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Intestinal microbiome analysis of Laiwu piglets with diarrhea through 16s RNA gene sequencing

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Abstract

Laiwu pigs are known to be highly resistant against diseases, but their piglet's can still have diarrhea that can reduces their growth and consequently their market value later. To confirm that the imbalance of intestinal micro flora might have caused the mild diarrhea in Laiwu piglets, 16S rRNA technology was used to analyze bacterial communities at various taxonomic levels and determine the abundance and differences associated with dynamic changes of beneficial and pathogenic intestinal bacteria. The results showed that at the phylum level, Firmicutes, Bacteroidetes, and Proteobacteria were the dominant phyla in both diarrhea-symptomatic and healthy piglets. Compared with healthy ones, the relative abundance of Spirochaetes decreased ($P<0.05$) but that of Firmicutes and Fusobacteria increased ($P<0.05$) significantly in diarrhea-symptomatic piglets. In addition, linear discriminant analysis effect size (LEfSe) analysis showed that Fusobacterium and Prevotellas, genera associated with diseases such as colorectal cancer and irritable bowel syndrome, were highly abundant in the diarrhea-symptomatic piglet group, whilehealthypigletshad more Erysipelothrix, Psychrobacter, Treponcma, Jeotgalibaca, Acinetobacter, and Christensenellaceae in their intestines, suggesting that a decrease in the relative abundance of beneficial bacteria or an increase of pathogenic bacterial population may have been the cause of the mild diarrhea occurred in Laiwu piglets. Moreover, the increase of Fusobacterium and Prevotella population might have irritated Laiwu piglets to show symptoms of mild diarrhea, and theabundance of Erysipelothrix, Psychrobacter, Treponcma, Jeotgalibaca, Acinetobacter and Christensenellaceae in Laiwu pigs may well have contributed to their high resistance against diseases. The results that we have provided here should have shed some lights on how to manage intestinal microbiome, especially in piglets.

Keywords: Intestinal microflora, diarrhea piglets, healthy piglets, Laiwu pig, resistant against diseases

Introduction

Piglet Diarrhea refers to a series of symptoms such as gastrointestinal discomfort, loss of appetite, dysbiosis, pathogen infection, and abnormalities of digestive system. The severity of the symptoms can range from mild dyspepsia to severe diarrhea, which may lead to dehydration and malnutrition. Therefore, accurate diagnosis at the initial stage of detecting diarrhea symptoms in piglets is a key step for timely preventive and therapeutic measures.

In general, piglet diarrhea is usually caused by:

- Inappropriate diet such as overfeeding or early introduction of solid food
- Fluctuation of micro biome in the digestion system of piglets, especially of beneficial and pathogenic bacterial populations
- Damages to the intestinal mucosa due to invaded pathogens such as bacteria, viruses or parasites
- Changes of environmental conditions such as temperature and humidity (Kongsted et al., 2018; Jacobso et al 2022).

To determine the cause of piglet diarrhea, veterinarians are usually involved in the diagnostic processthrough symptoms observation, clinicalmanifestations, epidemic investigation, laboratory testing and other procedures. The whole process of etiological diagnosis is complex and time-consuming. Although the causes of diarrhea in piglets are different, they all occur (Gryaznova et al., 2022) due to dynamic changes of intestinal bacterial communities. The intestinal microbiota of pigs refers to the collection of a large number of bacteria, viruses, fungi and archaea that colonize the intestinal tracts of pigs. Bacteria account for the majority of the intestinal flora, which has been one of current researches worldwide (Zhou et al., 2021). It is also uncertain (Huang et al., 2019) whether intestinal bacteria preexisted in the uterus of sows before birth, however, the most acceptable theory has been to believe that piglets acquire microflora in their digesting system during or after birth through maternal birth canal, feeding, discharged feces and surrounding environment (Moeller et al., 2018). The intestinal microbiome in piglets mainly include aerobic bacteria, facultative anaerobes, and anaerobes (Wang et al., 2019) and the composition of the piglet gut microbiota is in a status of constant fluctuation due to affecting conditions such as genetics (Xu et al., 2020), sex (He et al., 2019), age, and diet (Wang et al., 2019) and these changes in dominant species and the population level of intestinal bacterial flora in piglets may possibly and directly cause diarrhea in piglets (Gryaznova et al., 2022). Small subunit ribosomal RNA (16S rRNA) encodes a subunit of ribosomal RNA that is responsible for the essential process involved in passing genetic messages to functional cell components through mRNA translation to proteins.

It is highly conserved in structure and functions and exists in all organisms. Therefore, its sequences have been widely used as a molecular marker (Johnson et al., 2019) in thephylogenetic classification and discrimination of prokaryotic microorganisms.

At present, the use of variable regions on 16S rDNA combined with high-throughput sequencing to achieve the discrimination and identification of microbial composition in a specific environment has solved the problem that is encountered because that many bacteria cannot be or difficult to cultured or identified in a laboratory setting (Chen et al., 2021). Up to date, 16S rRNA heavy genome sequencing analysis of intestinal bacteria in piglets has provided a robust and convenient tool to analyze the whole microbial communities in intestines of piglets, understand the structure and composition of their dynamics, and compare the differences in composition and population levels of microbiome in given samples, aiming to reveal possible causal changes in the microbiota and potential pathogenic bacteria and potential dysbiosis in piglets with diarrhea (Kong et al., 2022; Zhang et al., 2018). Laiwu pig is a local black swine species domestic to Shandong Province. It has the characteristics of high fecundity, good meat quality, tolerance to roughage feed, and strong disease resistance. Due to its unique resistance/tolerance

against many diseases, Laiwu pigs have gained much popularity for pig farming in China (Cui et al., 2019). The intestinal microbial flora of the piglets is relatively stable and resistant to metabolic diseases and diarrhea caused by common pathogenic bacteria and viruses (Wei et al., 2020; Lai et al., 2023).mild symptoms similar to diarrhea do occurs in Laiwu piglets, which leads to a hypothesis that the mild type of diarrhea in Laiwu piglets might have been caused by the abnormal fluctuation of intestinal microflora and directly affects the growth and market value of adult pigs (Zhou et al., 2021).

This study was designed to analyze a large number of 16S rRNA high- throughput sequencing data in derived from diarrhea samples of Laiwu piglets to reveal the differences and dynamic changes of intestinal microflora between healthy and diarrhea-symptomatic Laiwu piglets. A possible correlation and interaction of main beneficial and pathogenic intestinal microbes and their underlying mechanism are also to be understood. Ultimately, this study is expected to provide a theoretical basis and specific guidelines for prevention and treatment of diarrhea in Laiwu piglets by using beneficial bacteria.

Materials and Methods

Sample collection

The experimental Laiwu piglets were provided by Delis Group Laiwu Pig Breeding Farm (Laiwu, Shandong Province). Three Laiwu piglets, with or without diarrhea from the same litter produced by the same sow were used for fecal samples collection on the 10th day. Healthy piglets were labeled as ZC-LW, and diarrhea symptomatic piglets as FX- LW. Fecal samples from diarrheal piglets were tested by immunogold kits to ensure that there were neither virus nor parasites in fecal smears. Fecal sample were collected when piglets defecate, placed immediately in a sterile container, packed in a 5 m L centrifuge tube, and quickly frozen in liquid nitrogen before being further processed.

Reagents and instruments

D5625-02 Soil DNA Kit (Genomic DNA Extraction Kit) from Omega Biotek Inc., ABI GeneAmp® 9700 PCR instrument from Thermo Fisher Scientific (China) Co., LTD., HE-220 electrophoresis device with Tanon 3500 automatic digital gel imaging system from Shanghai Tiangneng Technology Co., LTD., and a 5 418 R benchtop high speed refrigerated centrifuge from Abender (Shanghai) International Trading Co.,LTD. were used throughout the study. DNA PCR-Free DNA library building kit (Illumina), Phusion® High-Fidelity PCRMaster Mix GC Buffer and Phusion® High-Fidelity DNA Polymerase (New England Biolabs), 2×Taq PCR Master Mix, SYBR Premix Ex Taq II (Tli RNase H Plus), Hind III restriction enzyme, p MD 19-T Vector Kit were all from Bao BioEngineering (Dalian) Co., LTD. Axygen AP-GX-50 DNA Gel Recovery Kit (Axygen Inc, USA) was also used.

Experimental Methods

Extraction of DNA from fecal samples

Total bacterial DNA extracted from the fecal samples was tested for integrity and purity by 1% agarose gel electrophoresis, and the DNA amount (OD260/280 ratio) was checked by the Nanodrop® ND-1000 spectrophotometer. Qubit Fluorometer was used for accurate quantification of DNA concentration, and samples were diluted to 1 ng/μL using sterile water and stored at low temperature. Amplification of the V4 region of the 16S rRNA gene The V4 region of 16S rRNA gene was selected as the target fragment for amplification and sequencing. First, specific primers with barcode at both ends were synthesized, and 338-806 bp of 16S rDNA was amplified. The primer sequence was: 338F:5'-ACTCCTACGGGAGGCAGCAG-3', 806R:5'-GGACTACHVGGGTWTCTAAT-3'. Amplification reactions were performed using TransStart Fastpfu DNA polymerase and a GeneAmp model 9700 PCR apparatus from ABI. The diluted bacterial genomic DNA was used as a template and the reaction system was set up as: 5×Fast Pfu Buffer 4μL, 2.5mmol/L dNTPs 2μL, forward primer (5μmol/L) 0.8μL, reverse primer (5μmol/L)

0.8μL, Fast Pfu Polymerase 0.4 μL, template DNA 10 ng, ddH2O supplemented to 20 μL. PCR reaction conditions were as follows: pre-denaturation at 95℃ for 3min followed by 27 cycles of denaturation at 95℃for 30s, annealing at 55℃ for 30s, and extension at 72℃ for 45s. The final extension was at 72℃ for 10 min.

PCR amplifications were examined by electrophoresis on 2% agarose gel and quantified using Invitrogen/Quanti Fluor™-ST fluorescence quantitative system. According to the concentration of the PCR products, the samples were mixed in equal quantities and detected by 2% agarose gel electrophoresis. The target DNA bands were recovered by gel recovery kit. Sequencing data processing and analysis Operational taxonomic unit (OTU) and OTU clustering of optimized sequences was performed using Usearch (Version 7.1). Firstly, non-repetitive sequences were extracted to reduce the amount of redundant computation during the analysis according to the online manual (http://drive5.com/usearch/manual/dereplicy.html). After removing non-repetitive single sequences, OTU clustering was performed on the non-repetitive sequences that had a 97% similarity, also removing chimeras during the clustering process. Finally, all optimized sequences were aligned to the OUT representative sequences, and the sequences with more than 97% similarity to the OTU representative sequences were selected to generate the OTU table.

Diversity index analysis

A series of statistical analysis indices were used to estimate species abundance and diversity of microbiome, especially of bacterial communities in samples, Index Chao (http:/ /www. mothur. org/wiki/Chao) and Ace (http://www.mothur.org/wiki/Ace); Shannon (http://www.mothur.org/wiki/Shannon) and Simpson (http://www.mothur.org) werecalculatedfortheindexofmicrobial diversity (Com-munity diversity)/wiki/Simpson). Where Chao is an index that estimates the number of OTUs contained in a sample using the chao1 algorithm; Ace is an index used to estimate the number of OTUs in the community, and the algorithm is different from Chao. Simpson was one of the indices used to estimate microbial diversity in samples. The higher the Simpson index value, the lower the diversity of the community. Shannon is one of the microbial diversity indices used to estimate microbial diversity in samples. Shannon and Simpson diversity indices are often used to reflect the alpha diversity index.

Taxonomic analysis

Taxonomic analysis was performed using the RDP classifier Bayesian algorithm (confidence threshold = 0.7) for 97% similarity level OTU representative sequences and in phylum, class, order, family, genus levels of community composition for each sample. Select Silva (Release 123 http://www.Arb-silva.de) and unite (Release 7.0, http://unite.ut.ee/index.php) as a reference genome database. Dendrogram analysis of multiple genomes similarity a diagram of hierarchical clustering was used to describe and compare the similarity and difference relationships among multiple samples. Firstly, the distance between samples was calculated using analgorithm describing community composition relationship and structure. That is, hierarchical clustering analysis was performed according to beta diversity distance matrix, and the beta diversity distance matrix was calculated by Qiime. Bray Curtis algorithm was used to calculate the distance matrix, and UPGMA (unweighted pair group method with arithmetic mean) algorithm was used to construct the tree structure, and the tree relationship form was obtained for visual analysis.

Results

OTU quantitative analysis

High-throughput sequencing of fecal samples from 6 piglets, 3 healthy and 3 with diarrhea symptom, was performed on the Illumina Novaseq sequencing platform, and a total of 477,800 high-quality sequences and 432,528 valid sequences were obtained, with an average length of 419- 423bp. Usearch software was used to cluster the sequences at 97.0% similarity level. A total of 731 OTUs was obtained and the number of OTUs in each group is shown in Figure 1. The results showed that the num-

ber of OTUs in diarrhea symptomatic piglets was less than that in healthy piglets.

Among them, there were 612 OTUs in the diarrhea symptomatic piglet group and thehealthy piglet group, with 11 OTUs unique to the diarrhea symptomatic piglet group and 108 OTUs unique to the healthy piglet group. In conclusion, the number of OTUs unique to healthy piglets was much higher than that of piglets with diarrhea symptoms. Microbial diversity analysis in intestines of healthy and diarrhea symptomatic piglets.

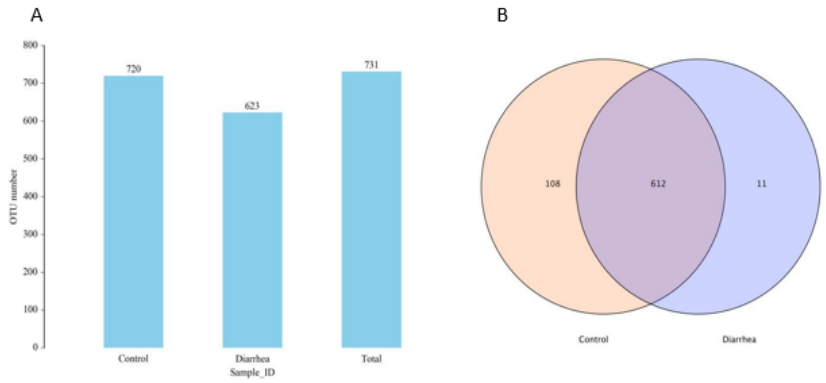

Figure 1: OUT analysis of healthy and diarrhea symptomatic piglet group (A) number of OTUs；(B) Overlapping OTUs.

Alpha diversity analysis

Alpha diversity analysis was performed to evaluate the abundance and diversity of intestine microbiota of Laiwu piglets and Alpha curves (Figure 2) increased exponentially to a plateau, indicating that the sample sequence is sufficient for Alpha diversity analysis. Also, the coverage index of all samples/sequences was greater than 0.99 that is minimal requirement for the bacterial diversity analysis. Alpha diversity analysis by QIIME2 software (Table 1) showed that Chao index, Ace index and Shannon index of diarrhea piglets group were higher than those of healthy piglets group, indicating that the abundance and diversity of diarrhea piglets were lower than those of healthy piglets, but the difference between the two groups was not significant.

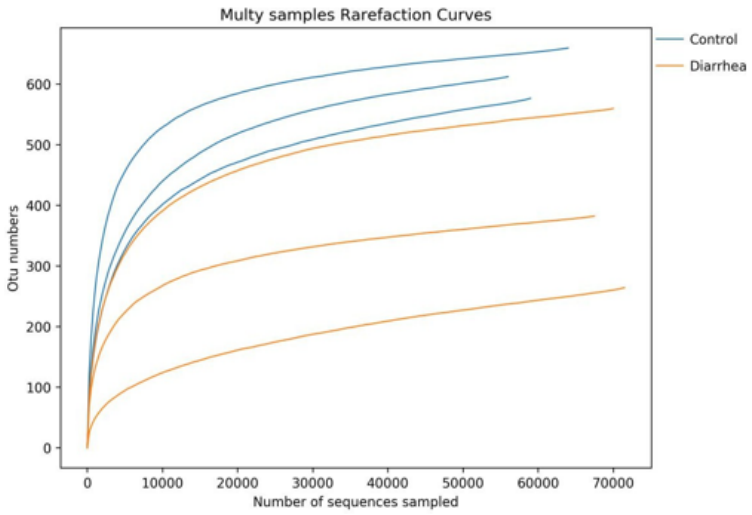

Figure 2: OUT numbers in various diluted samples

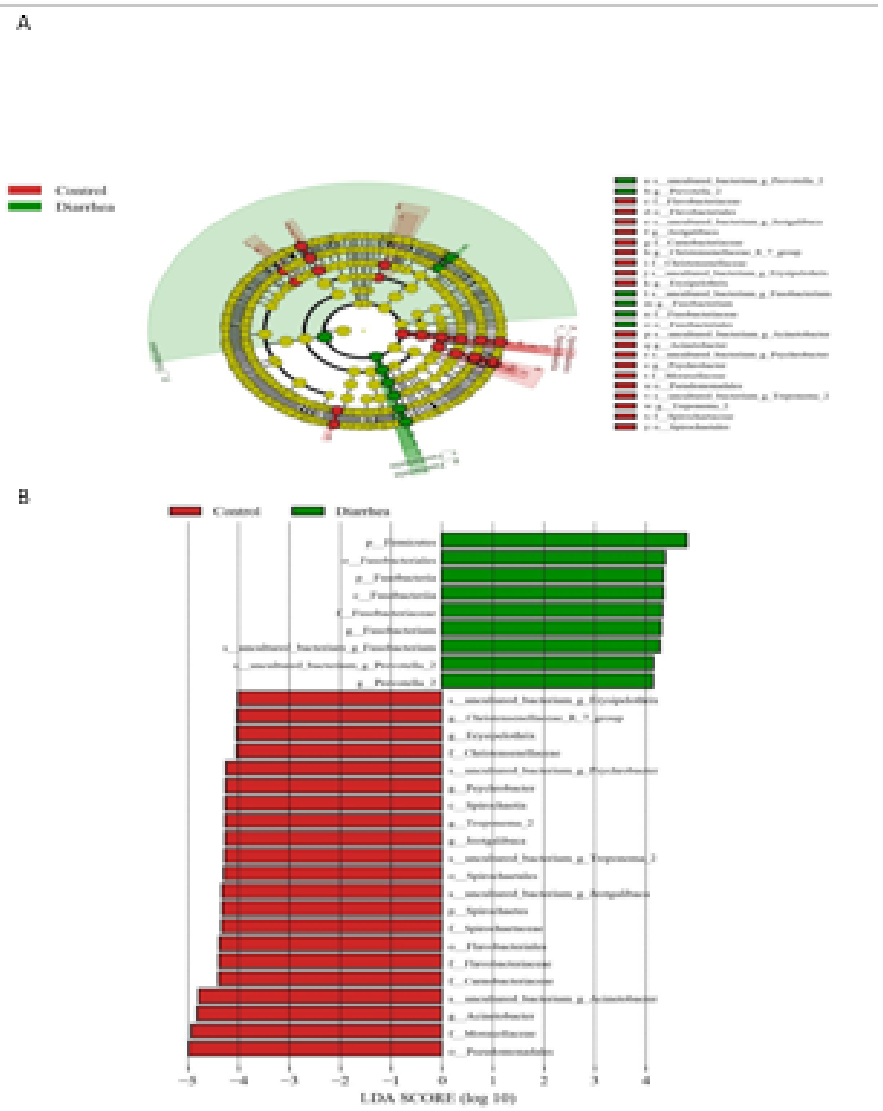

Figure 3: Differential microbiota in intestines of Laiwu piglets at different taxonomic levels

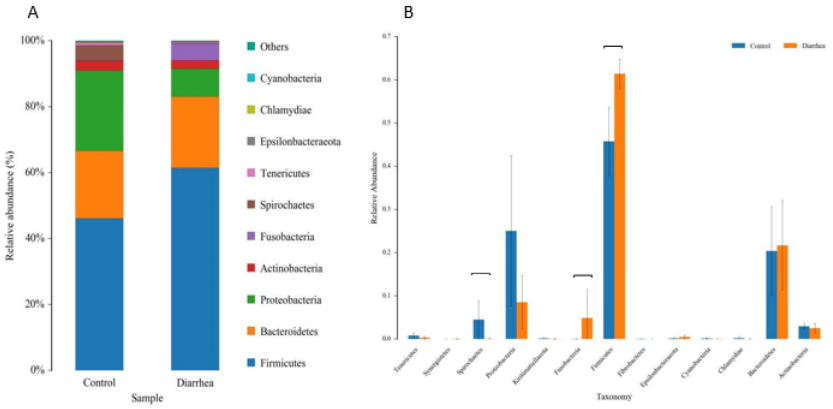

Figure 4: Microbial compositions at the phylum level

Beta diversity analysis

Principal coordinate analysis (PCoA) was performed by QIIME software (Figure 3) and the coordinates of the diarrhea piglet group were far from those of the healthy piglet group, indicating that microbial compositions of the diarrhea piglet group were

quite different from those of the healthy piglet group. Analysis of phylum flora structure between diarrhea piglets and healthy piglets the microbiome of fecal samples from 6 piglets were annotated to 13 phyla and 235 genera. At the phylum level (Figure 4A), Firmicutes, Bacteroidetes, and Proteobacteria were the dominant phyla in both diarrhea and healthy piglet group. As shown in Figure 4 B, compared with the healthy piglet group, the relative abundance of Spirochaetes in diarrhea piglet group was significantly reduced ($P < 0.05$), while the relative abundance of Firmicutes and Fusobacteria was significantly increased ($P< 0.05$).

Analysis of bacterial genera of healthy and diarrhea symptomatic Laiwu pigtets LEfSe analysis was used to evaluate taxonomic differences of intestinal microbiota between healthy and diarrhea symptomatic piglets. There were 30 significantly different taxonomic classification levels, including 3 phyla, 2 classes, 4 orders, 6 families, 8 genera and 7 species (Figure 5). At the genus level, Fusobacterium and Prevotellas were highly abundant in the diarrhea piglet group while Erysipelothrix, Psychrobacter, Treponcma, Jeotgalibaca, Acinetobacter, and Christensenellaceae were highly abundant in the healthy piglet group.

Discussion

There are a large number of microbial communities in the gastrointestinal tract of humans and animals. These microorganisms are the result of long-term co-evolution with hosts, and their composition and population level are closely related to the health of their residing hosts (Athanasopoulou et al., 2023; Kuznetsova et al., 2022). Studies

Of the gut microbiome have extended from their association initially with simple digestive problems or obesity (Henao-Mejia et al., 2012) and recently with the defective immune system (Upadhyay et al., 2012), cancer occurance (Louis et al., 2014), and even the health (Koch et al., 2016; Vatanen et al., 2016) conditions carrying on to their offspring. The early establishment and maintenance of intestinal microbiota affect the later health and growth rate of adult pigs. Factors such as genetic features, weaning, diet, antibiotic use, and prebiotic addition are crucial for diversity, composition, and succession of intestinal microbiota in piglets (Vatanen et al., 2016; Robin et al., 2019; Zhang et al., 2020). The dominant phylum of intestinal microbial communities of piglets usually include Firmicutes, Bacteroidetes, Proteobacteria and so on. Compared with healthy piglets, the abundance and diversity of diarrhea piglets were relatively reduced, and the composition of the microbiota was significantly different (Han et al., 2019). Zhou, et al. found that Fusobacteria were only detected in the diarrhea but not in the non-diarrhea piglet group (Zhou et al., 2022), which is consistent with ou findings. However, the detection of Spirochaetes in in healthy piglets with our study is contradictory to the finding by Zhou, et al., which might have been due to the differences (Hu et al., 2023) in the composition of intestinal microbiome between different breeds of pigs. Yu et al. showed that dietary supplementation of weanling piglets with high zinc oxide or antibiotics significantly promoted the number of ileal Spirochetes, Tenmicutes, Uroarchaeota, Verrucomicrobia and TM7, and reduced the number of Chlamydia (Ting et al., 2017). In the study by Xue, et al., the abundance of Spirochaetes, Proteobacteria, Fusobacteria, and Synergistetes in the fecal samples of piglets with fecal microbiota transplantation was significantly higher than that in the CO group at the age of 10 days (Xue et al., 2018). It is suggested that the decrease of Spirochaetes and the increase of Fusobacteria may be related to the intestinal dysfunction of piglets.

At the genus level, LEfSe analysis showed that Fusobacterium and Prevotella were more abundant in diarrhea piglets than those in healthy piglets. A significant increase of Fusobacterium is likely associated with the imbalance of intestinal microbiota in piglets with diarrhea (Kong et al., 2022). Fusobacterium is one of symbiotic bacteria in humans and animals and its abundance in feces is believed to be closely related to the presence of colorectal cancer (Amitay et al., 2017). Also, its presence may inhibit the production of butyrate and result in the CRC microbiome enriched in Fusobacterium and reduced butyrate productivity inside hosts (Hertel et al., 2021). Prevotellas has been assumed to be associated with proinflammatory functions (Larsen et al., 2017; Iljazovic et al., 2021), and studies have shown that it may interact with other bacteria to induce visceral hypersensitivity and exacerbate IBS symptoms by promoting carbohydrate fermentation (Majorn et al., 2017). In addition, Erysipelothrix, Psychrobac-

ter, Treponcma, Jeotgalibaca,Acinetobacter, and Christensenellaceae were highly abundant in the healthy group. Among them, the genus Acinetobacter showed the greatest difference between the two groups. Acinetobacter baumannii is a common opportunistic pathogen that widely colonize the digestive tract, skin, respiratory tract, and urogenital tract, causing bacteremia, pneumonia, endocarditis, diarrhea, and urinary and skin infections (Dong et al., 2021; Polanco et al., 2008). Resistance of A. baumannii to antibiotics is one of the main causes of nosocomial infections (Zhao et al., 2012). In this study, non-named species of the genus Acinetobacter were highly abundant in the healthy piglet group, which may be beneficial bacterial species. Diarrhea in piglets can be severe and lead to death of weaned piglets. Diarrhea affects the composition and diversity of intestinal microbial communities, and abnormal intestinal microbiota is also prone to induce symptoms of diarrhea. Studies have shown that fecal microbiota transplantation has a protective effect against diarrhea in weaned piglets. For example, Lactobacillus gasseri LA39 and Lactobacillus frumentias can be used as alternative treatment to replace antibiotics in preventing diarrhea in mammals (Hu et al., 2018). Bacillus subtilis Y-15, B. amyloliquefaciens DN6502 and B. licheniformis SDZD02 have been used to treat pig diarrhea through reconstiting beneficial intestinal bacteria in piglets (Yue et al., 2020).

In addition, feed additives such as probiotics, plant extracts, animal proteins, etc. can also be used to reduce intestinal injury-related diarrhea symptoms of piglets by regulating thecomposition, diversity and metabolic pathways of intestinal microbiome (Kong et al., 2022; Qin et al., 2022; Jin et al., 2017). In conclusion, the dynamic changes of beneficial and pathogenic bacteria in the composition and diversity of intestinal microbiota may be the main cause of diarrhea, especially the mild one in Laiwu piglets. The microbial imbalance or fluctuation due to the decrease of the relative abundance of beneficial bacteria or the increase of pathogenic bacteria may directly result in diarrhea of Laiwu piglets. It is also speculated that diarrhea in piglets may be related to the genera Fusobacterium and Prevotella, whileErysipelothrix, Psychrobacter, Treponcma, Jeotgalibaca, Acinetobacter, and Christensenellaceae are somehow related to disease resistance of Laihu pigs. More studies needs to be further explored and more evidence collected to reveal and understand the mechanisms involved in dynamic changes of intestinal microbiome and their association with piglet diarrhea and other digestive diseases to ultimately provide guidelines for the prevention and treatment of diarrhea in piglets through managing intestinal microbiome.

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Author Contribution

Cui JX conceived and designed the experiments, Tian H analyzed the experimental data, Sun XA edited it. Cui JX, Zhao NJ wrote this manuscript. All authors read and approved the final manuscript.

Competing Interests

The authors declare that there are no competing interests associated with the manuscript.

Ethics approval

This work was not need approved by the Institutional Animal Care and Use Ethics Committee of Weifang Science and Technology University and carried out in accordance with the “Guidelines for Experimental Animals” of the Ministry of

Science and Technology (Beijing, PR China).

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