Changes in Gut Microbiota by the *Lactobacillus casei* Anchoring the K88 Fimbrial Protein Prevented Newborn Piglets From Clinical Diarrhea

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In the last 20 years, accumulating evidence indicates that the gut microbiota contribute to the development, maturation, and regulation of the host immune system and mediate host anti-pathogen defenses. *Lactobacillus casei* (L.*casei*) is a normal flora of the gastrointestinal tract in mammals and, as a great mucosal delivery vehicle, has wide use in bioengineering. However, the diarrhea prevention role of commensal intestinal microbiota interfered by the recombinant *L.*casei* (r*L.*casei*) in newborn piglets is not well understood. In our study, newborn piglets orally fed with the r*L.*casei* surface displayed the fimbrial protein K88 of enterotoxigenic *Escherichia coli* (ETEC) and their feces were collected for a period of time after feeding. The next-generation sequencing of these fecal samples showed that the relative abundance of *L.*casei* was significantly increased.

The oral administration of r*L.*casei* altered the intestinal microbial community as evidenced by altered microbial diversity and microbial taxonomic composition. Remarkably, the functional enhancing of the intestinal bacterial community by r*L.*casei* was positively correlated with membrane transport, replication, and repair (p < 0.05). The specific antibody detection indicates that high levels of anti-K88 secretory immunoglobulin A (sIgA) were induced in fecal samples and systemic immunoglobulin G was produced in serum. The diarrhea rate in piglets caused by ETEC K88 was decreased by about 24%. Thus, the oral administration of r*L.*casei* not only activated the mucosal and humoral immune responses in vivo but also contributed to shape the intestinal probiotics in newborn piglets and to significantly reduce the diarrhea rates of newborn piglets.

Keywords: r*Lactobacillus casei*, ETEC K88, 16S rRNA sequencing, newborn piglets, gut microbiota, diarrhea
1 INTRODUCTION

Enterotoxigenic *Escherichia coli* (ETEC), one of the main pathogenic bacteria, can cause fatal diarrhea and edema in neonatal and weaning piglets, which leads to high morbidity and mortality around the world, especially in developing countries (Moon et al., 1979; Dorsey et al., 2010; Ikwap et al., 2016). The pathogens ETEC are non-invasive bacteria that colonize the small intestine via pili or fimbriae, where they produce enterotoxin leading to severe diarrhea that is fatal to piglets. The pili adhesins known to be important in ETEC infection to neonatal animals are K88 (F4), K99 (F5), 987P (F6), and F41 (F7) which are the key antigens inducing neutralizing antibodies (Moon et al., 1977; Morris et al., 1983; Sahagun-Ruiz et al., 2015).

It has been reported that the intestinal microbiota could resist the invasion of pathogenic microorganisms and assist the host immune system to eliminate exogenous pathogenic microorganisms (De Filippo et al., 2010; Wu et al., 2011; Gresse et al., 2017). All of the host’s diet, lifestyle, external environment, and genetic susceptibility affect the composition of the intestinal microbiota (Chen et al., 2018; Li et al., 2018). However, over time, the host intestinal microbiota remained stable (Guevarra et al., 2019). *Lactobacillus casei*, a normal resident of the gastrointestinal tract of mammals, has been extensively studied over the past few decades for its probiotic properties in clinical and animal models (McFarland et al., 2018; Riaz Rajoka et al., 2018). Also, it may be a good choice for mucosal immunization (Friedman et al., 2000; Lee et al., 2000; Shata and Hone, 2001; Wang et al., 2016), because it is safe, cheap, stable, and easily administrated and exhibits advantageous properties (Pouwels et al., 1998; Seegers, 2002; Praveen et al., 2004; Tarahomjoo, 2012). The potentiality of *L. casei* to deliver heterologous antigens to the mucosal immune system has been investigated during the last decades (Pouwels et al., 1996; Ho et al., 2005; Kuczkwoska et al., 2017; LeCureux and Dean, 2018). Previously, for surface display of the antigens ETEC K88 and K99 on *L. casei*, we have developed the strategy of generating *Lactobacillus casei/E. coli* shuttle expression vector with the PgsA gene as an anchoring matrix (Wen et al., 2012). However, we do not know whether this live recombinant *L. casei* affects the intestinal flora of animals, especially piglets. In this study, we mainly investigated the changes of immunoglobulin and intestinal flora after orally immunizing to piglets the recombinant pLA-ETEC K88/*L. casei*. The bioinformatics analysis may clear in focus the potential for utilizing this recombinant *L. casei* to interfere the intestinal physiological characteristics in newborn piglets and can reveal the specific effects on the intestinal flora.

2 MATERIALS AND METHODS

2.1 Bacterial Strains and Growth Conditions

Recombinant strain pLA-ETEC K88/*L. casei* was constructed and stored in our laboratory (Wen et al., 2012). Briefly, The 851-bp DNA fragment encoding the fimbrial protein K88 (GenBank: M29375.1) was amplified from the ETEC strain by PCR, and then the PCR product was inserted into the vector pLA to construct the recombinant plasmid pLA-K88. Electroporation of *Lactobacillus casei* (ATCC-334) was carried out according to the transformation condition which was 2.0 kV/cm, 200 μF, 25 μF, by using a Gene Pulser (Bio-Rad, Richmond, CA). It grew anaerobically at 37°C with 34 mg/ml of chloromycetin (Cm; Sigma) in MRS broth medium (Difco).

2.2 Animals, Diets, and Sampling

A total of 48 newborn piglets from 5 litters (Landrace, newborn average litter weight, 1.90 ± 0.05 kg) were obtained from the Sanhe farm (Qiqihar, Heilongjiang Province, China). All pigs in this study were chosen from one delivery room and had similar genetic backgrounds and husbandry practices. These piglets were allocated randomly to two groups (Ctrl: no feeding pLA-ETEC K88/*L. casei*, OA: feeding pLA-ETEC K88/*L. casei* on days 1–5 for the 28-day experiment. The piglets in the two groups were placed in three pens with a similar environment, the room temperature was maintained at 30°C, and the humidity was maintained constant at 65%–75%. The 24 piglets in the Ctrl group were fed the basic diet without any probiotics in the 28-day experiment. For the treatment group, 24 piglets in the OA group were treated with the protocol described by Li-Juan Wen (Wen et al., 2012). Briefly, pLA-ETEC K88/*L. casei* (5 × 10^11 CFU/ml) cells were orally administered daily on days 0–5 and treated according to animal protocols approved by the Institutional Animal Care and Use Committee (IACUC). All of the newborn piglets used in this study were weighed at birth, days 15 and 28 of age. The weights were calculated according to groups ctrl and OA no matter with or without diarrhea.

For the next-generation sequencing, five piglet samples were chosen randomly in each group. Piglets’ fecal samples (200 mg) were collected in a cryopreservation tube (Axygen) on days 15 and 28 for different groups. The Ctrl group samples and OA group samples were both collected on days 15 and 28 and named Ctrl15, Ctrl28, OA15, and OA28. After sample collection, the samples were quickly placed into the sterile tubes, then thrown into liquid nitrogen for half an hour and stored at -80°C until sequencing.

For special antibody detection, the piglets’ fecal samples (200 mg) were collected in the cryopreservation tube (Axygen) on days 0 (preimmune), 5, 10, 15, 20, and 28 for the Ctrl group and OA group. After collection, the samples were quickly placed into the sterile tubes, then thrown into liquid nitrogen for half an hour and stored at -80°C until antibody detection. The blood samples were collected from the front cavity vein of piglets on days 0 (preimmune), 5, 10, 15, 20, and 28 for the Ctrl group and OA group. Serum was isolated from the blood and stored at -20°C until analysis.

The incidence of diarrhea was recorded twice a day (monitoring time: 9:00 a.m. and 4:00 p.m.) according to the method of Ou et al. (Ou et al., 2007). In detail, scores were assessed as 0 = normal, solid feces; 1 = slight diarrhea, soft and loose feces; 2 = moderate diarrhea, semi-liquid feces; or 3 = severe diarrhea, liquid and unformed feces. Diarrhea was defined as a score of 2 or 3 per day, and the incidence of diarrhea (%) was calculated as (number of piglets with diarrhea)/(total number of
experiment piglets) \times 100\%. On days 0 (preimmune) and 28, the piglets were weighed.

According to the score of piglet diarrhea, we found that six piglets had obvious symptoms of diarrhea, and then piglet feces were collected.

2.3 PCR
In order to determine the diarrhea symptoms in the newborn piglets caused by ETEC K88, we conducted PCR-specific detection of feces collected from diarrheal piglets. We firstly extracted DNA from the feces samples, then used the designed K88 gene primers (5′-CGCGGATCTTT TTGAATGTGA T T T A T G - 3′, 5′- CGGGGTACCTTACTCTTT GAATCTGTC-3′) to amplify by PCR. The PCR products were detected by 1% agarose gel.

2.4 ELISA
The specific antibodies IgG, IgA in serum, and slgA in fecal samples were determined by enzyme-linked immunosorbent assay (ELISA), as described previously (Bai et al., 2020). The optical density was measured at 450 nm by using an ELISA auto-reader (Molecular Devices, San Jose, CA, USA) and visualized by R software (version 3.6.1) with ggplot2 package.

2.5 Next-Generation Sequencing of the 16S rRNA Gene
Genomic DNA was extracted from each group at each time point. The 16S rRNA genes were amplified via a pair of the universal bacterial 16S primers 338F (5′-ACTCTCCTACGGGAG GCAG CAG-3′) and 806R (5′-GGACTACHVGGGTWTCT TAAT-3′), covering the V3–V4 regions of the 16S rRNA gene. The sequencing library was quantified by Qubit and qPCR, and the barcode V3 and V4 PCR amplicons were sequenced using the Illumina MiSeq platform (Shanghai Personal Biotechnology Co., Ltd., Shanghai, China). All the raw sequencing data were submitted to the NCBI Sequence Read Archive (SRA) database under accession nos. SRP282258 and SRP344853.

2.6 Sequence Processing and Bioinformatics Analysis
2.6.1 Taxonomy classification
The sequences used in the subsequent analysis (effective tags) were obtained by successively splicing raw sequence reads using FLASH software (version 1.2.7) (Lozupone and Knight, 2005). After quality filtering by the QIIME quality-control process (Caporaso et al., 2010), the repetitive sequences were removed from the effective tags to acquire representative sequences by using USEARCH software (version v5.2.236) (Caporaso et al., 2010). The representative sequences showing 97% identity were then clustered as operational taxonomic units (OTUs) using UCLUST (Edgar, 2010). The taxonomy of each 16S rRNA gene sequence was analyzed by RDP Classifier against the SILVA (Release 115) 16S rRNA gene database (Quast et al., 2013). OTUs with abundance values less than 0.001% of the total sequencing samples were removed (Bokulich et al., 2013), and this abundance matrix with rare OTUs removed was used for subsequent series of analyses.

2.6.2 Alpha and Beta Diversity Analysis
Alpha diversity can be used to measure the diversity and abundance of microbes. Various alpha diversity indexes, such as Chao1, Shannon, Simpson, and ACE, were calculated on the basis of QIIME software (version 1.7.0), and boxplots were drawn via R software (version 3.6.1) with the ggplot2 package. To compare the construction of the microorganisms between the different samples, weighted UniFrac distances were calculated by using QIIME (version 1.7.0) (Lundberg et al., 2013). In addition, the samples were clustered basing on the weighted UniFrac distance matrix using the principal coordinate analysis (PCoA) (Ramette, 2007) and non-metric multidimensional scaling (NMDS), then visualized by R software (version 3.6.1) with the ggplot2 package. Also, the samples could be clustered based on both the UniFrac distance matrix using the unweighted pair-group method with arithmetic mean (UPGMA) implemented in QIIME (version 1.7.0) (Lozupone and Knight, 2005; Geng et al., 2018).

2.6.3 Random Forest Regression Analysis
In order to screen the bacterial genera that contribute most to the impact of intestinal flora, we regressed the relative abundances of bacterial taxa for all groups using the R algorithm (Random Forest) in default parameters which are described by Jingying Zhang (Zhang et al., 2018). Random forests are a classical and efficient machine learning algorithm based on the decision tree, which belongs to the non-linear classifier and can deeply mine the complex non-linear interdependence among variables, especially for microbiota data that often present discrete and discontinuous distribution (Breiman, 2001; Wang et al., 2018b). We used the “Random Forest” package in R software to build a Random Forest classification list by characteristic importance which was determined in 100 iterations. The number of marker taxa was identified using 10-fold cross-validation implemented with the “rfcv ()” function in the R package “Random Forest” with five repeats (Breiman, 2001; Zhang et al., 2018). Then the mean decrease in accuracy (the contribution of each genus to the model prediction accuracy) of every genus was visualized by R software (version 3.6.1) with the ggplot2 package.

2.6.4 Microbial Function Prediction
To find out the potential functional profiles of the bacterial community, the sequences were clustered into OTUs at 97% similarity by using a closed-reference approach in the Greengenes 13.5 database via QIIME (version 1.7.0). The resulting OTUs were used for the prediction of microbial function in PICRUST (Langille et al., 2013) according to the online protocol. Briefly, after the OTUs were normalized by 16S rRNA gene copy number, the metagenome for each sample was predicted, and the accuracy of the metagenome predictions was assessed. The relative abundance of each KEGG level was visualized by R software (version 3.6.1) with the ggplot2 package. The differences in KEGG level between the control and
experimental groups were assessed by STEMP software (Langille et al., 2013).

2.7 Statistical Analysis
The Student’s t-test was used to evaluate differences in fecal microbiome between the control and experimental groups, growth details, differences in the relative abundances of the genes involved in the KEGG pathway, and correlation analysis. Differences were considered significant when the p value was <0.05.

3 RESULTS

3.1 Growth Performance, Systemic, and Mucosal Immune Responses Induced by Oral Immunization With the Recombinant L.casei
The 48 newborn piglets were grouped as Ctrl group (24 piglets) and OA group (24 piglets). The piglets in the Ctrl group were fed the normal diet without any probiotics and those in the OA group fed pLA-ETEC K88/L.casei at days 0–5. On day 0, there was no significant difference in average body weight of piglets between the Ctrl group and OA group (p > 0.05) (Figure 1A). However, there was a significant difference in average body weight between the Ctrl group and OA group on day 28 (p < 0.05) (Figure 1B). These results indicated that the recombinant L.casei can improve and promote the growth and development of newborn piglets.

The specific anti-K88 IgG antibody in serum samples from immunized piglets was determined (Figure 1C). After the oral immunization, the anti-K88-specific IgG level was higher in the OA group than that in the Ctrl group and the highest value was found on day 15.

In order to determine the mucosal immune response, the specific slgA level in fecal samples and the IgA level in serum samples were measured by ELISA (Figures 1D, E). The results showed that the levels of the specific slgA and IgA were all higher than the control, suggesting that the systemic and mucosal immune responses were induced by oral immunization with pLA-ETEC K88/L.casei.

3.2 Statistics of Diarrhea Rate and PCR Identification of Diarrhea Samples
During the 28 days of the experiment, in the Ctrl group, there were 6 piglets that developed diarrhea (levels 2–3) caused by ETEC K88, which was confirmed by PCR detection (Figure 2B), and the diarrhea incidence was 25% (Figure 2A). However, in the OA group there was none. Thus, pLA-ETEC K88/L.casei improved the clinical performance of piglets by reducing incidence of diarrhea and morbidity.

3.3 α-Diversity and β-Diversity in Piglet Intestinal Flora
The intestinal flora in the three groups of piglets was analyzed by sequencing the bacterial 16S rRNA gene amplicons (V3+V4 region). After removing the low-quality sequences, 1,050,897 clean tags were identified as a total of 73,146 OTUs presenting in these samples. In α-diversity, the Chao1 and ACE indexes revealed...
the minor change but not statistical significance in the OA15 group when compared with the Ctrl15 group. Most interestingly, the Chao1 and ACE values of the α-diversity index were statistically significant in the OA28 group in comparison with the Ctrl28 group (p < 0.05). The Shannon and Simpson indexes of the α-diversity index were statistically significant in the OA28 group in comparison with the Ctrl28 group (p < 0.05) (Figure 3). These data suggest that after the recombinant \textit{L. casei} treatment, there was no clear difference in inter-group for the richness and variation of the intestinal flora on day 15. However, there was a significant difference in inter-group for the richness and variation of the intestinal flora on day 28.

In analyzing the degree of similarity between each group, principal component analysis (PCA) based on OTU relative abundances was taken for this experiment. In the coordinate system, the closer the two points, the higher the similarity was. The analysis results showed that the relative dispersion between different groups was relatively concentrated in the same group. According to the results of PCA analysis, there were significant differences between the Ctrl15 group and treatment group (OA group) (Figure 4A). Weighted UniFrac distances were used to estimate β-diversity and to compare among the three groups. The PCOA plot of the weighted UniFrac distances showed that the OA groups tended to separate from the Ctrl group (Figure 4B). Similar results were also observed in the analysis via non-metric multidimensional scaling (NMDS) (Figure 4C).

To determine the degree of similarity among the samples, a clustering tree of the samples was constructed (Figure 5). We found out that the treatment component was divided into two clusters, and the Ctrl15 component was divided into one cluster. However, three samples of the OA15 group were in the OA28 cluster. It was supposed that the intestinal microflora of piglets in the OA28 group was similar to that in the OA15 group.

### 3.4 Characterization of the Intestinal Flora of Piglets After the Recombinant \textit{L. casei} Treatment

We measured the relative abundance of intestinal flora in levels of phylum (Figure 6A), class (Figure 6B), family (Figure 6C), and genus (Figure 6D). The abundance of the two major phyla \textit{Firmicutes} and \textit{Proteobacteria} in the intestinal flora was 80.06% and 4.72% of the total abundance in the Ctrl15 group, respectively. However, after the recombinant \textit{L. casei} treatment, the phyla \textit{Firmicutes} was decreased in the OA15 group which constituted 72.26% and \textit{Proteobacteria} was increased to 9.43%. Notably, in the OA28 group, the phyla \textit{Proteobacteria} was decreased compared to the OA15 group, which constituted 8.88% of the total abundance. The abundance of the phyla \textit{Firmicutes} was increased on day 28 (Figure 6A).

At the Class level (Figure 6B), the intestinal flora was dominated by class \textit{Clostridia} which constituted 29.23% of the total abundance in the Ctrl15 group. After the recombinant \textit{L. casei} treatment, the class \textit{Bacilli} was significantly increased in the OA15 group which constituted 72.26% of their total abundance, and \textit{Gammaproteobacteria} was dramatically increased to 10.29% in their total abundance.
At the Family level (Figure 6C), we took the top 10 taxons of total abundance. The intestinal flora was dominated by the families Ruminococcaceae and Lactobacillaceae, which constituted 65.75% and 7.56% of the total abundance in the Ctl15 group. After feeding the recombinant *L. casei*, the family Lactobacillaceae was increased in the OA15 group which constituted 37.02% of their total abundance and the family Ruminococcaceae was dramatically decreased to 9.62% in their total abundance. However, surprisingly, Lactobacillaceae decreased to 20.27% in total abundance in the OA28 group.

At the Genus level (Figure 6D), we took the top 10 taxons of total abundance. After the recombinant *L. casei* treatment, *Lactobacillus* was markedly elevated in the OA15 group which constituted 37.02% of their total abundance and the family Ruminococcaceae was dramatically decreased to 9.62% in their total abundance. However, surprisingly, *Lactobacillaceae* decreased to 20.27% in total abundance in the OA28 group. Altogether, these results indicate that the recombinant *L. casei* intervention altered the microbiota composition in the piglet gut.

Basing on the OTU relative abundance of each sample, we analyzed the differences of bacterial abundances in genus level among groups Ctl15, Ctl28, OA15, and OA28 by using STAMP software with two-sided Student’s t-test (Figures 7A, B). The genus was ordered by effect sizes. In the Ctl15 vs. OA15 group, the mean percentage of the genus *Lactobacillus* in the OA15 group was significantly higher than that in the Ctl15 group (p < 0.05) (Figure 7A). In the Ctl28 vs. OA28 group, the mean proportions of the genera *Lactobacillus* and *Treponema* in the OA28 group were upregulated in comparison to the Ctl28 group (p < 0.05) (Figure 7B). However, it is not what we expected; the mean percentage values of the genus *Treponema*, *Oscillospira*, *Streptococcus*, and *Catenibacterium* in the OA28 group were upregulated when compared to those in the OA15 group (Figure 7C). This phenomenon also appeared to be consistent with the analysis results of the species classification tree (Figure 7D) and heat maps (Figures 7E, F) basing on OTU relative abundance between each sample.
The relative abundances of bacterial taxa and random forest regression analysis were carried out in each group to screen the key species affecting the change of intestinal flora. If a genus was removed, the model could predict the magnitude of the increase in error rate to determine its importance value which was ranked according to its importance to the model. The higher the value is, the greater the contribution of the genus to the prediction accuracy of the model. We took the top 20 taxons of the genus to predict via the model and visualize by R software (Figure 8). The genus *Lactobacillus* was significantly higher than other genera, indicating that the intervention of *L. casei* played an important contribution in the change of intestinal flora.

### 3.5 Microbial Function Prediction

16s rRNA marker gene sequences were used to predict the functional profiling of microbial communities by using Kyoto Encyclopedia of Genes and Genomes (KEGG), which were obtained through a phylogenetic prediction investigation of communities via reconstruction of unobserved states (PICRUSt). All the analysis results are shown in Figure 9A. We discovered that after the *L. casei* treatment, the relative abundances of the genes involved in membrane transport, replication, and repair were significantly increased, but in lipid metabolism, metabolism of cofactors and vitamins, metabolism of terpenoids and polyketides, and metabolism of other amino acids were significantly declined (*p* < 0.05) (Figure 9B). In the OA28 group, the relative abundances of the genes involved in membrane transport, replication, and repair were significantly increased (*p* < 0.05) (Figure 9C). In the OA15 vs. OA28 group, the relative abundances of the genes involved in cell motility and environmental adaptation were significantly decreased (Figure 9D).
3.6 Correlation Analysis Between Intestinal Microflora Composition Changes and Body Function Indexes

The Spearman rank correlation analysis was used to study the potential relationship between intestinal microbial composition and body function in piglets (Figure 10). The genus *Lactobacillus* abundance enhancement was positively correlated with the increased levels of translation, nucleotide metabolism, replication and repair, serum IgG value, serum IgA value, and fecal sIgA value \((p < 0.05)\) but was negatively correlated with the fecal score \((p < 0.05)\). The genus *Collinsella* abundance enhancement was also positively related to the increased levels of translation, nucleotide metabolism, replication and repair, and serum IgA value \((p < 0.05)\). However, the genus *Treponema* abundance enhancement and the increased levels of carbohydrate metabolism were negatively correlated \((p < 0.05)\). *Bacteroides* abundance enhancement was negatively correlated with the lipid metabolism increased level \((p < 0.05)\).

4 DISCUSSION

The animal body is colonized by a diverse community of microorganisms collectively referred to as the microbiota that mediate colonization resistance directly and indirectly against infectious pathogens. Probiotics have been used extensively as animal feed additives. There are several benefits set for probiotics: preventing infections (Hu et al., 2017; Lin and Zhang, 2017; Azad et al., 2018), potential antitumor activity (So et al., 2017; Yu, 2018), promoting food absorption, and repairing the intestinal epithelial barrier (Chang et al., 2018; Kim et al., 2018; Huang et al., 2019). Our studies on *L. casei*, one of the probiotics, as a live vehicle for the delivery of heterologous antigens to the mucosa have been conducted for over 20 years. In the present work, we investigated the changes of intestinal flora, growth of piglets, specific humoral immunity, and mucosal immunity under the intervention of recombinant pLA-K88/L. casei, which provided important insights into the diversity and function of piglets gut microbiota. We tried to find out about the therapeutic modulation of the gut microbiota in piglets to prevent or treat diarrhea diseases by recombinant *L. casei*.

In this study, we found that pLA-ETEC K88/L. casei were sufficient to elicit elevated sIgA responses in mucosal tissues as well as the systemic IgG antibody responses. The treatment of recombinant *L. casei* to newborn piglets induced the anti-K88-specific serum IgG, serum IgA, and fecal sIgA, suggesting that they could be the potential mucosal vaccine against ETEC infection. However, its molecular mechanism still needs to be confirmed experimentally, such as what T cell responses caused by the mucosal vaccine play an important role.

![FIGURE 5](Qin et al. rL.casei Prevented Piglets From Diarrhea Frontiers in Cellular and Infection Microbiology | www.frontiersin.org March 2022 | Volume 12 | Article 842007)
In order to evaluate the diversity of the bacterial community in newborn piglets, a series of $\alpha$-diversity indexes were calculated. We found out that the richness and diversity of intestinal flora in the L. casei-treated group were significantly increased on day 28. Similar results were also reported in some studies demonstrating that L. casei can increase the $\alpha$-diversity index of the microbial ecosystem in mice (Liew et al., 2019). However, there are a very limited number of studies that have analyzed the $\alpha$-diversity index of the microbial ecosystem in piglets under the intervention of recombinant L. casei. In $\beta$-diversity, microbiota structure ($\beta$-diversity indices) was changed after pLA-K88/L. casei treatment. The OA groups have formed distinct clusters, and these groups tended to separate from the Ctrl15 group. One possible explanation for this discrepancy is that it was caused by the intervention effects of pLA-K88/L. casei. Another possibility is that the Lactobacillus peptides secreted by probiotics regulated the change of intestinal flora (Hu et al., 2018; Wang et al., 2020).

More generally, our results indicate that the oral administration of pLA-K88/L. casei can decrease the relative abundance phyla of Proteobacteria. Thus, we speculate that the intervention of recombinant L. casei to piglets may relieve diarrhea symptoms caused by ETEC, which was proved by the decreased diarrhea rate. In previous research in our lab, pLA-K88/L. casei can protect mice against ETEC-K88 challenge, and 85% protective efficiency was obtained to provide a first line of protection at infectious pathogen entry ports (Wen et al., 2012). However, we still need corresponding experiments to prove the immune protection rate in piglets. Furthermore, our results also showed that the oral administration of recombinant L. casei not only significantly increased the relative abundance of L. casei but also altered the intestinal microbiota in the feces of newborn piglets, as evidenced by altered microbial diversity, microbial taxonomic composition, and bacterial functional profiles. Surprisingly, either family or genus, the richness of Lactobacillus was decreased in the OA28 group. One possible reason is that decreasing antimicrobial peptides owing to the low-abundance Lactobacilli caused by the oral tolerance mechanism further resulted in changes in relative abundance of the dominant genus. It is generally believed that the gut immune system normally does not respond to food antigens and the gut’s native bacteria, which is called oral tolerance.

**FIGURE 6** | The intestinal flora detail of piglets after L. casei treatment, microbiota at the Phylum level (A), Class level (B), Family level(C) and Genus level (D) were shown by bar plot. The top 20 taxons of Family and Genus abundance were taken to analyse the effect of probiotic L. casei on intestinal flora composition in piglets.
Based on the OTU relative abundance of each sample, the differences of bacterial abundances among the Ctrl15 group, Ctrl28 group, and the first feeding groups OA15 and OA28 were analyzed by using STAMP software with two-sided Student’s t-test. Ctrl15 group vs. OA15 group (A), Ctrl28 group vs. OA28 group (B), OA15 group vs. OA28 group (C). GraPhlAn tools were used for visualization of the hierarchical tree (D). The hierarchical tree shows the hierarchical relationship of all taxons (represented by nodes) from phylum to genus (arranged from inner circle to outer circle in turn), and the node size corresponds to the average relative abundance of the taxon. The top 20 taxa of relative abundance will also be identified by letters in the figure (arranged from phylum to genus in order from outer layer to inner layer). The top 30 taxa of genus abundance were taken to analyze the differences between each sample (E) and each group (F) by heat maps which were drawn by R software (version 3.6.1) with the ggplot2 package.

Based on the relative abundances of bacterial taxa, random forest regression analysis was taken and the top 20 taxa of genus in mean decrease in accuracy were visualized by R software.
This tolerance state is closely associated with various components of intestinal epithelial cell integrity, dendritic cell presentation, CD4+CD25+Treg cells, and other factors, which may influence the balance of the microenvironment in gut. Furthermore, previous studies reported that probiotics stimulated Paneth cells to produce antimicrobial peptides and regulated the relative stability of intestinal flora (Chen et al., 2017; Lueschow et al., 2018; Wang et al., 2018a). On all accounts, solving this problem needs our further experimental studies. Also, further research is required to clarify the molecular basis of the microbiota variation in a larger scale of piglets.

Our results demonstrated that the OA15 cluster was close to the Ctrl28 cluster (Figure 5). The possible reason was that supplemented probiotics were part of the “transient microbiota” in the pigs’ body for a relatively short period, which was consistent with our previous findings. The permanent colonization of the supplemented probiotics is largely hindered by the resident flora, suggesting that continuous addition of the probiotics might be an option for farm applications. Interestingly, our data suggested that after administration of pLA-K88/L.casei, the functional alteration of the intestinal microbiota was characterized by significantly increasing and positive correlating...
with membrane transport, repair, and translation. This indicated that the \textit{L.\,casei} intervention to piglets could enhance the host transport of intracellular substances and cell proliferation and repair, which can restore the intestinal mucosal barrier, relieve diarrhea, and enhance immune function to an improved clinical outcome of diarrheal disease (Wang et al., 2017; Riaz Rajoka et al., 2018; Wang et al., 2019).

Thus, oral administration of pLA-K88/\textit{L.\,casei} facilitates the functional maturation of the intestinal microbiota in newborn piglets. To sum up, this study revealed that the recombinant \textit{Lactobacillus casei} was worthwhile to promote the shape of intestinal flora in newborn piglets, although further experimental studies are still needed to uncover the recombinant \textit{L.\,casei} impact on host microbiome interaction and on the molecular mechanism and cellular pathway of repairing the host intestinal mucosal barrier. This beneficial superimposed effect of the specific immunity and probiotic role will be a strategy for preventing piglets from diarrhea.

**ETHICS STATEMENT**

The animal study was reviewed and approved by the Animal Care and Ethics Committee of Heilongjiang Bayi Agricultural University (HBAU-2019003).

**AUTHOR CONTRIBUTIONS**

L-YY, XH, GW, and LL contributed to the conception and design of the study. DQ, YB, LY, and YH conducted the experiments and analyzed the experimental results. YQ and JW performed the statistical analysis. DQ, YB, and L-YY wrote the first draft of the manuscript. All of the authors contributed to the manuscript revision and read and approved the submitted version.

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