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Characteristics of the intestinal microbiome of sows in spontaneous and induced estrus

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ABSTRACT - In this study, a mixture of estradiol benzoate, progesterone, and testosterone propionate was injected into Diannan small-ear sows to induce estrus. The 16S rRNA technology was used to comparatively analyze the differences in fecal microbial composition and diversity between induced and spontaneous estrus in Diannan small-ear sows. The most abundant phylum in the sows in estrus were Firmicutes, Bacteroidetes, Actinobacteria, and Proteobacteria. There was a significant negative correlation between Firmicutes and Bacteroide and a significant positive correlation between *Bacteroides* and *Bifidobacterium*. The relative abundance of *Stenotrophomonas*, *Neisseria*, *Anaerofustis*, and *Terrisporobacter* in the sows during induced estrus was significantly higher than that during spontaneous estrus. Taken together, induced estrus affects the relative abundance of specific microbes in the feces of Diannan small-ear sows, but it does not affect the overall composition and diversity. These results provide fundamental knowledge about the gut microbiota of sows with induced estrus.

Keywords: 16S rRNA, Diannan small-ear pig, fecal microbial, induced estrus

1. Introduction

The fertility of sows is affected by many factors, including breed, weight, nutrition, and exposure to boars (Li et al., 2018). Chinese indigenous pig breeds are characterized by early sexual maturity and high fecundity. For example, Meishan sows, which are an excellent native breed in China, are known for their high reproductive performance (Li et al., 2020). Meishan gilts enter puberty at approximately four months of age, while European breed gilts typically reach puberty between 200 and 220 days of age (Evans and O'Doherty, 2001). Diannan small-ear pigs also possess the aforementioned characteristics. They grow in the southern region of Yunnan Province, with a subtropical climate, and have strong adaptability to environmental changes (Wu et al., 2020a).

The reproductive performance of sows affects pig production efficiency, and estrus is a key factor affecting the reproductive performance of sows. In the modern swine industry, sows typically enter estrus between three and five days post-weaning, with no more than 90% returning to estrus by day 7 post-weaning (Poleze et al., 2006). To make sows return to estrus as soon as possible, hormone induction is usually used. The use of hormones to induce estrus in sows has a long history (Kilgour and

Choquenot, 1994). The follicular development cycle of gilts can be regulated with hormones (De Rensis and Kirkwood, 2016). Studies have shown that the dose of estradiol benzoate is positively correlated with the duration of estrus (Dial et al., 1983).

The gut microbiota is influenced by the physiological state and hormones of the animals. Gut microbes play a role in lipid disorders caused by estrogen deficiency (Guo et al., 2023). In our previous study, the intestinal microbial composition and microbial metabolism of Diannan small-ear sows were found to be significantly different between diestrus and metestrus (Guan et al., 2022). *Lactobacillus* and *S24-7* were abundant in the feces of sows that returned to estrus normally, while *Streptococcus luteciae* was more abundant in sows that did not return to estrus normally (Zhang et al., 2021). Prevotella and Treponema are abundant in the intestines of sows in normal estrus, while Lachnospiraceae is more abundant in sows that do not show puberty (Wang et al., 2021). Changes in intestinal bacteria may lead to retinol metabolism disorders, leading to estrus failure (Wang et al., 2021).

Recently, there have been few studies on the gut microbiome of hormone-induced estrus in sows. Therefore, 16S rRNA sequencing technology was used to analyze the fecal microbiota of Diannan small-ear sows during induced estrus and spontaneous estrus to explore the microbiome differences between the two groups.

2. Material and Methods

2.1. Ethics statement

The Diannan small-ear sows were raised in Kunming, Yunnan, China (25°03' N, 102°72' E, 1.89 km). Research on animals was conducted according to the institutional committee on animal use (case number: 20210513). Animals were maintained and processed in accordance with the institutional guidelines for the care and use of animals.

2.2. Experimental animals and fecal collection

Twelve Diannan small-ear sows were used in this study. They were raised on a corn-soybean formula diet with free access to water. Dietary composition is detailed in Table 1. Sows were fed twice a day. All experimental sows were in parity 2. After the piglets were weaned on day 21, fecal samples were collected from the anuses of six spontaneous estrus sows (DC group) 3-4 d after weaning and placed into 5-mL sterile tubes. On the day of weaning, 12 sows were given a single dose injection of 2 mL of a mixture containing estradiol benzoate, progesterone, and testosterone propionate (Tristerone, Shanghai Full Woo Biotechnology Co., Ltd.). Among these induced-estrus sows, fecal samples were collected from six sows that came into estrus 3-4 d after hormone injection (DB group). We observed

Table 1 - Dietary compositions

Ingredient	Content (%)	Dietary nutrition level	Value
Corn	65.16	Digestive energy (MJ/kg)	13.96
Soybean meal	15.76	Crude protein (%)	17.08
Wheat bran	10.52	Crude fiber (%)	3.20
Fish meal	4.20	Ca (%)	1.06
$Ca(HCO_3)_2$	2.10	P (%)	0.47
Soybean oil	1.05		
Salt	0.16		
Premix[1]	1.05		
Total	100.00		

[1] Premix provided the following per kg of diet: vitamin A, 10,000 IU; vitamin D, 3240 IU; vitamin E, 10 IU; vitamin K, 0.6 mg; vitamin B1, 1.0 mg; vitamin B2, 3.8 mg; vitamin B6, 1.0 mg; vitamin B12, 10.01 mg; nicotinic acid, 28.0 mg; biotin, 0.08 mg; folic acid, 0.2 mg; Cu, 4.81 mg; I, 0.14 mg; Fe, 83.98 mg; Mn, 3.02 mg; Se, 0.24 mg; Zn, 84.07 mg.

the sows in estrus/non-estrus every day at 08:00 h and 16:00 h. If we observed that the sow's vulva was red, swollen, and with increased secretions, we used a railing to separate the boar and the sow, and had one person press the back of the sow. When the sow remained in locked stance, it was considered to be in estrus. The boars and sows in this experiment were housed in separate buildings. All samples were immediately frozen in liquid nitrogen and stored at −80 °C until use.

2.3. 16S rRNA gene sequencing

The CTAB/SDS method was used to extract total fecal genomic DNA. The concentration and integrity of DNA samples were determined using a Nanodrop-1000 (Thermo Fisher Scientific, United States) and 1% agarose gel electrophoresis. The specific primers 338F (5'-ACTCCTACGGGAGGCAGCA-3') and 806R (5'-GGACTACHVGGGTWTCTAAT-3') were used to amplify the V3-V4 hypervariable region of the 16S rRNA gene. All PCR reactions were carried out in 30-μL reactions with 15 μL of Phusion High-Fidelity PCR Master Mix (New England Biolabs, United Kingdom), 0.2 μM of forward and reverse primers, and about 10 ng template DNA. Thermal cycling consisted of initial denaturation at 98 °C for 1 min, followed by 30 cycles of denaturation at 98 °C for 10 s, annealing at 50 °C for 30 s, and elongation at 72 °C for 30 s; finally, 72 °C for 5 min. The same volume of 1× loading buffer (contained SYB green) was mixed with PCR products and the electrophoresis was operated on 2% agarose gel for detection. The mixture of PCR products was purified using the GeneJETTM Gel Extraction Kit (Thermo Fisher Scientific, United States). After purification, the PCR products were used for library construction. Sequencing libraries were generated using Ion Plus Fragment Library Kit 48 rxns (Thermo Scientific, United States). The library quality was assessed on the Qubit@ 2.0 Fluorometer (Thermo Scientific). The library was sequenced on an Ion S5™ XL platform, and single-end reads of 400 bp/600 bp were generated.

2.4. Data analysis

Single-end reads was assigned to samples based on their unique barcode and truncated by cutting off the barcode and primer sequence. Raw reads were quality filtered using the quality control process of Cutadapt (Martin, 2011) (version 1.9.1) to obtain high-quality clean reads. Chimeric sequences were detected using the UCHIME algorithm (Edgar et al., 2011) by comparing reads to the Silva database (Quast et al., 2013). Subsequently, the chimera sequences were removed (Haas et al., 2011). Sequence analysis was performed using Uparse software (version 7.0.1001) (Edgar, 2013).

The sequence condition for being assigned to the same operational taxonomic unit (OTU) is that the similarity is ≥ 97%. The representative sequences of each OTU are screened and annotated with taxonomic information using the Silva database (Mothur algorithm). Based on the abundance of the species, the correlation coefficient values (Spearman correlation) of each phylum/genus were calculated, the correlation coefficient matrix was obtained, and the filtering conditions were set: the cutoff value (> 0.6) set to filter out weakly related connections; node self-joining was filtered out; connections with node abundance less than 0.005% were removed. According to the relevant value of filtration, taking bacteria as nodes and values as edges, we used Graphviz-2.38.0 to draw network diagrams.

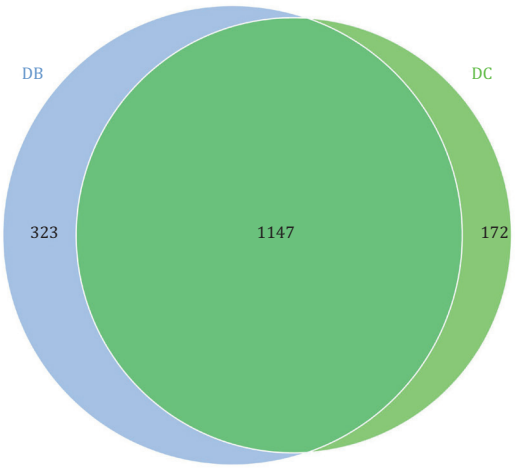
Alpha diversity indices in our sample were calculated using QIIME (version 1.7.0) and displayed using R software (version 2.15.3). Alpha diversity difference between groups was calculated by Welch's t-test. Unweighted pairwise mean arithmetic (UPGMA) clustering was performed using QIIME software (version 1.7.0) as a hierarchical clustering method to analyze beta diversity. Principal Coordinate Analysis (PCoA) was performed to get principal coordinates and visualize from complex, multidimensional data. A distance matrix of weighted or unweighted UniFrac among samples obtained before was transformed to a new set of orthogonal axes, by which the maximum variation factor is demonstrated by first principal coordinate, and the second maximum one by the second principal coordinate, and so on. The PCoA analysis was displayed by WGCNA package, stat packages, and ggplot2 package in R software (version 2.15.3).

The linear discriminant criterion (LDA Score) filtering value of LEfSe software was set to 2 (Segata et al., 2011) to conduct species analysis of differential species among groups. The Tax4Fun software was compared with the SILVA database for functional prediction. Tax4Fun functional prediction was achieved by the nearest neighbor method based on the minimum 16S rRNA sequence similarity by extracting the KEGG database prokaryotic whole genome 16S rRNA gene sequence and aligning it to the SILVA SSU Ref NR database using BLASTN algorithm (BLAST Bitscore >1500) to establish a correlation matrix and map the prokaryotic whole genome functional information of the KEGG database annotated by UProC and PAUDA to the SILVA database to implement the SILVA database function annotation. The sequenced samples were clustered out of the OTU using the SILVA database sequence as a reference sequence to obtain functional annotation information. Analysis of function difference between groups was calculated by Welch's t-test.

3. Results

3.1. Analysis of basic sequencing information for fecal samples

An average of 77,463 clean reads per sample was acquired. All sequences were assigned to 1,487 OTU with a species similarity of ≥97%. Based on the results of the OTU analysis obtained through clustering, the Venn diagram was used to analyze the shared and unique OTU in the DB and DC groups (Figure 1). A total of 1,470 and 1,319 OTU were observed in the DB and DC groups, respectively. The two groups shared 1,147 OTU. The number of unique numbers in the DB and DC groups were 323 and 172, respectively.

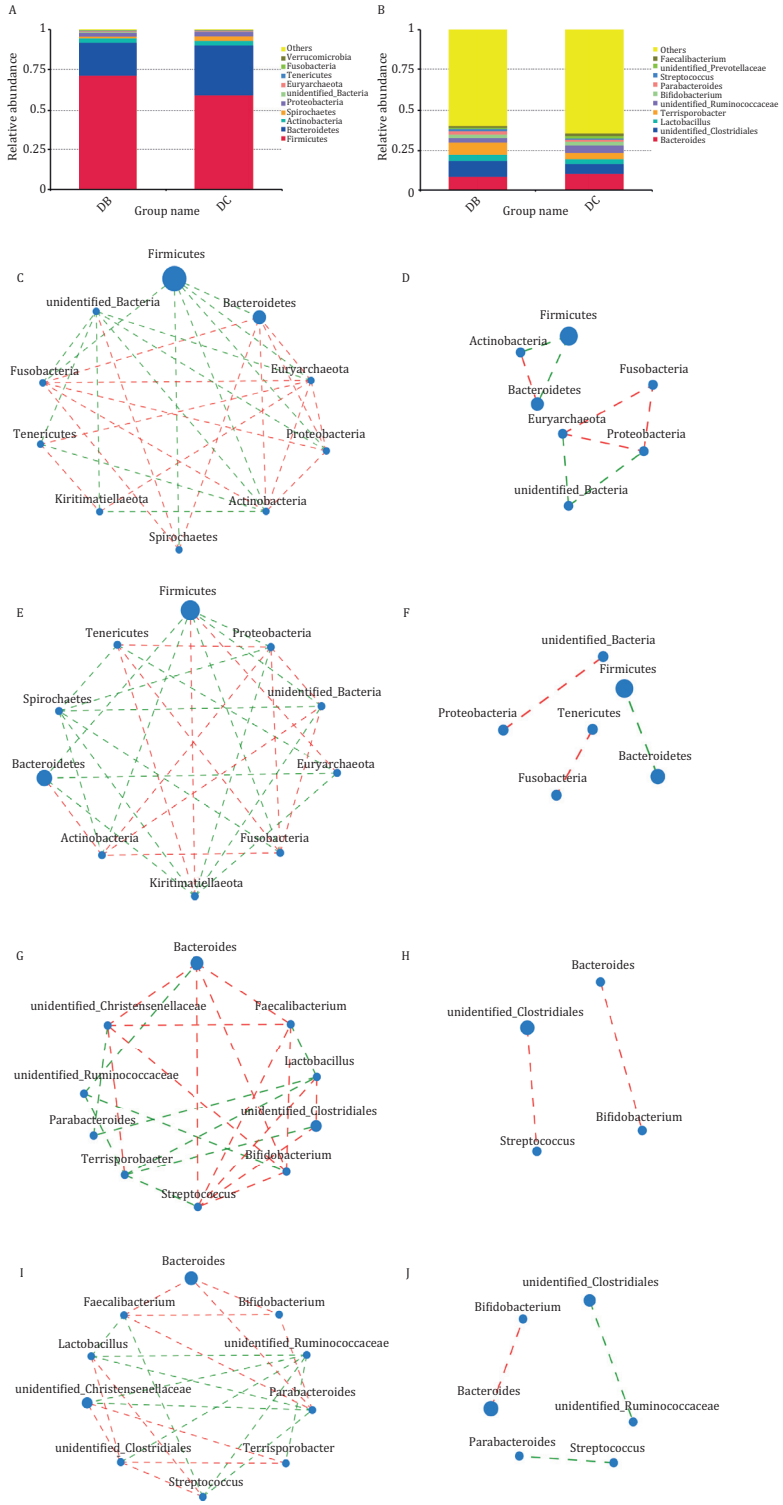


OTU - operational taxonomic unit.

Figure 1 - Venn diagram depicting the overlap of OTU in Diannan small-ear sows during induced (DB) and spontaneous (DC) estrus.

3.2. Comparison of gut microbial composition between induced and spontaneous estrus of Diannan small-ear sows

At the phylum level, the most abundant bacteria in the DB group were Firmicutes (71.32%), Bacteroidetes (20.71%), Actinobacteria (2.77%), and Proteobacteria (2.27%) (Figure 2A and Table 2). In the DC group, the most abundant bacteria were Firmicutes (59.35%), Bacteroidetes (31.05%), Actinobacteria (2.82%), and Proteobacteria (2.77%) (Figure 2A and Table 2).



A: The top ten phyla in terms of relative abundance. B: The top ten genera in terms of relative abundance. C: Spearman correlation network of the top ten phyla by relative abundance in the sows during induced estrus. D: At the phylum level, the Spearman correlation network showed significant differences in the sows of induced estrus ($P<0.05$). E: Spearman correlation network of the top ten phyla by relative abundance in the sows during spontaneous estrus. F: At the phylum level, the Spearman correlation network showed significant differences in the sows of spontaneous estrus ($P<0.05$). G: Spearman correlation network of the top ten genera based on their relative abundance in the sows during induced estrus. H: At the genus level, a Spearman correlation network was constructed to identify significant differences in the sows with induced estrus ($P<0.05$). I: Spearman correlation network of the top ten genera based on their relative abundance in sows during spontaneous estrus. J: At the genus level, the Spearman correlation network showed significant differences in the sows experiencing spontaneous estrus ($P<0.05$).
The red dotted line represents a positive correlation; the green dotted line represents a negative correlation; the size of the point indicates the abundance of the species.

Figure 2 - The gut microbial composition and Spearman correlation network of Diannan small-ear sows during induced (DB) and spontaneous (DC) estrus.

At the genus level, the relative abundances of *Bacteroides*, *unidentified_Clostridiales*, *Lactobacillus*, *Terrisporobacter*, and *unidentified_ Ruminococcaceae* ranked in the top five. The relative abundances of the DB group were 9.31, 9.60, 3.81, 7.39, and 2.70%, respectively (Figure 2B and Table 3). The relative abundances of the DC group were 11.11, 5.95, 2.97, 3.77, and 4.50%, respectively (Figure 2B and Table 3).

Spearman correlation networks were constructed for the top ten phyla based on their relative abundance in the DB (Figure 2C) and DC (Figure 2E) groups. There was a significant negative correlation between Firmicutes and Bacteroidetes in the DB (Figure 2D) and DC (Figure 2F) groups ($P<0.05$, $r = -0.94$). Spearman correlation networks showed dominant genera in the DB (Figure 2G) and DC (Figure 2I) groups. In the DB (Figure 2H) and the DC (Figure 2J) groups, there was a significant positive correlation between *Bacteroides* and *Bifidobacterium* ($P<0.05$, $r = 0.99$).

Table 2 - Top ten phyla in terms of relative abundance

Taxonomy	DB (%)	DC (%)
Firmicutes	71.32	59.35
Bacteroidetes	20.71	31.05
Actinobacteria	2.77	2.82
Spirochaetes	1.00	2.75
Proteobacteria	2.27	2.77
unidentified_Bacteria	0.56	0.16
Euryarchaeota	0.46	0.38
Tenericutes	0.38	0.31
Fusobacteria	0.13	0.06
Verrucomicrobia	0.04	0.11

DB - sows in induced estrus; DC - sows in spontaneous estrus.

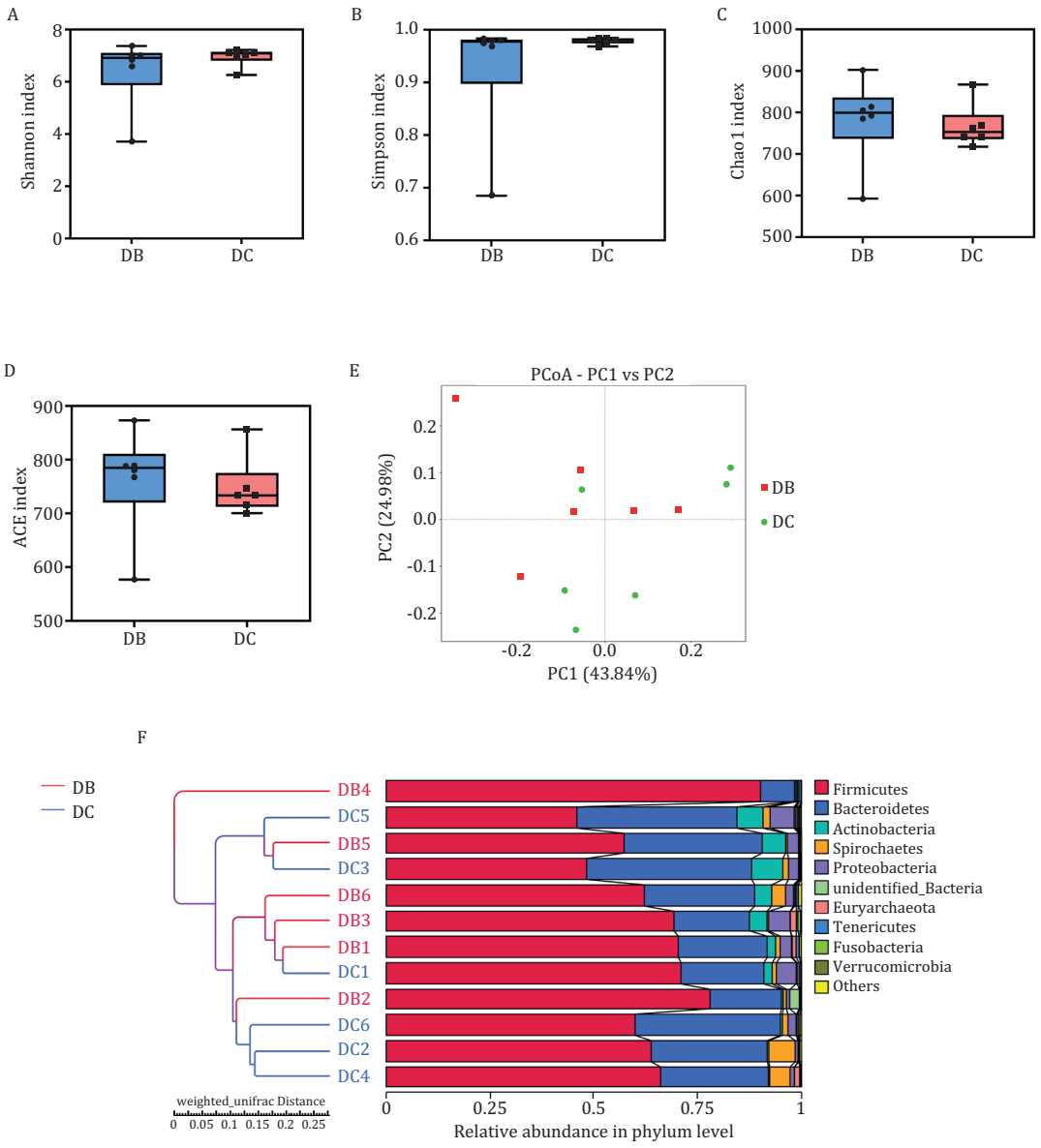
Table 3 - Top ten genera in terms of relative abundance

Taxonomy	DB (%)	DC (%)
Bacteroides	9.31	11.11
unidentified_Clostridiales	9.60	5.95
Lactobacillus	3.81	2.97
Terrisporobacter	7.39	3.77
unidentified_Ruminococcaceae	2.70	4.50
Bifidobacterium	2.11	2.29
Parabacteroides	2.05	1.21
Streptococcus	1.29	0.69
unidentified_Prevotellaceae	0.77	1.54
Faecalibacterium	1.08	1.57

DB - sows in induced estrus; DC - sows in spontaneous estrus.

3.3. Gut microbial diversity in Diannan small-ear sows during induced and spontaneous estrus

We compared the diversity of gut microbiota between sows in spontaneous and induced estrus. There was no significant difference in the Shannon index (Figure 3A), Simpson index (Figure 3B), Chao1 index (Figure 3C), and ACE index (Figure 3D) between the two groups ($P>0.05$). PCoA (Figure 3E) and UPGMA (Figure 3F) based on weighted_unifrac distance showed clustering of fecal samples in terms of β-diversity. The DB and DC groups are not two independent regions, and the cluster branches of the two groups are not completely separated. This indicates that the microbial composition of both groups is similar.

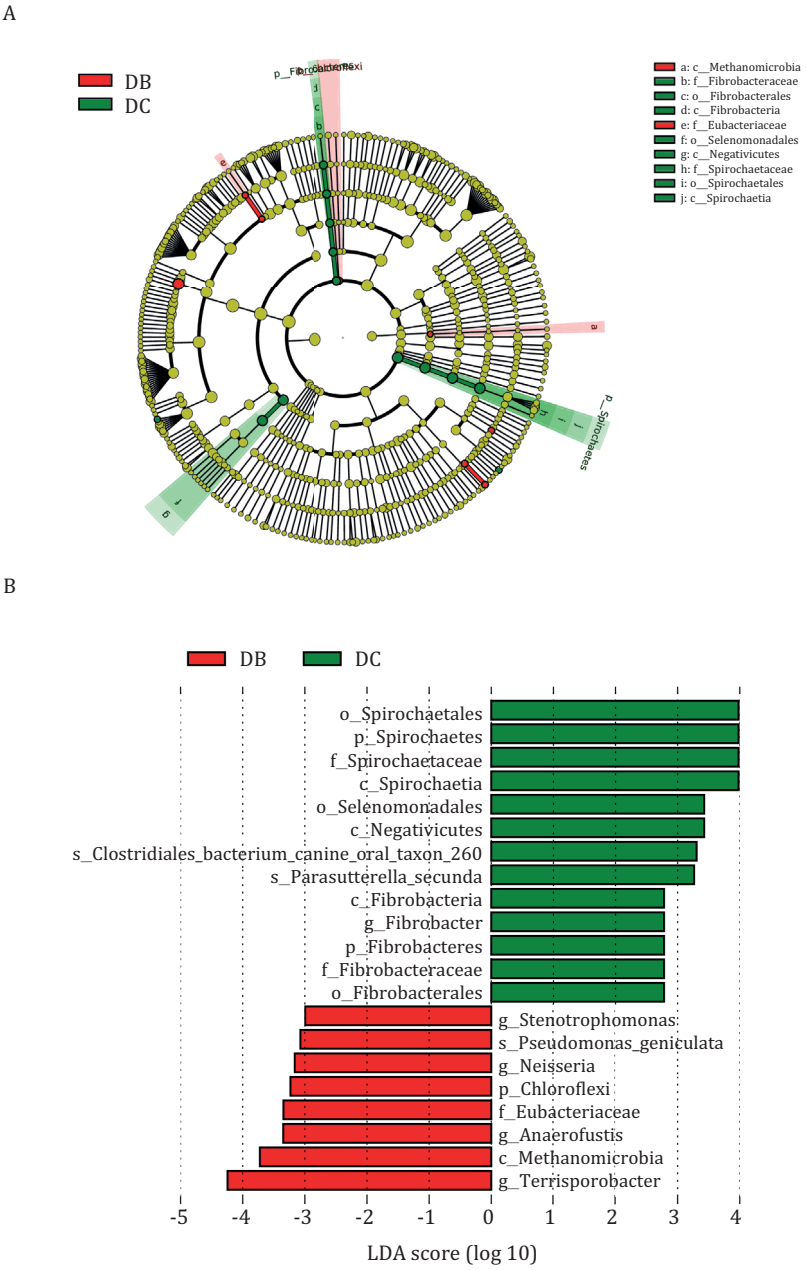


A: Shannon index; B: Simpson index; C: Chao1 index; D: ACE index; E: PCoA analysis; F: UPGMA analysis.

Figure 3 - Gut microbial diversity in Diannan small-ear sows during induced (DB) and spontaneous (DC) estrus.

3.4. LEfSe differential abundance analysis between induced and spontaneous estrus in Diannan small-ear sows

Based on LEfSe analysis, there were 21 biomarkers with LDA scores ≥ 2 in the DB and DC groups. Among them, the microorganisms that showed significant differences in the DB group mainly belonged to Chloroflexi, while those in the DC group belonged to Fibrobacteres and Spirochaetes (Figure 4A). In the phylum level, Spirochaetes and Fibrobacteres had a lower abundance in the DB group, while Chloroflexi was higher than the DC group (Figure 4B). At the genus level, the relative abundance of *Stenotrophomonas*, *Neisseria*, *Anaerofustis*, and *Terrisporobacter* was higher in the DB group, while *Fibrobacter* was lower in the DC group.

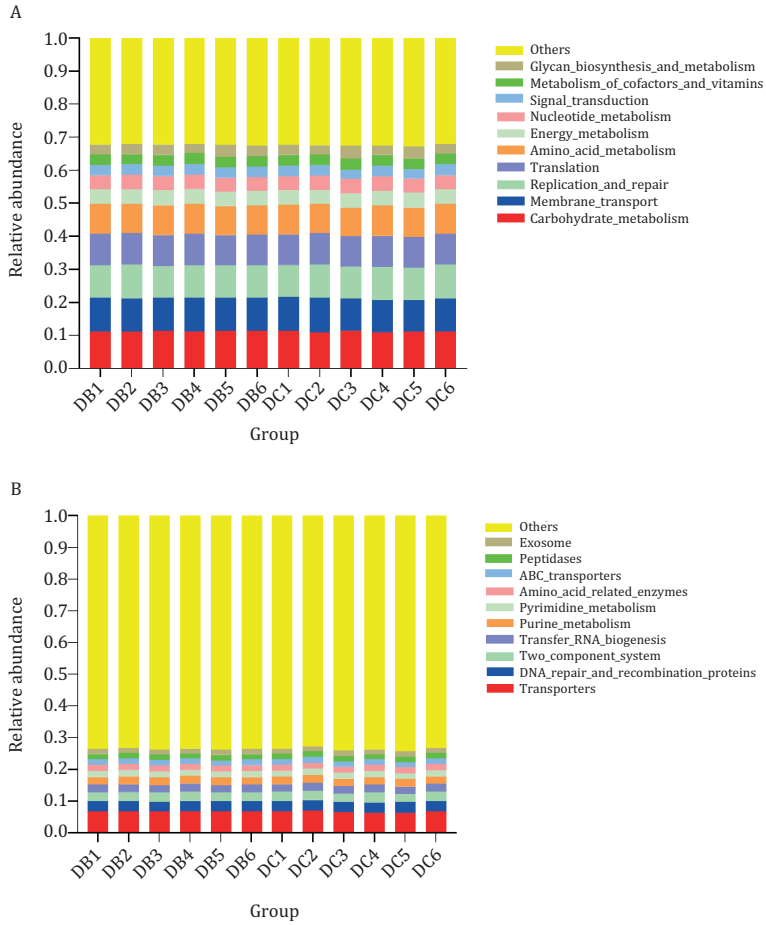


[1] These differences were determined using the criteria of an LDA>2 and P<0.05.
A: Cladogram plot of microbiome differences; B: histogram of LDA score in microbiome differences.

Figure 4 - Histograms of significant differences1 in the microbiome of Diannan small-ear sows during induced (DB) and spontaneous (DC) estrus.

3.5. Functional analysis of gut microbiota in induced and spontaneous estrus in Diannan small-ear sows

The potential functional capacities of the fecal microbiome were predicted using Tax4Fun. At level 2 of KEGG pathways, we selected the 10 most enriched pathways, including carbohydrate metabolism, membrane transport, replication and repair, translation, amino acid metabolism, energy metabolism, nucleotide metabolism, signal transduction, metabolism of cofactors and vitamins, and glycan biosynthesis and metabolism (Figure 5A). At level 3 of KEGG pathways, the 10 most enriched pathways include transporters, DNA repair and recombination proteins, two-component systems, transfer RNA biogenesis, purine metabolism, pyrimidine metabolism, amino acid-related enzymes, ABC transporters, and peptidases (Figure 5B).



A: Level 2; B: level 3.
DB - sows in induced estrus; DC - sows in spontaneous estrus.

Figure 5 - Predicted abundance of function annotations for the KEGG pathways in the feces of Diannan small-ear sows.

4. Discussion

Compared with spontaneous estrus, induced estrus did not change the composition and diversity of intestinal microorganisms in Diannan small-ear sows, but the relative abundance of several bacterial genera changed significantly. In this study, 16S rRNA sequencing technology was used to investigate the intestinal microorganisms present in the feces of the sows. The composition and diversity characteristics of intestinal microbiota in Diannan small-ear sows were examined during induced and spontaneous estrus, and the correlation and function of the flora were explored. The relative abundance of *Stenotrophomonas*, *Neisseria*, *Anaerofustis*, and *Terrisporobacter* in the feces of the sows with induced estrus was higher than that of sows with spontaneous estrus, while the relative abundance of *Fibrobacter* was decreased.

The findings of this study suggest that the composition of bacterial phyla in the feces of the sows during induced estrus and spontaneous estrus was similar, with the most abundant phyla being Firmicutes, Bacteroidetes, Actinobacteria, and Proteobacteria. At the genus level, the dominant relative abundances were *Bacteroides*, *unidentified_Clostridiales*, *Lactobacillus*, and *Terrisporobacter*. The dominant phyla in Meishan gilt during estrus were Firmicutes, Bacteroidetes, Spirochaetes, Proteobacteria, Tenericutes, and Actinobacteria, among which *Prevotella*, *Treponema*, and *Lactobacillus* were the dominant genera (Xu et al., 2019). In the fecal microbiota of multiparous Large White × Landrace pigs (with a parity of

3 or 4) from weaning to estrus, Firmicutes, Bacteroidetes, and Proteobacteria were the dominant phyla (Xu et al., 2020). Firmicutes, Bacteroidetes, and Fusobacteria were the three dominant phyla in the fecal samples of Dholes, and the dominant genera were *Fusobacterium*, *Bacteroides*, and *Clostridium* (Wu et al., 2020b). In the feces of buffaloes in estrus, Clostridiales was found to be the most abundant, and only Bacteroidales were present exclusively during estrus (Sharma et al., 2021). The relative abundance of Firmicutes and Bacteroidetes in the feces of these animals during estrus was the highest, followed by one of Actinobacteria, Proteobacteria, and Fusobacteria in third place.

Actinobacteria is the third dominant bacterial phylum in southern Yunnan small-eared pigs during the estrus period. Actinobacteria are pivotal in the maintenance of gut homeostasis, and *Bifidobacteria* in particular are widely used as probiotics (Binda et al., 2018). There was a significant positive correlation between *Bacteroides* and *Bifidobacterium*. Previous studies have supported the potential of *Lactobacillus* to enhance intestinal metabolic capacity, maintain intestinal flora balance, and modulate the host immune system (Valeriano et al., 2017). *Bifidobacteria* ferment to produce short-chain fatty acids (SCFA), which have many health-promoting properties, including the maintenance of intestinal barrier integrity and anti-inflammatory functions (Sadeghpour Heravi and Hu, 2023). *Bacteroides* and *Bifidobacteria* have co-evolved to utilize various diets and host-derived glycans. They coordinate different glycan utilization systems to maintain gut microbial symbiosis and improve the fitness of their own or other communities (Singh, 2019).

Differential flora analysis showed that the relative abundance of *Stenotrophomonas*, *Neisseria*, *Anaerofustis*, and *Terrisporobacter* in Diannan small-ear sows during induced estrus was significantly higher than that during spontaneous estrus. *Stenotrophomonas* are straight rod-shaped, non-fermenting bacteria that can utilize monosaccharides or polysaccharides as carbon sources. *Stenotrophomonas maltophilia* is an opportunistic human pathogen that normally spares healthy individuals; however, it is associated with high morbidity and mortality in severely immunocompromised and frail individuals (An and Berg, 2018). Non-pathogenic *Neisseria* can cause invasive infections. However, *Neisseria lactamica*, a nonpathogenic commensal, has been shown to inhibit the colonization of *Neisseria meningitidis* (Dorey et al., 2019).

Some sex hormones can affect the composition of gut microbes. Studies have reported the direct effects of sex hormones on bacterial metabolism, growth, and the expression of virulence (García-Gómez et al., 2013). Importantly, studies show that the expression of steroid nuclear receptors, including estrogen receptor-β, can determine the composition of the intestinal microbiota (Mulak et al., 2014). 16S rRNA sequencing of feces from estrus-synchronous Simmental cows revealed alterations in the structure, composition, and function of the gut microbiota, and these changes were mediated by reproductive hormones, specifically estradiol (Wu et al., 2022). Fluctuations in reproductive hormone concentrations, particularly progesterone, lead to reduced fecal microbiome diversity during pregnancy and lactation (Mallott et al., 2020). In an *in vitro* study, progesterone stimulated the growth of *Lactobacillus reuteri* (Sovijit et al., 2021). Studies have shown that progesterone promotes the growth of *Bifidobacterium* during late pregnancy (Nuriel-Ohayon et al., 2019). Induction of estrus affects specific bacterial taxa in the fecal microbiota of Diannan small-ear sows but does not alter the overall community structure.

5. Conclusions

The findings of the present study suggest that Firmicutes, Bacteroidetes, Actinobacteria, and Proteobacteria are dominant in Diannan small-ear sows in estrus. There is a significant negative correlation between Firmicutes and Bacteroidetes and a significant positive correlation between *Bacteroides* and *Bifidobacterium*. The relative abundance of *Stenotrophomonas*, *Neisseria*, *Anaerofustis*, and *Terrisporobacter* in Diannan small-ear sows is significantly higher during induced estrus than during spontaneous estrus. Sex hormone-induced estrus alters the relative abundance of specific microbes in the feces of Diannan small-ear sows, but it does not affect the overall composition and diversity.

Conflict of Interest

The authors declare no conflict of interest.

Author Contributions

Data curation: Yi, L. and Li, Z. **Funding acquisition:** Zhao, Y. and Zhao, S. **Resources:** Guan, X. **Software:** Cheng, W. and Xie, Y. **Visualization:** Li, Q. and Zhu, J. **Writing – original draft:** Yi, L. and Li, Z. **Writing – review & editing:** Zhao, Y. and Zhao, S.

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