
TVFA (mg/g)	114.68 ± 7.47^{a}	34.47 ± 1.67^{c}	60.56 ± 5.03^{b}	<0.001
BVFA (mg/g)	9.94 ± 0.68^{a}	1.81 ± 0.10^{c}	4.66 ± 0.43^{b}	<0.001
Acetic acid/TVFA (%)	52.49 ± 0.97^{b}	54.88 ± 3.66^{ab}	60.13 ± 1.80^{a}	0.074
Propionic acid/TVFA (%)	35.31 ± 1.27	34.42 ± 2.89	29.87 ± 1.32	0.113
Isobutyric acid/TVFA (%)	2.88 ± 0.23^{a}	1.81 ± 0.17^{b}	2.56 ± 0.07^{a}	<0.001
Butyric acid/TVFA (%)	4.36 ± 0.47^{b}	6.25 ± 0.85^{a}	3.87 ± 0.49^{b}	0.017
Isovaleric acid/TVFA (%)	3.76 ± 0.35^{a}	2.10 ± 0.14^{c}	2.81 ± 0.07^{b}	<0.001
Pentanoic acid/TVFA (%)	1.20 ± 0.07^{a}	0.55 ± 0.05^{c}	0.76 ± 0.06^{b}	<0.001
BVFA/TVFA (%)	6.65 ± 0.56^{a}	3.91 ± 0.27^{c}	5.37 ± 0.09^{b}	<0.001

TVFA = acetic acid + propionic acid + butyric acid + pentanoic acid + isobutyric acid + isovaleric acid.
BVFA = isobutyric acid + isovaleric acid.
a, b, c – Values within a row with different letters differ significantly at $P < 0.05$.
SLA: Landrace pig; SLW: Yorkshire pig; SMIN: Min pig.

ronment of the intestine. The effects were mainly demonstrated on species diversity, species abundance and metabolites. The rich bacterial diversity can maintain a positive intestinal environment and dynamic balance of intestinal flora. Li et al. revealed that the difference in the intestinal environment contributes to the dramatic bacterial diversity. Moreover, the host plays a crucial role in bacterial diversity [20]. Lan reported that significant differences were observed in the species diversity of intestinal faecal microbiota between sheep and rabbits [58]. In the present study, the results of Alpha-diversity indexes revealed that no significant difference was found in the OTU level on the four indexes among SLA, SLW, and SMIN, and only the Simpson index of SLW was markedly lower than that of SLA and SMIN (Fig. 2). Therefore, we infer that this may occur because SLA, SLW, and SMIN belong to the same species and feed on the same diet; however, the results of the diversity indexes display the difference between SMIN and the other two groups (Fig. 3). We speculate that this is because both SLA and SLW are imported breeds, which are not native to China. In our experiment, the Venn diagram 5 of microbial diversity further indicates that the difference in core microbiota of SMIN is much greater than that of SLA and SLW.

The structure of intestinal flora is directly determined by the difference in host breeds to a certain degree. For example, in the mammalian intestine, Firmicutes and Bacteroidetes account for the vast majority [59,60,61,62], followed by Ruminococcaceae, Prevotellaceae and Clostridiales [61,63,64]. This is closely related to the genes of each host, that is, the host gene can affect the apparent character and the development of intestinal bacteria. Therefore, it causes differences in bacterial structure. Groenen reported that numerous significant differences were observed between domestic and wild pigs [65]; however, the difference in bacterial structure can also influence the host by changing the function of intestinal bacteria. The study by Ley reported that the energy and fat absorbed by humans and mice had a strong relationship with the ratio of Firmicutes and Bacteroidetes [66]. Thus, Firmicutes can efficiently absorb the food calories. Therefore, we infer that the massive fat that can be served in the muscle of Min pig is due to the presence of Firmicutes. This is in accordance with our experimental results (Fig. 6). In contrast, Bacteroidetes contain GHs and PLs, which can degrade polysaccharides that exist in the cytoderm [67]. Hence, Landrace pigs can use the nutrients present in the feed efficiently through Bacteroidetes and can

improve feed utilisation. Our results are in accordance with these results; however, Bacteroidetes are also opportunistic pathogens. When certain factors such as the environment or diet are adversely affected, they are more likely to turn into harmful bacteria and further harm animals. In our test, the proportion of Bacteroidetes in SLA was higher than that in SMIN. To some extent, thus, we presume that this can explain the phenomenon behind the week resistance of Landrace pig compared to that of Yorkshire pig and Min pig. Furthermore, *Clostridium* is prevalent in the gastrointestinal tract, and it has both positive and negative effects on animals [68,69,70]. Rajilic-Stojanovic and de Vos found that although some members of *Clostridium* species are generally regarded as pathogenic, their density can be detected at 10^7–10^{11} cells/g in the intestinal content of healthy body [71]. In our experiment, the content of *Clostridium* in SMIN was higher than that in SLA and SLW (Fig. 7). Thus, we infer that *Clostridium* possesses some unknown properties that need to be further explored.

The high abundance of functional genes in the metabolic pathway of membrane transport indicates that the communication channels between intestinal flora and the external world are more extensive and their frequency of transportation is high. Intestinal flora exchanges substances with the external world through their cell membranes. Gill found that some intestinal flora could form a protective barrier through contact with intestinal epithelial cells, which can effectively prevent the invasion of pathogens and offer protection for the intestinal environment [72]. Moreover, Hooper reported that intestinal flora could produce bacteriostatic substances and activate non-specific defence functions by combining with intestinal epithelial cells [73]. Moreover, Jaehme found that numerous bacteria and archaea could harness various families of transporters to gain vitamins [74]. In our experiment, PICRUSt analysis revealed that the abundance of functional genes in the metabolic pathway of membrane transport in Min pig was significantly higher than that in Yorkshire pigs (Fig. 8). We infer this as due to the abundance of membrane transport genes in the metabolic pathway, Min pigs can absorb nutrients and discard the waste as well as maintain the dynamic balance of intestinal flora and retain a high level of immunity to a certain degree. Thus, Min pigs can represent strong disease resistance. Furthermore, Ma's results also indicated that Min pigs harbour strong disease resistance [75].

Notably, northeast China is located in the latitude area and has a large number of freeze months. Min pig is a special breed found in this region. Compared with other kinds of pigs, the Min pig can live here well and adapt to the local climate as it has unique features. In our experiment, we detected that the abundance of functional genes in the metabolic pathway of environmental adaptation in Min pig was significantly higher than that in Yorkshire pigs (Fig. 8a). We speculate that the functional genes of environmental adaptation regulate the structure of intestinal flora so that the Min pig can survive in extreme cold environment. Hence, the Min pig can exhibit cold resistance.

Carbohydrate is the principal energy source of animals. Min pigs have a strong ability to utilize carbohydrates, especially the crude fiber in the diet. Wang reported that the contents of glutamic-pyruvic transaminase, glutamic oxaloacetic transaminase, lactate dehydrogenase and creatine kinase increased in Min pigs and large Yorkshire pigs fed different levels of crude fiber diet (9, 12 and 15%), but the increase in Min pigs was small, which indicated that Min pig was more suitable for the diet with high crude fiber content [76] . The research of Zhao and Gong indicated that adding 5% roughage to Min sow diet during pregnancy and lactation had little effect on litter size, live weight, lactation and fertility, which proved that Min pig has strong resistance to roughage [77] . The intestinal flora can assist their host to degrade carbohydrates and generate energy, which can be absorbed by the host for intestinal peristalsis and feed digestion [78]. Different bacteria harbour distinct abilities to utilise carbohydrates. Thus, the composition of intestinal flora plays a crucial role in the rate of carbohydrate degradation. Bacteria that have a strong ability to degrade carbohydrate indicate that they have high digestibility and high energy utilisation rate for feed. According to our results, the abundance of functional genes in the metabolic pathway of carbohydrate transport and metabolism in Min pig was significantly higher than that in Yorkshire pigs (Fig. 8b). We infer that the expression of these genes can make the Min pig better absorb and utilise the nutrition and energy in feed. This may be directly related to the resistance of the crude feed of Min pig. Moreover, the decrease in short-chain fatty acids can influence the structure of intestinal bacteria, particularly changes in the proportion of *Lactobacillus*, thereby increasing the risk of colitis [79]. It is apparent that a high proportion of acetic acid in the intestine can reduce intestinal pH value and then decrease the risk of diseases. Our results indicated that the percentage of acetic acid in Min pig was significantly higher than that in the two other groups, which may explain why Min pig possesses strong disease resistance (Fig. 8b).

5. Conclusions

The structure of the intestine is closely related to pig breeds. The structure of intestinal flora at the phylum and genus levels was significantly different among Min pig, Yorkshire pig and Landrace pig, respectively, although there was a certain similarity in the species diversity of faecal flora. In addition, the VFAs among the three groups were also significantly different. Min pigs possess specific intestinal flora structure to meet their requirements of physiology and metabolism, which might result in strong disease resistance, cold resistance, crude feeding resistance and intramuscular fat deposition. In order to reveal the mechanisms, the relationship among intestinal flora, host apparent properties and metabolites can be further explored by using the methods of metagenomics and metabonomics in future.

Conflict of interest

The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

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Data availability

The datasets generated for the study can be found in the NCBI database: SRR11613170, SRR11613171, SRR11613172, SRR11613173, SRR11613174, SRR11613175, SRR11613176, SRR11613177, SRR11613178, SRR11613179, SRR11613180, SRR11613181, SRR11613182, SRR11613183, SRR11613184, SRR11613185, SRR11613186, SRR11613187.

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Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.ejbt.2021.05.002.

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