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Gut microbiota contributes to protection against porcine deltacoronavirus infection in piglets by modulating intestinal barrier and microbiome

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Abstract

Background Gut microbiota plays a critical role in counteracting enteric viral infection. Our previous study demonstrated that infection of porcine deltacoronavirus (PDCoV) disturbs gut microbiota and causes intestinal damage and inflammation in piglets. However, the influence of gut microbiota on PDCoV infection remains unclear.

Results Firstly, the relationship between gut microbiota and disease severity of PDCoV infection was evaluated using 8-day-old and 90-day-old pigs. The composition of gut microbiota was significantly altered in 8-day-old piglets after PDCoV infection, leading to severe diarrhea and intestinal damage. In contrast, PDCoV infection barely affected the 90-day-old pigs. Moreover, the diversity (richness and evenness) of microbiota in 90-day-old pigs was much higher compared to the 8-day-old piglets, suggesting the gut microbiota is possibly associated with the severity of PDCoV infection. Subsequently, transplanting the fecal microbiota from the 90-day-old pigs to the 3-day-old piglets alleviated clinical signs of PDCoV infection, modulated the diversity and composition of gut microbiota, and maintained the physical and chemical barrier of intestines. Additionally, metabolomic analysis revealed that the fecal microbiota transplantation (FMT) treatment upregulated the swine intestinal arginine biosynthesis, FMT significantly inhibited the inflammatory response in piglet intestine by modulating the TLR4/MyD88/NF-κB signaling pathway.

Conclusions PDCoV infection altered the structure and composition of the gut microbiota in neonatal pigs. FMT treatment mitigated the clinical signs of PDCoV infection in the piglets by modulating the gut microbiota composition and intestinal barrier, downregulating the inflammatory response. The preventive effect of FMT provides novel targets for the development of therapeutics against enteropathogenic coronaviruses.

Keywords Gut microbiota, PDCoV, Fecal microbiota transplantation, Metabolomic

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Introduction

Coronaviruses are enveloped, positive-sense, and single-stranded RNA viruses, which belong to the *Coronaviridae* family in the *Nidovirales* order. Coronaviruses are classified into four genera: *Alphacoronavirus* (*α*-CoV), *Betacoronavi*rus (*β*-CoV), *Gammacoronavirus* (*γ*-CoV), and *Deltacoronavirus* (*δ*-CoV) [1]. In recent decades, the *β*-CoVs—severe acute respiratory syndrome CoV (SARS-CoV), Middle East respiratory syndrome CoV (MERS-CoV), and SARS-CoV-2—have caused severe respiratory diseases with high morbidity and mortality in 2003, 2012, and 2019 [2], respectively, posing serious threats to human health. Porcine deltacoronavirus (PDCoV), a member of *δ*-CoV, was first documented in pigs in Hong Kong in 2012 [3]. After the initial detection, this virus has been found in numerous countries worldwide [4–6]. PDCoV infection can cause acute watery diarrhea, vomiting, and dehydration in piglets, and can lead to death in nursing piglets [7, 8]. Apart from pigs, PDCoV can also infect calves, chickens, and turkeys in experiment settings [9, 10]. More recently, PDCoV was detected and isolated from three Haitian children with acute febrile illness [11], highlighting the potential of PDCoV to cause cross-species infections.

Gut microbiota and its metabolites are implicated in various metabolic, immune, gastrointestinal, and mental diseases [12, 13]. The homeostasis of gut microbiota can be disrupted by pathogens, resulting in inflammation or diarrhea [14]. This disturbance compromises the immunity of intestinal mucosa, the largest mucosal surface of the body, and reduces the production of mucins and antimicrobial proteins, which are essential for establishing physical and biochemical barriers against enteric pathogen [15]. Influenza infection can alter gut microbiota composition through the gut-lung axis [16], and disorders of gut microflora can diminish the host's antiviral immune response, exacerbating lung damage from infections [17]. In patients with coronavirus disease 2019 (COVID-19) and influenza A virus (H1N1), the diversity and abundance of gut microbiota significantly decrease, while the abundance of opportunistic pathogens, such as *Streptococcus*, *Rothia*, *Veillonella*, and *Rothia*, increase significantly [18]. Notably, the PDCoV and porcine epidemic diarrhea virus (PEDV) infection disrupt the gut microbiota homeostasis of pigs, and these changes are closely linked to inflammatory responses [19].

Meanwhile, gut microbiota regulates host immune responses to counteract viral infections [20]. Metabolites such as tyrosine, secreted by gut microbiota, induce the production of type I interferons and inflammasome-dependent cytokines, thereby providing protection against influenza [21]. Butyrate produced by gut microbiota down-regulates the expression of SARS-CoV-2 receptor angiotensin-converting enzyme 2 (ACE2), resulting in reduced viral entry [22]. Short-chain fatty acids (SCFAs) inhibit inflammatory response through activating anti-inflammatory immune cells or inhibiting inflammatory signaling pathways [23]. Tight junction proteins (TJs), which help maintain an intact gut barrier, can be influenced by the composition of the gut commensal microbiota [24, 25]. Interestingly, exopolysaccharides from *Lactobacillus plantarum* promote intestinal homeostasis by modulating the proliferation and differentiation of intestinal stem cells (ISCs) [26]. *Lacticaseibacillus rhamnosus* relieves diarrhea caused by rotavirus infection by maintaining intestinal mucosal barrier function [27]. However, the interactions between pathogenic infections, microbial communities, and host immune responses remain largely unknown.

Fecal microbiota transplantation (FMT), a method to regulate the composition of gut microbiota, has gained significant attention over the past decade and has shown therapeutic efficacy for gut diseases such as inflammatory bowel disease (IBD) [28], irritable bowel syndrome [29], and neurological diseases [30] in humans. Similar to humans, pigs possess a rich community of microorganisms in the gastrointestinal tract (GIT), and variation in microbiota can affect the host's gut health[31]. It has been shown that FMT can alleviate weaning stress through modulating microbiota structure, thereby improving intestinal function and growth performance in piglets [32, 33]. Despite FMT appears to be an effective therapeutic against some diseases, whether it can be used against enteric coronavirus infections is unknown.

Our previous study showed that PDCoV infection disturbs gut microbiota, causing intestinal damage and inflammation in piglets [34, 35]. The alteration of the gut microbiota following PDCoV infection seems to correlate with disease severity in piglets [36]. Moreover, the diversity and richness of gut microbiota gradually increase during pig development [37]. Clinically, the diarrhea caused by PDCoV predominantly occurs in the suckling piglets, rather than in older pigs [38]. We hypothesized that gut microbiota and its metabolites play a crucial role during PDCoV infection. In the current study, we examined the structural and functional changes of gut microbiota following PDCoV infection in pigs at different growth stages (8-day-old and 90-day-old), and we proposed that the gut microbiota might represent a critical influencing factor for PDCoV infection. Consequently, we explored the impact of early-life FMT intervention on gut microbiota and gut health in piglets infected with PDCoV. Finally, we analyzed the changes in gut microbiota and metabolites, as well as the upregulated pathways, to elucidate how FMT improves intestinal homeostasis

and reduces inflammatory responses, thereby alleviating PDCoV infection.

Materials and methods

Cells and virus

The LLC-porcine kidney (LLC-PK) cell line was purchased from the Institute of China Veterinary Medicine Inspection and cultured in Minimum Essential Medium (MEM, Gibco, USA) containing 5% fetal bovine serum (FBS), 1% nonessential amino acids (NEAA, Gibco, USA), 1% antibiotic–antimycotic (Gibco, USA), and 1% HEPES (Gibco, USA). The PDCoV HNZK-02 passage 5 (P5) (GenBank accession number MT260149) with a virus titer of 1×10^6 $TCID_{50}$/0.1 mL was used in this study [39].

Preparation of fecal microbiota suspension

The healthy 90-day-old pigs without evident gastrointestinal infections and not subjected to any treatments within 3 months were used as donors for gut microbiota transplantation. Detection of specific pathogens, including PEDV, transmissible gastroenteritis virus (TGEV), PDCoV, porcine circovirus type 2 (PCV-2), porcine reproductive, and respiratory syndrome virus (PRRSV), *Actinobacillus pleuropneumoniae*, *Brucellosis*, and *Mycoplasma hyopneumoniae* were negative in donor pigs. The fecal suspension was prepared as previously described with a few modifications [40]. Briefly, the freshly passed stool specimen was diluted 20-fold and homogenized in sterile potassium phosphate buffer saline (PBS, 0.1 M) containing 10% (v/v) glycerol. The solution was left standing for 5 min at 4 °C, then the supernatant was distributed to cryotubes and stored at − 80 °C.

Animal experimental design

Eight 8-day-old, eight 90-day-old, and twenty 3-day-old healthy pigs (Duroc × Landrace × Yorkshire) were all purchased from a commercial pig farm in Henan Province, China. No obvious clinical signs were observed in these piglets, and these piglets were confirmed to be negative for PEDV, TGEV, PDCoV, PCV-2, and PRRSV with viral-specific PCRs [10]. Eight 8-day-old piglets and eight 90-day-old pigs were used in the animal experiment I, and twenty 3-day-old piglets were used in the animal experiment II. Pigs were randomly assigned to the control and experimental groups. Each experimental group was housed in a separate room, and all environmental conditions (temperature, humidity, etc.) and diet were controlled consistently. The research protocols for animal experiments were approved by the Animal Care and Use Committee of Henan Agricultural University (Zhengzhou, China) and were performed in accordance with the "Guidelines for Experimental Animals" of the Ministry of Science and Technology (Beijing, China).

The design of animal experiment I: Eight 8-day-old piglets were randomly divided into two groups (group A and group B), and eight 90-day-old pigs were randomly divided into two groups (group C and group D). The piglets in groups A ($n=4$) and C ($n=4$) were orally inoculated with PDCoV at a dosage of 1.0×10^8 $TCID_{50}$/piglet. The piglets in groups B ($n=4$) and D ($n=4$) were treated with the same volume of MEM. Piglets were observed and evaluated daily for temperature changes, body weights, and clinical signs until 3 days post inoculation (dpi). All piglets were euthanized for necropsy examinations on 3 dpi.

The experimental design of animal experiment II: As shown in Fig. S1A, the 3-day-old piglets were used as the recipients for microbiota transplants. These piglets were randomly allocated into two groups, including the control group (Con, $n=10$) and the FMT group (FMT, $n=10$). The pigs in the FMT groups were inoculated orally with fecal microbiota suspension (1.5 mL per piglet), the piglets in the control group were orally administrated with the same volume of PBS, and they were fed once every other day from the first day to the 9th (12-day-old). The body temperature and weight of all piglets were recorded daily. On day 10, the half of 13-day-old piglets in the control group ($n=5$) and FMT group ($n=5$) were infected with PDCoV of 2.0×10^8 $TCID_{50}$/piglet, which was determined as the minimal effective dosage by preliminary experiment, and the other halves of the control and FMT groups were treated with an equal volume of MEM. Clinical symptoms of all piglets were observed daily. Rectal swabs were collected for PDCoV detection on 12, 24, 48, and 72 h post inoculation (hpi). All piglets were euthanized for necropsy examinations on 3 dpi.

Intestinal morphology of pigs

Duodenum, jejunum, ileum, and colon were collected after piglets were euthanized. To detect the goblet cells and inner mucus layer, the colon was fixed in Carnoy's fixative. These tissues were embedded in paraffin and cut into sections, and the colon was stained with alcian blue (AB) and periodic acid-Schiff (PAS). The duodenum, jejunum, and ileum of piglets fixed in 4% paraformaldehyde were used for the histopathology and immunohistochemistry analysis as described previously [36]. All sections were examined using the light microscope. The scanning electron microscope (SEM) was used to observe the morphology of the jejunum and ileum villi. Briefly, the jejunum and ileum samples were fixed with 2.5% glutaraldehyde, dehydrated gradient with 30–100% ethanol, treated with isoamyl acetate for 15 min, and then dehydrated samples with Critical Point Dryer. Specimens are

attached to metallic stubs and sputter-coated with gold for 30 s and observed with SEM (Hitachi SU8100, Japan).

Evaluation of intestinal permeability and gastrointestinal function using ELISA

Sera from all the experimental piglets were collected and then stored at −20 °C before testing. The levels of Ghrelin (GHRL), glucagon-like peptide-1 (GLP-1), CCK, D-lactic acid (D-Lac), and diamine oxidase (DAO) in serum were detected using ELISA Kits (mIBio, China) according to the instructions provided by the manufacturer.

Real-time quantitative PCR (qRT-PCR)

Total RNAs from the tissue of pigs were extracted and cDNA was constructed. The mRNA expression level of multiple genes was detected by qRT-PCR with the specific primers (Table S1). SYBR Green PCR Master (Takara, Japan) was used for qRT-PCR on a CFX real-time fluorescence quantitative PCR system. The relative expression of each gene was expressed and analyzed statistically using the $2^{-\Delta\Delta Ct}$ method.

Western blot

The total protein of pigs' colon and jejunum tissues were extracted using the T-PER Tissue Protein Extraction Reagent (Thermo), then the protein concentration was performed using the BCA Quantitation Kit. The procedure of western blot is based on our previous study [39]. The antibodies used in this study included anti-MUC2, ZO-1, occludin, TLR4, NF-κB, and Phospho-NF-κB (Proteintech, China).

Colonic and jejunum lumen microbiome analysis and functional metagenomics prediction

The colonic and jejunum contents collected from the experimental piglets were used to extract DNA using the OMEGA Soil DNA Kit (Omega). After quality inspection, the extracted genomic DNA and universal primers were used to amplify the V3-V4 regions of the 16S rRNA gene. The purified PCR product was quantified using the Quant-iT PicoGreen dsDNA Assay Kit (Invitrogen, USA), and the paired-end 250 bp sequencing was performed using the Illumina NovaSeq platform with NovaSeq 6000 SP Reagent Kit (500 cycles). The high-quality reads were clustered into the operational taxonomic units (OTUs) based on 97% sequence similarity. Alpha diversity (Chao, Simple, and Shannon) was calculated using the OTUs richness. Beta diversity analysis was determined using principal coordinate analysis (PCoA) based on Bray–Curtis distance, which was conducted to assess the relationships among the different groups. Bacterial taxa leading to differences between groups were identified by LEfSe. Phylogenetic investigation of communities by reconstruction of unobserved states (PICRUSt2) was used to predict the functional profiles of microbial communities.

Untargeted metabolomics of colonic content using HILIC-LC–MS/MS

Metabolome analysis of the colonic-content samples was conducted by ultrahigh-performance liquid chromatography/mass spectroscopy (UHPLC/MS/MS; positive mode and negative mode). The (−20 °C) methanol/acetonitrile/aqueous solution (2:2:1, v/v) of pre-chilled was added to the stool sample, homogenized, and then centrifuged at 14,000 × g at 4 °C for 20 min. The supernatants were treated with vacuum-drying and reconstituted with 75% acetonitrile, and then centrifuged at 14,000 × g at 4 °C for 15 min. The supernatants were used for the MS analyses.

The LC separation was performed with a UPLC BEH Amide column. The mobile phase was composed of buffers: (A) (water + 25 mM ammonium acetate + 25 mM ammonium hydroxide) and (B) (acetonitrile). Samples were detected in both ESI positive and negative modes. Analyses were performed using an UHPLC coupled to a quadrupole time-of-flight (AB SCIEX TripleTOF 6600). SIMCA-P 14.1 (Umetrics, Umea, Sweden) was used for principal component analysis (PCA) and partial least squares-discriminant analysis (PLS-DA). Single dimensional statistical analysis includes Student's *t*-test and fold change. The volcano plot was obtained by R software. In addition, the metabolic pathways were searched using the KEGG (http://www.genome.jp/kegg/) and NIST (http://www.nist.gov/index.html).

Statistical analysis

Statistical analyses were performed with SPSS 24.0 software and the GraphPad Prism 8.0 software was used to make charts. Data between the two groups were identified using a Student's *t*-test, and the three groups were used by one-way ANOVA software. Significant differences are considered significant at $*p < 0.05, **p < 0.01$.

Results

Pathogenicity of PDCoV in pigs of different age

To explore the pathogenicity of PDCoV in pigs, 8-day-old ($n = 4$) and 90-day-old ($n = 4$) pigs were orally inoculated with 10^8 $TCID_{50}$/pig of the PDCoV HNZK-02. The 8-day-old piglets infected with PDCoV started developing clinical signs at 12 hpi, including diarrhea, loose yellowish stools, and loss of appetite. No obvious signs were observed in 90-day-old pigs and the control groups. The 8-day-old piglets exhibited significant intestine lesions, including thin-walled, transparent, gas-distended dilatation filled with yellow fluids. In contrast, the 90-day-old

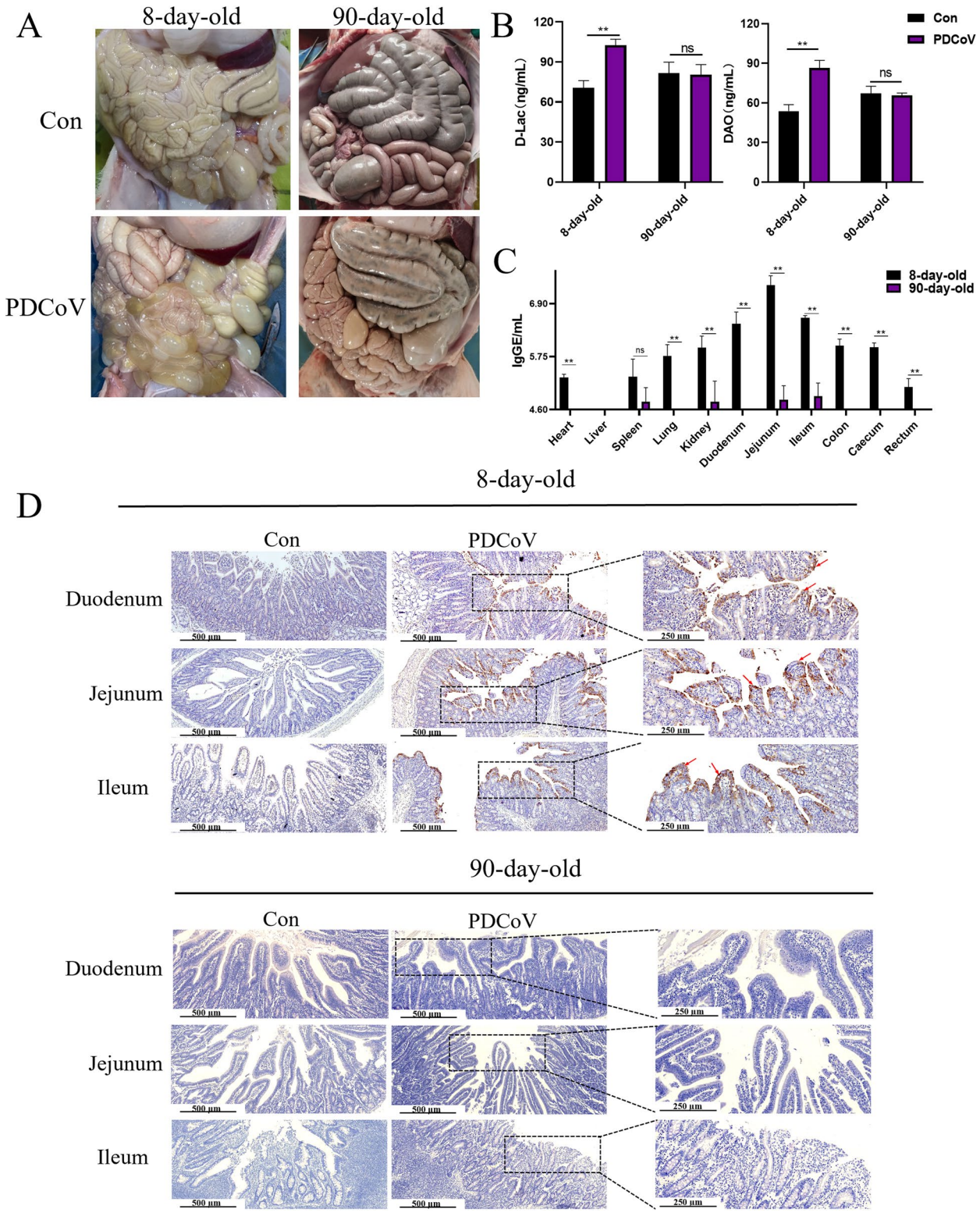

Fig. 1 The pathogenicity of PDCoV in 8-day-old and 90-day-old pigs. **A** Clinical symptoms and intestinal changes of pigs. **B** The DAO and D-Lac in sera were collected from 8-day-old and 90-day-old pigs challenged with PDCoV (PDCoV) and MEM (Con). **C** Viral distribution in various tissues in 8-day-old and 90-day-old pigs infected with PDCoV. **D** Immunohistochemical analysis of duodenum, jejunum, and ileum by staining with a monoclonal antibody against PDCoV N protein. Scale bars are shown in each picture. *$p < 0.05$; **$p < 0.01$

pigs displayed no obvious pathological intestinal lesions (Fig. 1A). Next we examined the effect of PDCoV infection on intestinal permeability of pigs by measuring the levels of serum D-Lac and DAO, which are common indicators of intestinal permeability [36]. The amount of D-Lac and DAO in sera increased significantly in 8-day-old piglets after PDCoV infection, while no obvious change was seen in 90-day-old pigs (Fig. 1B), indicating PDCoV infection affected the intestinal permeability of 8-day-old pigs, but not 90-day-old pigs. Notably, PDCoV could be detected in the heart, lung, spleen, kidney, and all the segments of the intestine in 8-day-old piglets. In contrast, PDCoV was only found in the spleen, ileum, and jejunum in 90-day-old pigs (Fig. 1C). Immunohistochemical analysis showed that a large amount of PDCoV antigen was detected in the jejunum, duodenum and ileum of the 8-day-old pigs compared to those of the 90-day-old pigs and mock groups (Fig. 1D). Taken together, these results demonstrated that PDCoV infection caused severe clinical symptoms and intestinal damage of 8-day-old piglets, but not in 90-day-old pigs.

Characterization of gut microbiota in pigs at different growth stages

To explore the dynamic distribution of gut microbiota in pigs at different growth stages, we analyzed the gut microbiota of 8-day-old and 90-day-old healthy pigs using 16S rRNA gene sequence. Principal coordinate analysis (PCoA) and the hierarchical clustering analysis, based on the weighted-unifrac distance, were employed to assess the differences in bacterial community structure between these two groups. The PCoA indicated a significant separation between these two groups (Fig. 2A), and the hierarchical cluster tree showed the 8-day-old and 90-day-old pigs belonged to distinct branches (Fig. 2B). These findings suggest notable differences in the composition of gut microbiota between 8-day-old piglets and 90-day-old pigs. Furthermore, the number of OUTs in 90-day-old pigs (1793) was substantially higher compared to that in 8-day-old piglets (651) (Fig. 2C). Alpha diversity analysis revealed that the diversity indices of Chao 1, Shannon, and Simpson for 90-day-old pigs were significantly higher than those for 8-day-old piglets (Fig. 2D), suggesting that the richness and evenness of microbial communities in colonic content vary significantly with developmental stage.

To further analyze the differences in gut microbial composition and distribution between 8-day-old and 90-day-old pigs, we examined the relative abundance of microbiota at the phylum and genus levels using the heatmap of bacterial distribution (Top 10) (Fig. 2E). At the phylum level, *Verrucomicrobia*, *Firmicutes*, *Spirochaetes*, and *Tenericutes* were significantly increased in the 90-day-old pigs. At the genus level, the 90-day-old pigs exhibited higher levels of *Blautia*, *Streptococcus*, and *Gemmigelevels*, while lower levels of *Ruminococcus*, *Lactobacillus*, *Bacteroides*, *Oscillospira*, and *Phascolarctobacterium* were observed. However, the opposite was seen in 8-day-old piglets. Significant differences in microbiota compositions were observed as well between pigs of different ages when LEfSe analysis was employed (Fig. S1B). Fifteen potential microbial biomarkers were identified in the 8-day-old, dominated by *Bacteroidetes*, *Proteobacteria*, and *Bacteroidia*. Conversely, the gut microbiome of the 90-day-old pigs included 16 microbial biomarkers, predominantly *Bacilli*, *Clostridia*, and *Erysipelotrichi* (Table S2). These results showed there is a dynamic shift in the gut microbiota of pigs at different growth stages.

The diversity analysis of colonic microbiota in 8-day-old and 90-day-old pigs after PDCoV infection

To explore the effects of PDCoV infection on the gut microbiota of pigs of different ages, the characteristics of the gut microbiome in 8-day-old and 90-day-old pigs challenged with PDCoV were analyzed using a 16S rRNA gene sequence. Venn diagram revealed a decrease in the number of OTUs in 8-day-old piglets (352) compared to control groups (626). In contrast, 90-day-old pigs exhibited a slight increase in OUT numbers compared to non-treated pigs (Fig. 3A). Alpha diversity analysis was used to assess the variety of the gut microbiota between mock-treated and PDCoV-infected pigs. PDCoV infection significantly reduced community richness and diversity in 8-day-old piglets, as evidenced by decreased Chao 1, Shannon, and Simpson indices. However, the 90-day-old pigs showed a significant increase in Shannon and Simpson indices, with no significant differences observed in the Chao 1 index (Fig. 3B). Beta diversity analysis indicated that distinct clustering of microbiota composition between control and PDCoV-infected groups in both age groups (Fig. 3C). Furthermore, the hierarchical clustering revealed that the control and PDCoV-infected groups former separate branches in both 8-day-old and 90-day-old pigs (Fig. 3D). These findings demonstrate that PDCoV infection alters the composition of the colonic microbial community. Specifically, the PDCoV infection significantly reduced community richness and diversity in 8-day-old piglets (Chao1, Shannon, and Simpson indices, $p < 0.05$). In 90-day-old pigs, community diversity was significantly increased (Shannon and Simpson indices, $p < 0.05$), while community richness showed no significant change (Chao1 index, $p > 0.05$) following PDCoV infection. Based on these results, we conclude that the alterations in gut microbiota can affect the pathogenicity of PDCoV in pigs.

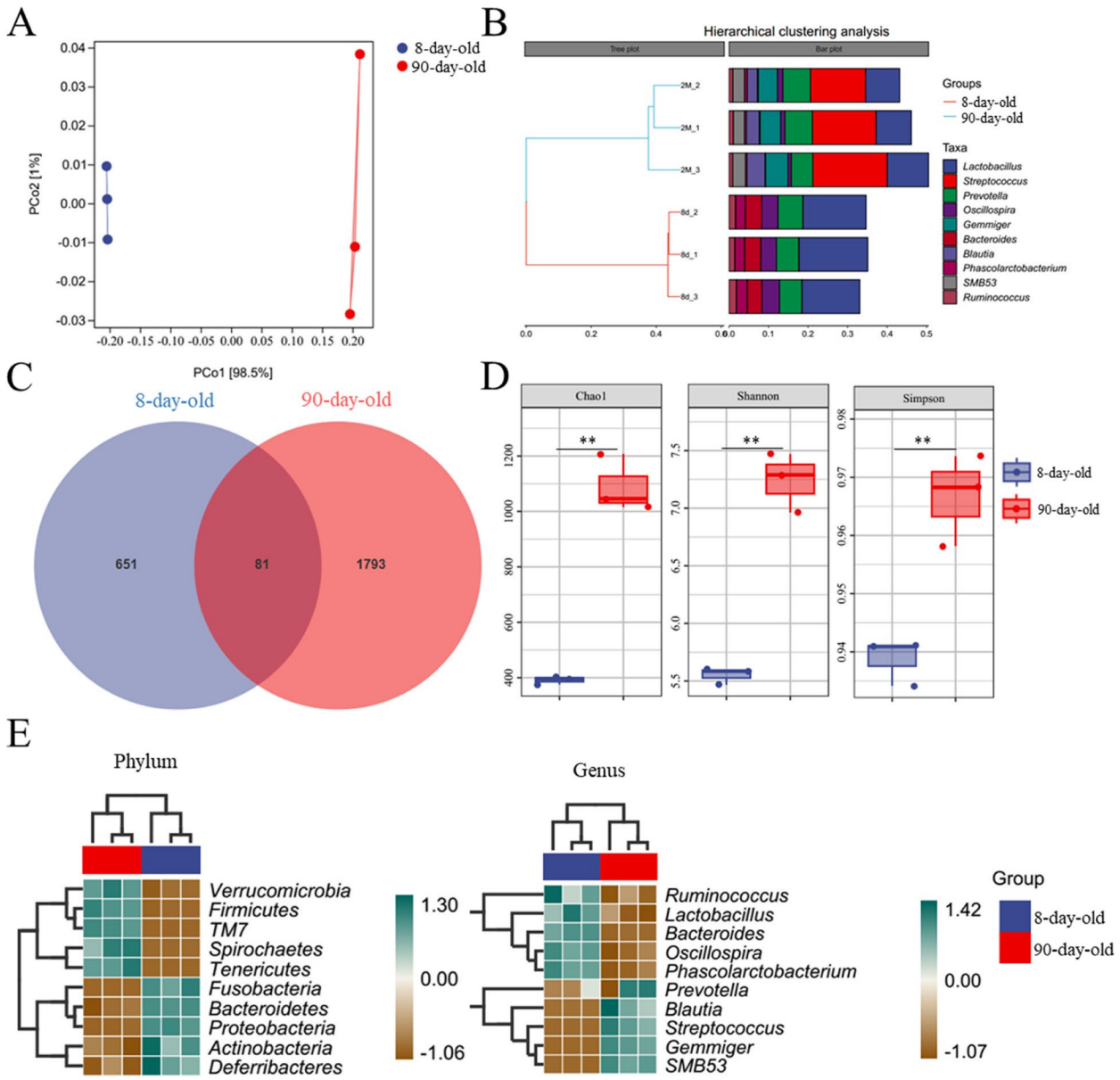

Fig. 2 Community structure of colonic microbiome of the 8-day-old and 90-day-old pigs. **A** The principal coordinate analysis (PCoA) of the colonic microbiota based on the weighted unifrac in 8-day-old and 90-day-old pigs. **B** The hierarchical clustering analysis of the colonic microbiota. The panel on the left is a hierarchical clustering tree. The composition of the two samples is similar when the branch length between the samples is shorter. **C** Venn diagram of shared OTUs based on the sequences with more than 97% similarity ($n = 3$) in the 8-day-old and 90-day-old pigs. **D** The alpha diversity indexes (Chao1, Shannon, and Simpson) of the colonic microbiota in 8-day-old and 90-day-old pigs. **E** The Heatmap of bacterial distribution (Top 10) in the 8-day-old and 90-day-old pigs on the phylum and genus. *$p < 0.05$; **$p < 0.01$

(See figure on next page.)

Fig. 3 Community structure of colonic microbiome in 8-day-old and 90-day-old pigs administrated with or without PDCoV. **A** Venn diagram of shared OTUs based on the sequences with more than 97% similarity ($n = 3$), 8-day-old (left) and 90-day-old pigs (right). **B** The alpha diversity indexes (Chao1, Shannon, and Simpson) of the colonic microbiota in 8-day-old (left) and 90-day-old pigs (right). **C** Principal coordinate analysis (PCoA) of the colonic microbiota based on the weighted unifrac in 8-day-old (left) and 90-day-old pigs (right). **D** The hierarchical clustering analysis of the colonic microbiota. The panel on the left is a hierarchical clustering tree. The composition of the two samples is similar when the branch length between the samples is shorter. *$p < 0.05$; **p 0.01

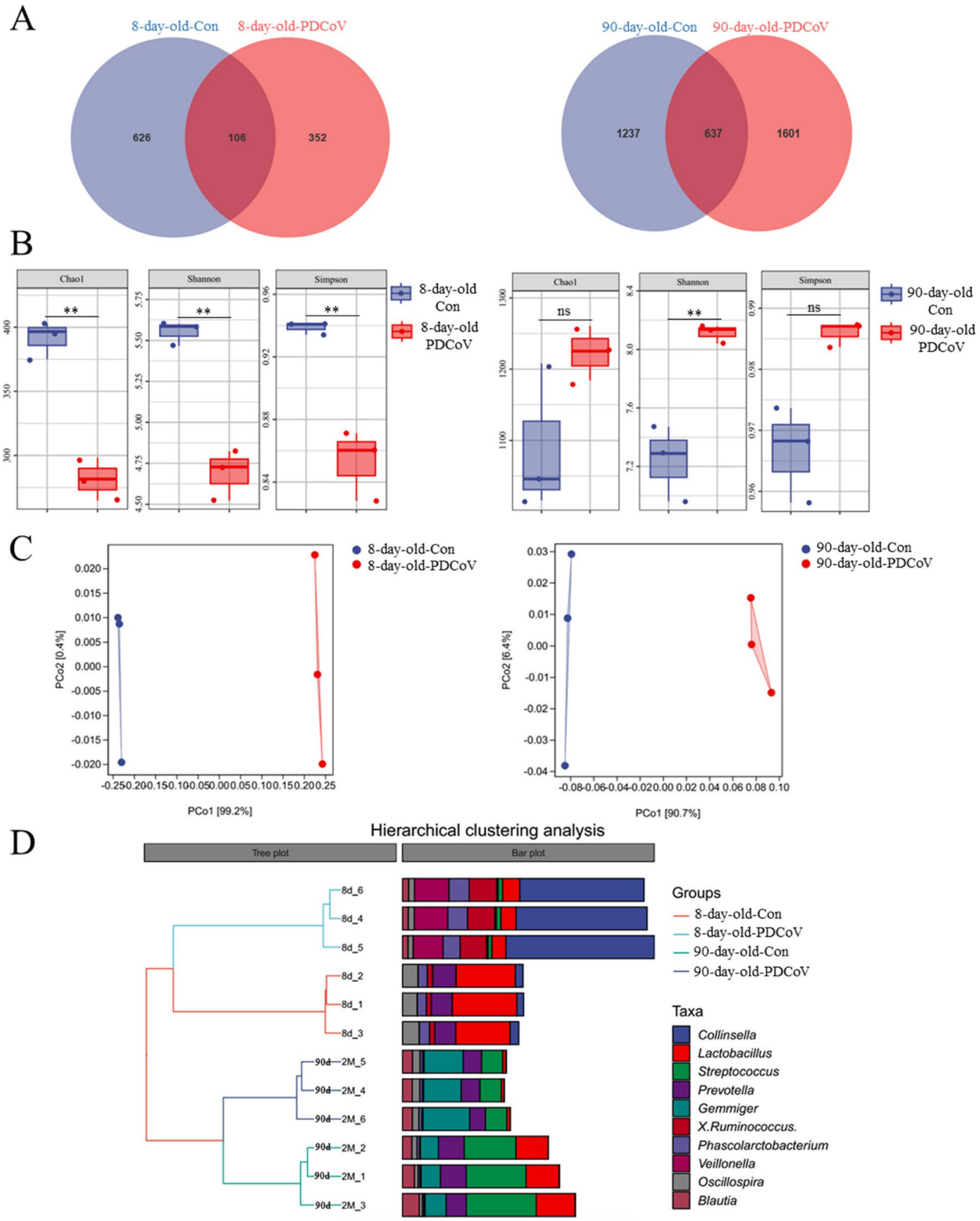

Fig. 3 (See legend on previous page.)

FMT alters diversity and structure of gut microbiota in jejunum and colon of the recipient piglets

To modulate the gut microbiota of piglets, we established the FMT model using 3-day-old recipient piglets and 90-day-old donor pigs. The recipient piglets were randomly divided into FMT and control groups, receiving oral inoculation of fecal microbiota suspension and PBS, respectively. Compared to the control group, the body weight and body temperature of the recipient piglets remained normal (Fig. S2A), with no obvious changes in the levels of GLP-1, CCK, and GHRL (Fig. S2B). These results indicate that the growth performance of the recipient piglets was not adversely affected by FMT. To assess the effects of FMT on gut microbiota composition and diversity, we analyzed the bacterial communities in the jejunum and colon contents by 16S rRNA gene sequencing. The results showed that Simpson, Chao1, and Shannon indices were significantly higher in the jejunum of the recipient piglets, and Simpson and Shannon indices were dramatically increased in the colon (Fig. 4A). Beta diversity analysis, based on the unweighted and weighted UniFrac, revealed a significant difference in microbiota composition between the colon and jejunum of recipient and control piglets (Fig. S2C). Additionally, the Venn diagram showed that the number of OTUs in the colon of the control and recipient piglets was 5779 and 5362, respectively, and 1257 OTUs were shared among the two groups. The number of OTUs in the jejunum of the control and recipient piglets was 821 and 1454, respectively, and 617 OTUs were shared among the two groups (Fig. 4B). Relative abundance analysis at the phylum level indicated that *Firmicutes*, *Bacteroidetes*, and *Proteobacteria* were the main intestinal phyla in all age groups. The relative abundances of *Bacteroidetes* and *Fusobacteria* were higher in the recipient piglets compared to the control piglets, whereas the control piglets had higher abundances of *Actinobacteria* and *Proteobacteria*. At the genus level, the recipient piglets exhibited higher levels of *Enterococcus*, *Shigella*, and *Weissella* level in the jejunum, and higher levels of *Lactobacillus*, *Prevotella*, *Mitsuolella*, and *Collinsella* in the colon (Fig. 4C and D). These observed differences in the gut microbial composition between control and recipient piglets suggest that FMT alters diversity and structure of gut microbiota in the colon and jejunum of recipient piglets.

To directly demonstrate that the FMT-induced microbial changes could modulate the metabolic functions of the gut microbiota, we conducted functional metagenomics prediction based on 16S rRNA gene sequencing of the colon using the PICRUSt2. A total of 112 KEGG pathways were identified in the two groups (Supplementary Table S3), encompassing cellular processes, metabolism, genetic information processing, environmental information processing, human disease, and organismal systems (Fig. S3A). The PCoA results indicated a distinct separation between the FMT and control groups (Fig. S3B). Specifically, FMT significantly increased the proportions of pathways related to tryptophan metabolism, glutathione metabolism, and cytochrome P450, while it decreased the proportions of pathways associated with NOD-like receptor signaling pathway, linoleic acid metabolism, and fructose and mannose metabolism (Fig. S3C), indicating that the changes in gut microbiota are closely related to alterations in metabolic functions.

Early-life gut microbiota intervention by FMT reduces the pathogenicity of PDCoV to piglets

To determine the effect of gut microbiota on the pathogenicity of PDCoV in piglets, we infected piglets from the FMT and control groups with PDCoV and evaluated the effect through clinical symptoms, pathological autopsy, fecal virus shedding, and viral tissue distribution. The results showed that the piglets infected with PDCoV exhibited an acute onset of yellow watery diarrhea, whereas the recipient piglets infected with PDCoV showed no obvious signs (Fig. 5A). Additionally, gut hormones such as GLP-1, CCK, and GHRL, which regulate food intake by terminating hunger and inducing satiety, were measured [41]. PDCoV infection significantly increased serum levels of CCK and GLP-1 and decreased level of GHRL, while FMT restored these hormone levels to near-normal (Fig. 5B). The body weight of piglets infected with PDCoV was significantly reduced compared to control piglets, whereas recipient piglets infected with PDCoV maintained stable growth (Fig. 5C). Fecal virus shedding and viral distribution in the intestines of PDCoV-inoculated piglets were evaluated using qRT-PCR [8]. The results suggested that FMT significantly reduced viral loads in the feces, duodenum, jejunum, ileum, and colon (Fig. 5D and E), consistent with the immunohistochemistry staining (Fig. 5F). Taken together, FMT can alleviate the pathogenicity of PDCoV in piglets.

FMT restored the composition and diversity of gut microbiota in the colon and jejunum of piglets infected with PDCoV

To determine the effect of PDCoV on gut microbiota in piglets and whether FMT could restore the abundance and diversity of gut microbiota, we analyzed the bacterial communities in colonic and jejunum contents of the control piglets, PDCoV-infected piglets, and PDCoV-infected recipient piglets. The results showed that in the colon, the abundance of phyla *Bacteroidetes* and *Proteobacteria* was significantly lower, while the phylum *Actinobacteria* was increased in PDCoV-infected piglets compared to

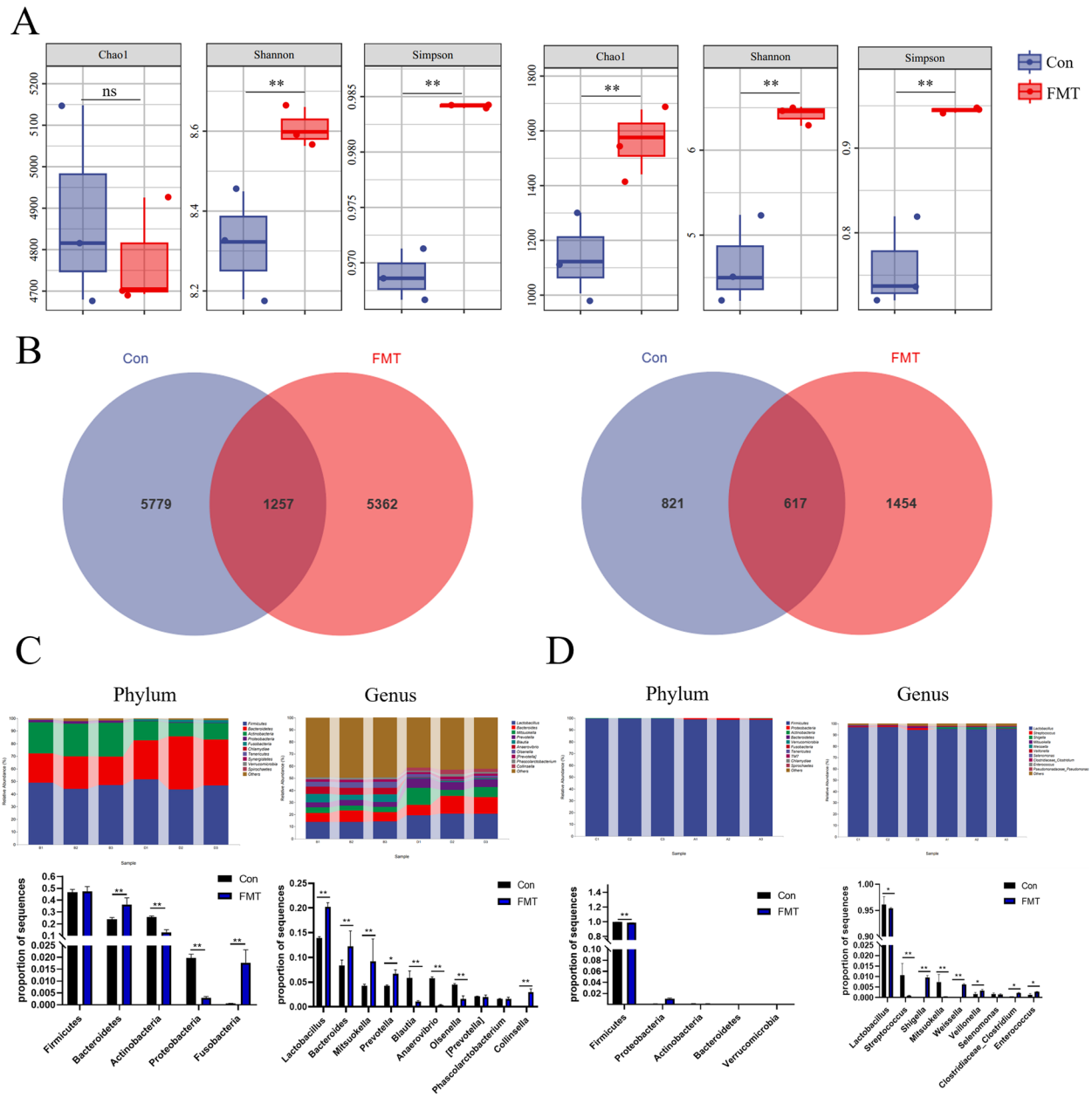

Fig. 4 The regulation of gut microbial composition in piglets treated with FMT. **A** The alpha diversity indexes (Chao1, Shannon, and Simpson) in the colonic (left) and jejunum (right) microbiota of the control and recipient piglets. **B** Venn diagram in the colonic (left) and jejunum (right) microbiota of the control and recipient piglets. **C**, **D** The regulation of gut microbial composition in piglets by FMT in colon (**C**) and jejunum (**D**). $^{*}p < 0.05$; $^{**}p < 0.01$

control piglets. However, after FMT treatment, the abundance of *Bacteroidetes* was restored to normal level, *Actinobacteria* was significantly reduced, and *Proteobacteria* was significantly increased. At the genus level, compared to the control group, PDCoV-infected piglets had higher levels of *Ruminococcus* and lower levels of *Lactobacillus*, *Bacteroides*, and *Prevotella* in the colon. Conversely, in PDCoV-infected recipient piglets, the abundance of *Lactobacillus*, *Campylobacter*, and *Prevotella* was significantly increased (Fig. 6A). In the jejunum, FMT significantly increased the abundance of phylum *Actinobacteria.* At the genus level, compared to the control and PDCoV-infected piglets, *Veillonella* and *Selenomonas* levels were increased, while there was a decreasing

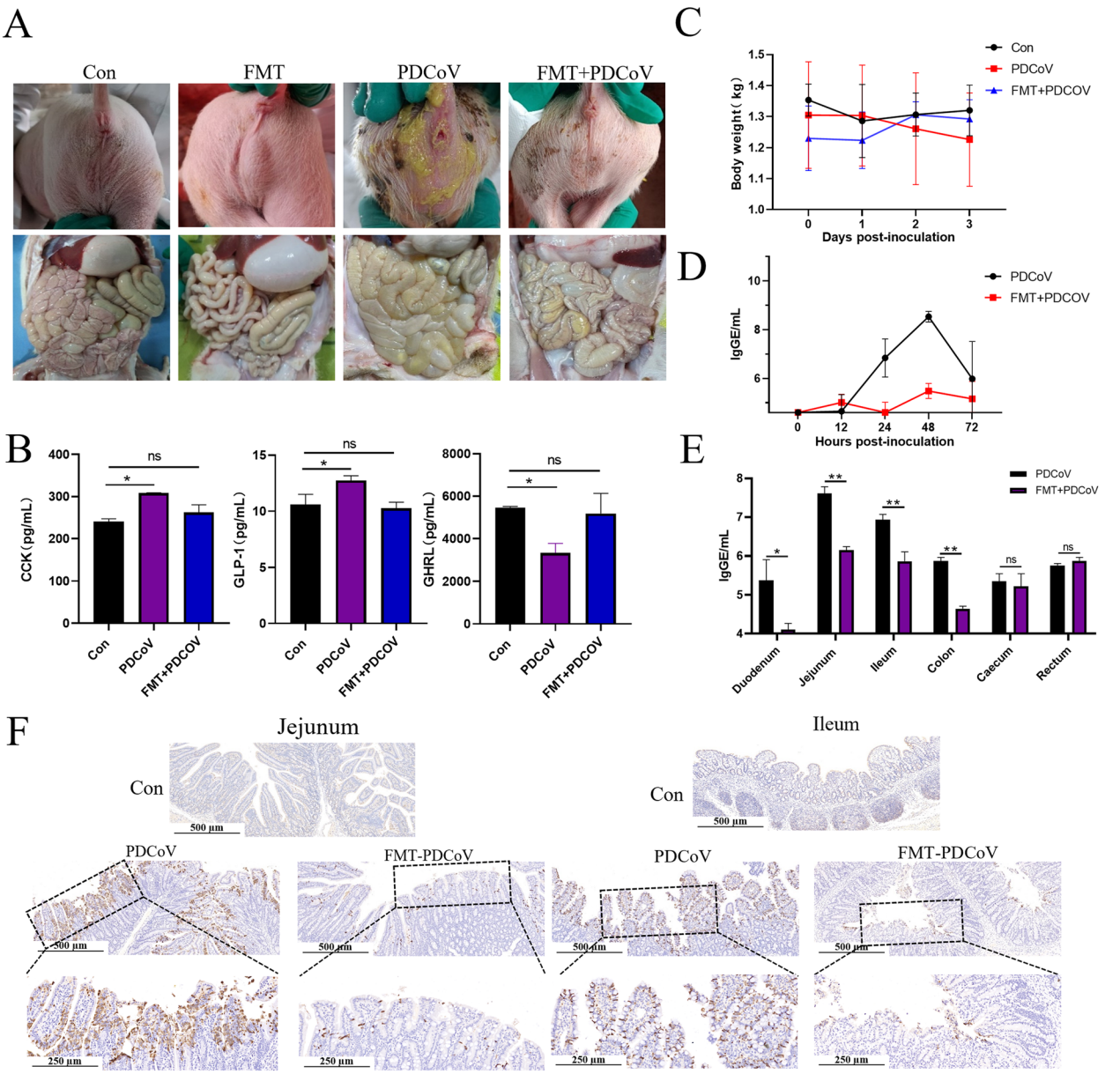

Fig. 5 Effects of FMT on pathogenicity in piglets infected with PDCoV HNZK-02. **A** Clinical symptoms and intestinal changes of piglets. **B** The CCK, GLP-1, and GHRL in serums were collected from the control (Con), PDCoV, and FMT-PDCoV groups. **C** The body weight. **D** Fecal virus shedding. **E** Viral tissue distribution. **F** Immunohistochemical analysis of jejunum and ileum. *$p < 0.05$; **$p < 0.01$

trend for *Lactobacillus*, *Streptococcus*, and *Mitsuokella* in PDCoV-infected recipient piglets (Fig. 6B). These results demonstrated that FMT could alter the composition of the gut microbiota affected by PDCoV infection.

In addition, the Venn diagram showed that the number of OTUs in control piglets, PDCoV-infected piglets, and PDCoV-infected recipient piglets were 775, 301, and 937, respectively, in jejunal contents, and 6001, 2264, and 5237, respectively, in colonic contents (Fig. 6C), suggesting that FMT could restore a number of OTUs affected by PDCoV infection. As shown in Fig. 6D, the results of Chao 1, Shannon, and Simpson diversity indices indicated that the PDCoV infection reduced the richness and diversity of gut microbiota, and FMT restored the richness as evidenced by the Chao 1 and Shannon indices, increased the diversity in the jejunum, and reduced the diversity in the colon as evidenced by the Simpson index. LEfSe analysis further indicated that in the jejunum, FMT increased the relative abundance of *Veillonella.* While in the colon, FMT increased the relative abundance of *Prevotella*, *Lactobacillus*, *Campylobacter*, and *Shigella* (Fig. 6E).

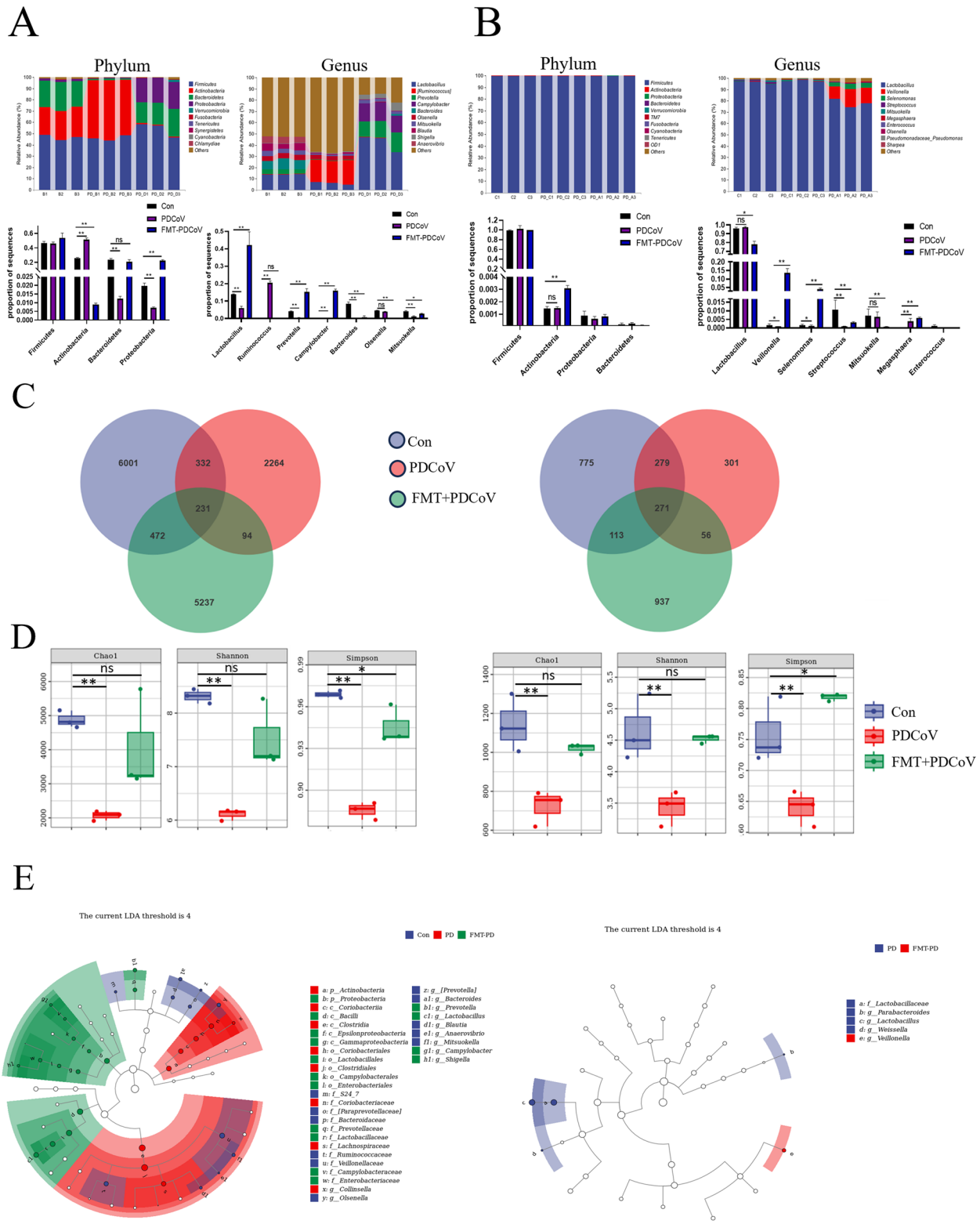

Fig. 6 The change of gut microbial composition in piglets infected with PDCoV after FMT. **A** The modulation of gut microbial composition in PDCoV-infected piglets by FMT in the colon. **B** The modulation of gut microbial composition in PDCoV-infected piglets by FMT in jejunum. **C** Venn diagram of shared OTUs based on the sequences with more than 97% similarity in the colonic (left) and jejunum (right) microbiota of the piglets of control, PDCoV, and FMT + PDCoV group. **D** The alpha diversity indexes (Chao1, Shannon, and Simpson) of the colonic (left) and jejunum (right) microbiota. *$p < 0.05$; **$p < 0.01$

FMT allowed to maintain the intestinal barrier function in PDCoV-infected piglets

To evaluate the protective effects of FMT on intestinal barrier function in PDCoV-infected piglets, we examined the intestinal villi using SEM and assessed goblet cells, mucins, and MUC2 protein levels PAS staining, AB-PAS staining, and western blot, respectively. Additionally, the integrity of the mechanical barrier was evaluated by analyzing DAO activity, D-Lac content, and intestinal TJ expression. The results showed that the villi of jejunal and ileum in the piglets infected with PDCoV suffered severe damage, characterized by villous atrophy, blunting, and even shedding. However, there was no significant difference in the ileum between the control and the PDCoV-infected recipient piglets regarding the extent of damage (Fig. 7A). These results demonstrate that the FMT effectively mitigates the damage to jejunal and ileum villi caused by PDCoV infection. The PAS staining and AB-PAS staining revealed that the PDCoV infection can significantly reduce the number of goblet cells and the thickness of the mucous layer in the colon. While compared with the PDCoV-infected piglets, the number of goblet cells in the colonic mucosa of the FMT recipient piglets was significantly increased, and the thickness of the colonic mucus layers was markedly enhanced compared to the PDCoV-infected piglets (Fig. 7B). Consisting with the staining, the protein expression of MUC2 protein expression also increased significantly (Fig. 7C). These findings indicated that FMT enhances the mucosal barrier and protects the intestinal epithelium against PDCoV-infection. Furthermore, compared to PDCoV-infected piglets, recipient piglets exhibited significantly reduced DAO activity and D-Lac in their serum (Fig. 7D), indicating that the FMT treatment decreases intestinal permeability. The expression of occludin and ZO-1, both at the protein and mRNA levels, were higher in the colonic tissue of recipient piglets than in the PDCoV-infected piglets (Fig. 7E). This suggests that the early-life gut microbiota intervention via FMT maintain the secretion of tight junction protein of intestinal against PDCoV challenge.

Functional changes of the gut metabolome in the colon of the recipient piglets following FMT

To determine the differential levels of metabolites in the intestinal lumen of PDCoV-infected piglets following FMT, we performed metabolomic analysis using LC–MS/MS. The typical total ion chromatograms (TICs) of the control piglets and recipient piglets are shown in Fig. S4A. A total of 2276 metabolites were identified (1608 in positive mode and 668 in negative mode) (Fig. 8A), including lipids and lipid-like molecules, organic acids and derivatives, and organoheterocyclic compounds (Fig. 8B). Score plots of orthogonal partial least squares-discriminant analysis (OPLS-DA) (Fig. 8C) and PCA (Fig. 8D) based on the identified untargeted metabolites revealed a significant separation between the clusters of piglets with or without FMT, illustrating significant differences in metabolites composition between the two groups. In addition, the permutation test was used to assess the robustness of the model, yielding $R^2 = 0.3272$ and $Q^2 = -0.6404$ for positive ion mode, and $R^2 = 0.8777$ and $Q^2 = -0.3536$ for negative ion mode (Fig. S4B), the model is stable.

The metabolic compounds between the two groups were compared based on the criteria of variable importance in the project (VIP) > 1 and $p < 0.01$. A total of 167 and 124 metabolites were identified as significantly different in the positive and negative ion modes, respectively. Specifically, in the positive ion mode, 129 metabolites increased and 38 decreased, while in the negative ion mode, 72 metabolites increased and 52 decreased (Fig. 8E). As shown in Fig. 9A, the top 40 metabolites with high VIP scores belong to categories such as lipids and lipid-like molecules, benzenoids, nucleosides, nucleotides and analogues, organic acid and derivatives, organic oxygen compounds, and organoheterocyclic compounds. These metabolites have great potential to distinguish between control and recipient piglets. To identify metabolite biomarkers for differentiating the gut metabolome between the two groups, 70 and 45 metabolites were found to be significant differentially expressed metabolites (DEMs) (log2 fold change > 1.5, adjusted p value < 0.05, VIP scores > 1.5) in positive and negative ion modes, respectively (Tables S4 and S5). Metabolite set enrichment analysis was used to identify key biomarker metabolic pathways. The results indicated that arginine biosynthesis was the most affected pathway ($p = 0.0007$), followed by biosynthesis of amino acids ($p = 0.0026$) and glycine, serine, and threonine metabolism ($p = 0.0219$) (Fig. 9B). Consequently, we further explored the role of arginine biosynthesis in PDCoV infection.

FMT attenuated the inflammation response through modulating TLR4/MyD88/NF-κB inflammasome pathway

Our and other previous studies have demonstrated that PDCoV infections lead to robust expression of pro-inflammatory cytokines, including IL-6, IL-8, and TNF-α [8]. The inflammatory response is closely linked to the TLR4/NF-κB inflammasome pathway [39, 42, 43]. Besides, it has been shown arginine biosynthesis can attenuate inflammation through the regulation of TLR4 and NF-κB [44], while PDCoV infection induces TLR4 and MyD88 upregulation [45]. We explored whether

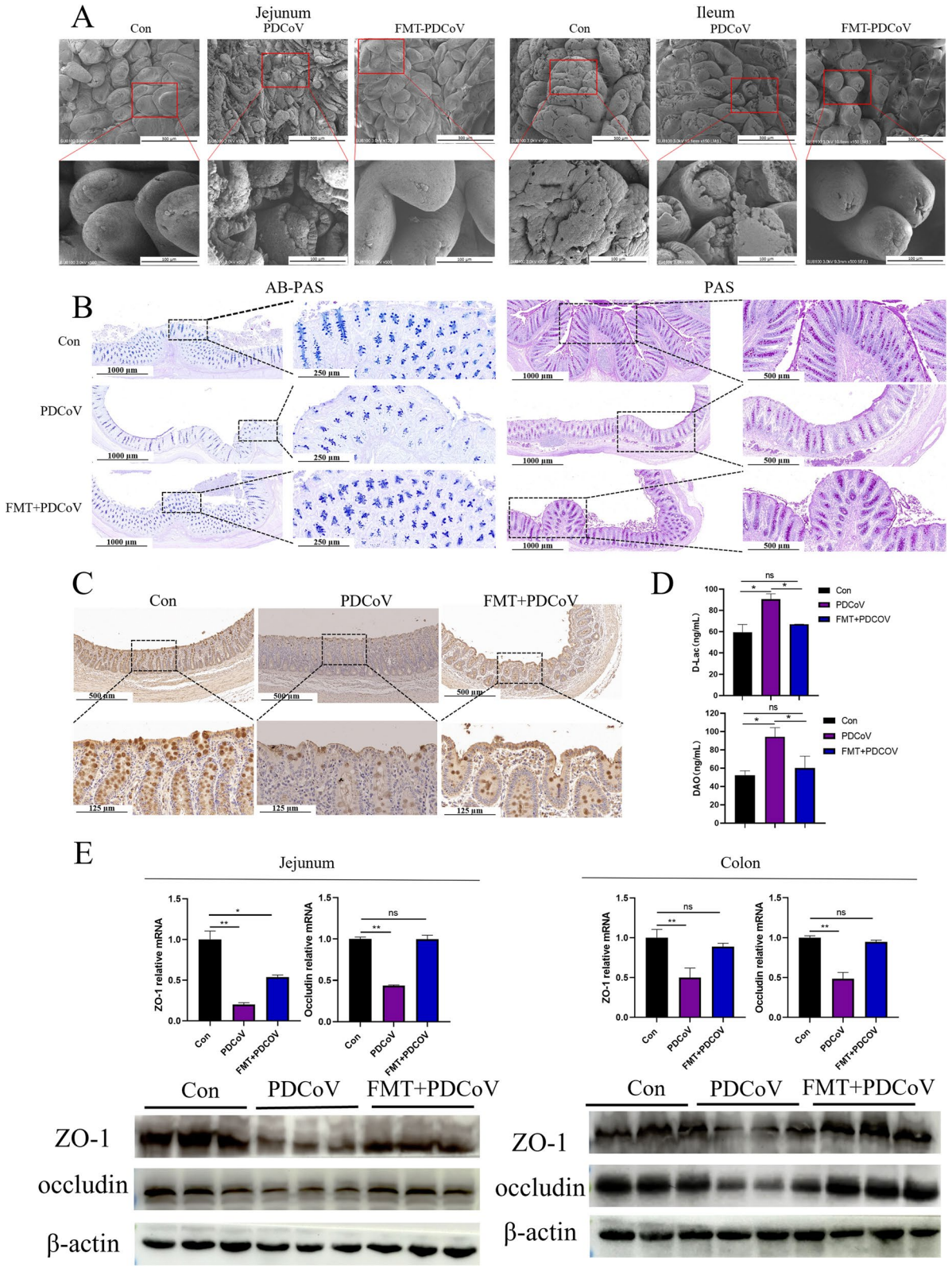

Fig. 7 Intestinal morphology and barrier analysis of piglets. **A** The morphology of jejunum and ileum villi was observed by SEM. **B** AB-PAS and PAS in colon. **C** Immunofluorescence images of the colon with MUC2 protein. Scale bars are shown in each picture. **D** The DAO and D-Lac in serums collected from the piglets of control, PDCoV, and FMT + PDCoV group. **E** The relative levels of protein and mRNA expression of ZO-1 and occludin in the jejunum (left) and colon (right). *$p < 0.05$; **$p < 0.01$

A

Detection mode	Number of metabolites
Postive mode	1608
Negtive mode	668

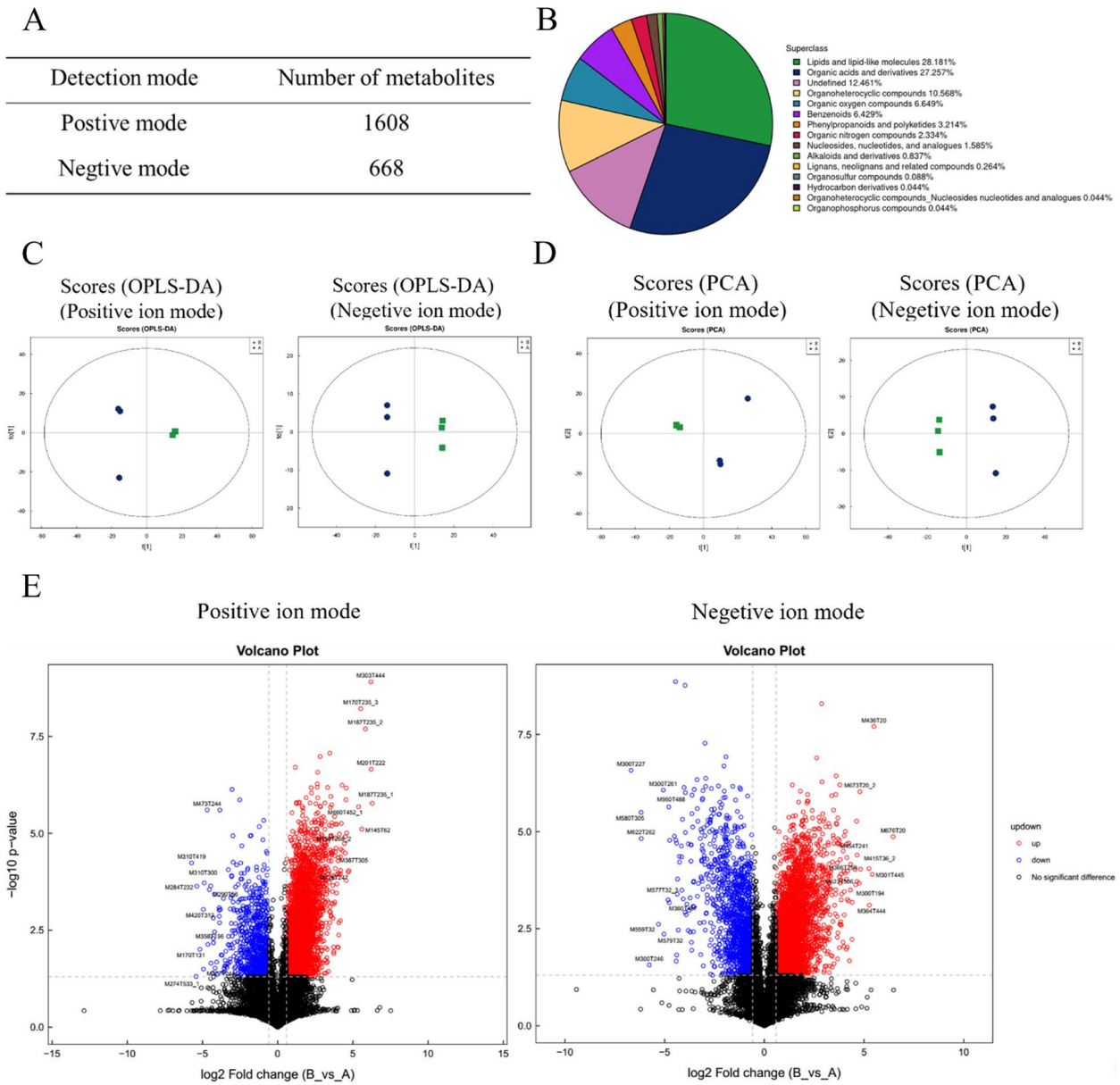

Fig. 8 Statistical comparison of metabolites of untargeted metabolomics of colonic contents from piglets and FMT-piglets. **A** The number of metabolites identified in positive and negative ion models. **B** The proportion of metabolites identified in each chemical classification. OPLS-DA score plot (**C**) and PCA analysis (**D**) revealed a significant separation between the clusters of the control and FMT piglets. Each dot in the score plots represents an independent sample. **E** Volcano map reveals significant changes of metabolites (FMT relative to Con) in colon contents following FMT. Expression of metabolites increased are shown in red and decreased are shown in blue. Each dot in the volcano plots represents a detected metabolite

the TLR4/NF-κB inflammasome pathway is involved in FMT-dependent regulation of the inflammatory cytokines. FMT significantly reduced the expression of typical inflammatory cytokines (IL-6, IL-8, and TNF-α) in piglets infected with PDCoV (Fig. 10A), suggesting that early-life gut microbiota intervention by FMT can help to alleviate the inflammatory responses caused by PDCoV. Subsequently, we measured the expression levels of TLR4, NF-κB, and *p*-NF-κB in the colon. As shown in Fig. 10B, the PDCoV infection resulted in increased protein expression of TLR4, MyD88, NF-κB, and *p*-NF-κB compared to the control group. However, FMT significantly decreased the expression of these proteins. Taken together, these results indicated that early-life gut

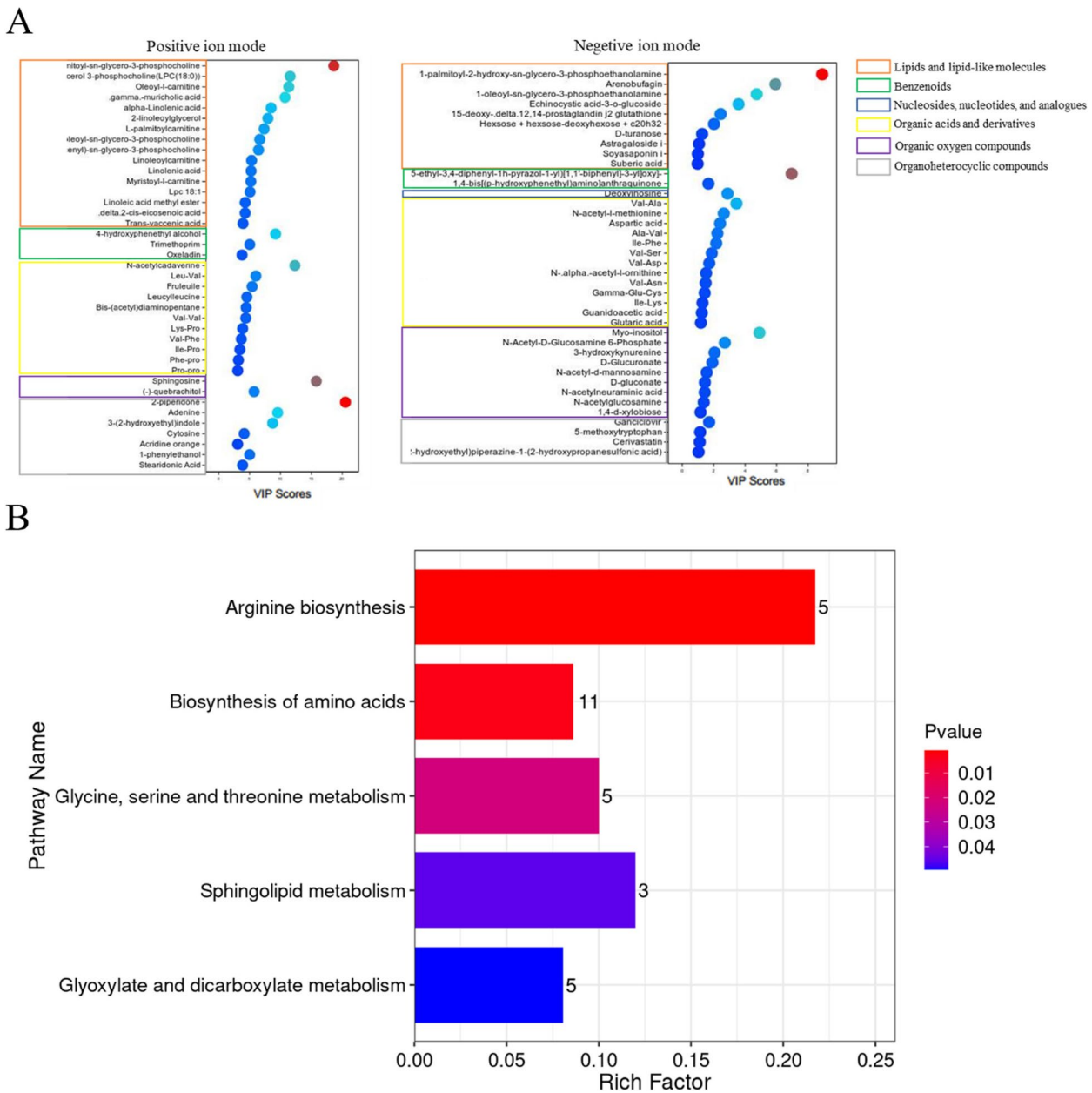

Fig. 9 **A** The top 40 metabolites with high discriminatory accuracy ranked by variable importance in projection (VIP) score in positive (left) and negative (right) ion mode. Classification of these compounds are shown by a color box. **B** Summary plot for metabolite set enrichment analysis. The size of a bar indicates the fold enrichment of each pathway and the color density of each bar indicates the extent of the difference

microbiota intervention via FMT inhibited the excessive inflammatory response induced by PDCoV in piglet, and the mechanism of inhibition was associated with suppression of the TLR4/NF-κB inflammasome pathway.

Discussion

As a newly emerged porcine enteropathogenic coronavirus, PDCoV primarily causes severe diarrhea, dehydration, and even death in nursing piglets, leading to significant economic losses in the pig industry. Furthermore, PDCoV infects a variety of animals, including humans, [46], indicating its potential for cross-species transmission and zoonotic risk. Currently, there are no effective drugs or vaccines available against PDCoV infection. Gut microbiota and its metabolites play an important role in maintaining intestinal homeostasis and are closely associated with gastrointestinal diseases [31]. This study provides evidence that the differential

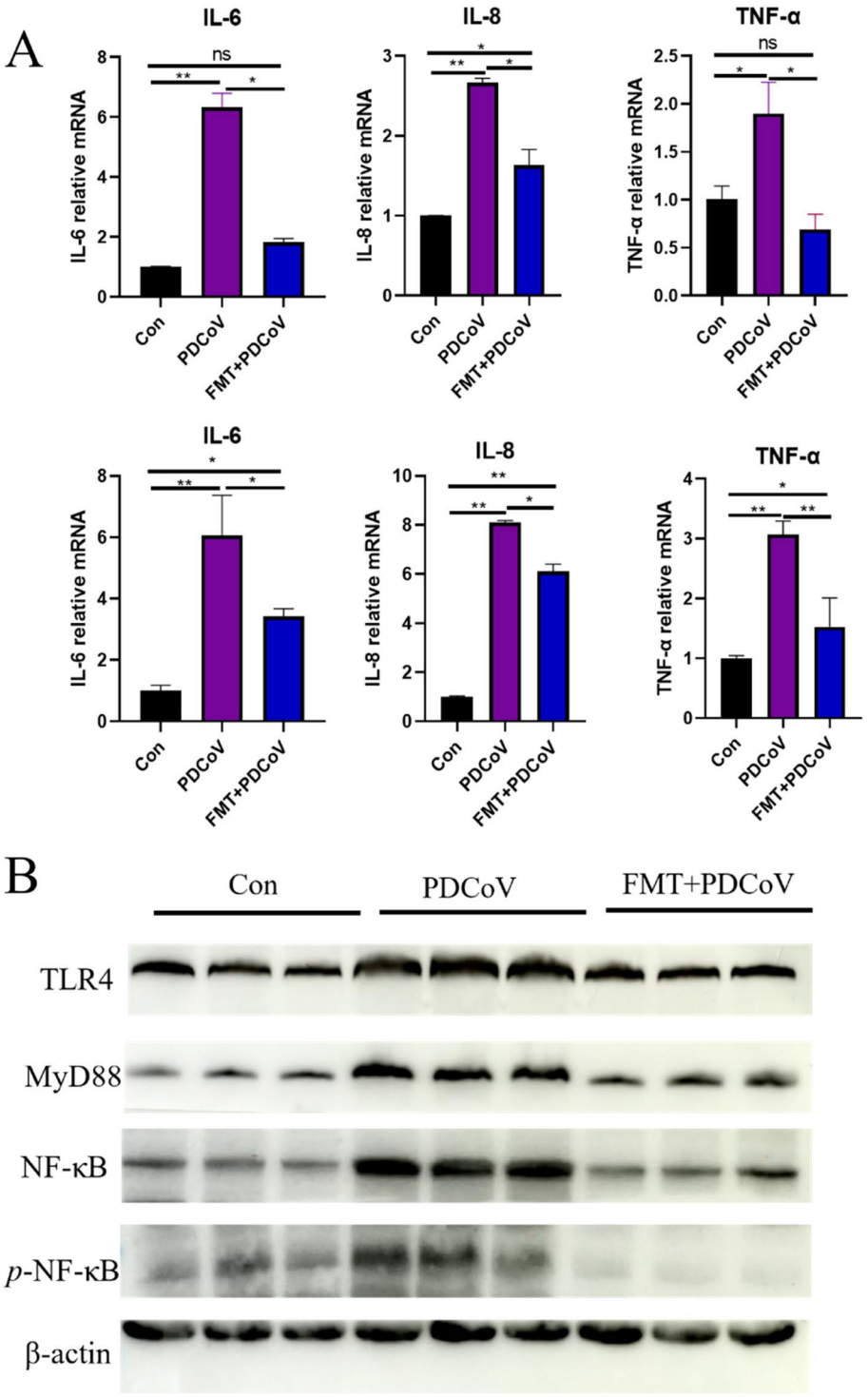

Fig. 10 Inflammatory response and the associated pathways. **A** Cytokines detection in jejunum (up) and colon (down) tissue in the piglets of control, PDCoV, and FMT-PDCoV group. **B** The protein expression of TLR4, MyD88, NF-κB, and *p*-NF-κB in colon tissues by western blot. *$p < 0.05$; ** $p < 0.01$

gut microbiota of 8-day-old and 90-day-old pigs influences the severity of PDCoV infection. The microbiota modulations likely reduce the piglet's susceptibility to PDCoV by maintaining intestinal health and microflora homeostasis of piglets. Moreover, enhanced arginine biosynthesis via FMT attenuated the PDCoV-induced inflammatory response by regulating the TLR4/MyD88/NF-κB pathway.

Previous study has shown that the diversity (richness and evenness) of gut microbiota in pigs increases progressively during development until a stable level is attained [37]. Notably, clinical observations indicate that PDCoV-induced diarrhea predominantly occurs in suckling piglets rather than older pigs [38]. We hypothesized that age-related differences in gut microbiota composition may influence the susceptibility of pigs to PDCoV. In our study, we observed that 8-day-old piglets infected with PDCoV exhibited pronounced clinical symptoms, whereas 90-day-old pigs infected with PDCoV showed no obvious pathological lesions, consistent with our previous study [8]. In addition, following infection with PDCoV, there was a significant decreased in community diversity in 8-day-old piglets, while 90-day-old pigs exhibited a remarkable increased in these parameters. Previous studies have indicated that increased microbial diversity benefits gut health [47, 48]. Therefore, we further analyzed the difference in the composition of gut microbiota between 8-day-old and 90-day-old pigs. Compared to the 8-day-old piglets, the abundance of the phyla *Firmicutes* and *Verrucomicrobia* was higher in the 90-day-old piglets. *Firmicutes* promote the development of intestinal epithelial cells and protect the intestinal tract from infection by producing large amounts of pyruvic and butyric acid [49]. *Verrucobacteria* are strongly associated with obesity, IBD, sleep disorders, type 2 diabetes, and anti-inflammatory responses [50]. Furthermore, 90-day-old pigs exhibited higher levels of *Blautia*, *Lactobacillus*, and *Gemmiger* and lower levels of *Ruminococcus*, *Bacteroides*, and *Oscillospira*. Among these, *Blautia* relieves inflammation and is linked with metabolic diseases [51], *Lactobacillus* regulates mucosal MUC2 expression and maintains intestinal integrity [52], and *Ruminococcus* is related to IBD, asthma, and depression [53]. Therefore, we hypothesized that gut microbiota can maintain intestinal health and reduce the susceptibility of pigs to PDCoV.

FMT has gained considerable attention over the past decade as it has proven to be a highly effective treatment for recurrent clostridioides difficile infections (rCDI) [54, 55]. It has also demonstrated some success in treating IBD, irritable bowel syndrome (IBS) [56], and decolonization of multidrug-resistant organisms to resolve disease [57]. The mechanism by which FMT works is not fully understood, but evidence suggests multiple mechanisms. One possible mechanism involves the transplantation or engraftment of donor species to the recipient microbiome, thereby complementing missing functions [58]. FMT has been shown to alleviate intestinal injury and reduce inflammation caused by weaning stress in piglets [32]. It can also significantly reverse pathogen-induced changes in the composition of gut microbiota in mice [59, 60]. In the present study, we found that FMT reduced the susceptibility to PDCoV in piglets, characterized by minimal clinical symptoms and intestinal injury. Additionally, FMT restored the composition and diversity of gut microbiota in piglets infected with PDCoV, consistent with observations in mice and piglets [32, 60]. Consisting with other studies [40, 61], we found that FMT improved the alpha diversity of the gut microbiota in colonic and jejunal contents in piglets, indicating the microbiota could effectively colonize the intestines. Specifically, FMT increased the abundance of the phyla Bacteroidetes, Fusobacteria, Lactobacillus, Mitsuokella, and Prevotella. These results demonstrate that FMT can elevate the relative abundance of beneficial genera.

The epithelial barrier of the intestine is composed of epithelial cells and intercellular junction proteins, which prevent the invasion of harmful substances, such as antigens, toxins, and microorganisms [62]. It has been reported that FMT can decrease the tight junction (TJ) and adherent junction proteins in E. coli K88 infected piglets [63]. We observed that the damaged villi caused by PDCoV infection were effectively repaired following FMT. Goblet cells, mucus, and mucin in the intestinal epithelium play important roles in the immune regulation of the intestine [64]. The secretion of TJ proteins increases the permeability of the epithelial barrier, with DAO and D-lactic acid as sensitive markers [65]. In this study, FMT increased the expressions of ZO-1, occludin, and MUC2 proteins, as well as the thickness of the mucus layer in PDCoV-infected piglets. Meanwhile, the levels of DAO and D-lactic acid in the serum of PDCoV-infected piglets were also reduced by FMT. Pro-inflammatory cytokines such as IL-6, IL-8, and TNF-a play pivotal roles in the immune response [66]. FMT diminished the elevated expressions of pro-inflammatory cytokines IL-6, IL-8, and TNF-α induced by PDCoV infection. Overall, our data suggest that early-life gut microbiota intervention via FMT maintains intestinal health and relieves intestinal barrier injury and inflammatory responses caused by PDCoV.

The gut microbiota-derived metabolites are essential in maintaining the gut homeostasis [67]. In exploring the connection between FMT-induced microbial changes and the modulation of the intestinal physiological function, we observed that FMT significantly

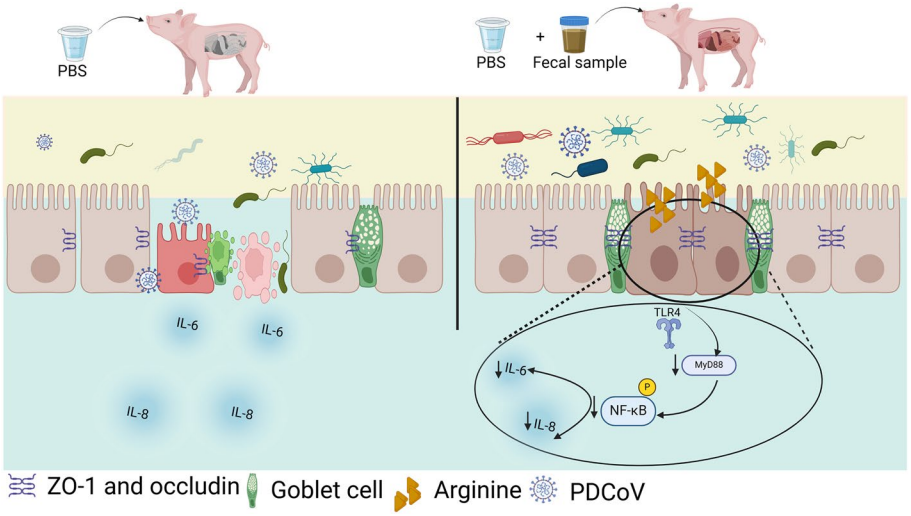

Fig. 11 Mechanisms of PDCoV infection alleviation by FMT. The fecal microbiota suspension from 90-day-old healthy pigs were transplanted into 3-day-old piglets, which could maintain the intestinal physical barrier, characterized by reduced levels of DAO and D-Lac in serum and the expression of ZO-1 and occludin in the intestine of 3-day-old piglets infected with PDCoV, and maintain the intestinal chemical barrier, characterized by the increased the number of intestinal goblet cells, the expression of MUC2 protein, and the thickness of the mucus layer. FMT significantly enhanced arginine metabolism, which may be related to that gut microbiota can reduce the inflammatory response induced by PDCoV by inhibiting the TLR4/MyD88/NF-κb signaling pathway.The mechanisms diagram was created in BioRender (https://BioRender.com/h95b651)

increased the proportions of pathways involved in tryptophan metabolism and cytochrome P450, while decreasing proportions of pathways associated with NOD-like receptor, linoleic acid metabolism, and fructose and mannose metabolism showed a trend to increase. Previous studies have shown that cytochrome P450 reduces intestinal injury caused by IBD [68], and metabolites derived from tryptophan metabolites regulate inflammation by limiting the activation of NF-κB [69]. Moreover, the NOD-like receptors are known to regulate cellular pathways that govern both the growth and the immune responses to stimuli, including the MAPK and NF-κB pathways [70]. These findings suggest that changes in microbiota-derived metabolites contribute to maintaining intestinal health, consistent with the observed improvement in intestinal epithelial integrity in PDCoV-infected piglets following FMT. Metabolite set enrichment analysis demonstrated that the arginine biosynthesis was the most significantly affected metabolic pathway by FMT. Studies have shown that arginine biosynthesis ameliorates multidrug-resistant pseudomonas aeruginosa induced pulmonary inflammation [44]. Spearman's correlation analysis further indicated that *Lactobacillus*, *Bacteroides*, *Mitsuokella*, and *Prevotella* were positively correlated with pathways such as arginine biosynthesis, biosynthesis of amino acids, glycine, serine and threonine metabolism, sphingolipid metabolism, and glyoxylate and dicarboxylate metabolism. Conversely, *Blautia*, *Anaerovibrio*, and *Olsenella* were negatively correlated with these pathways.

Arginine supplementation enhances immunity, anti-infective, and anti-oxidative responses [71]. Moreover, arginine and its metabolites are involved in the inflammatory response and may inhibit the secretion of proinflammatory factors. Notably, there are strong associations between arginine metabolites and hyper inflammation in COVID-19 patients [72]. In this study, we found that arginine biosynthesis was the most significantly affected metabolic pathway by FMT. The TLR4/MyD88/NF-κB is a classic pathway in the inflammatory response, and its activation can increase the production of proinflammatory cytokines and growth factors [73]. Previous studies have shown that arginine biosynthesis can improve inflammation by inhibition of the TLR4/NF-κB pathway [40]. Consistent with reported data, our results demonstrated that FMT significantly decreased the production of TLR4, NF-κB P65, *p*-NF-κB P65, and MyD88 in the recipient colon, indicating that gut microbiota can reduce the inflammatory response induced by PDCoV by inhibiting the TLR4/MyD88/NF-κb signaling pathway, which may be related to the arginine synthesis.

Conclusions

In conclusion, this study demonstrated that the gut microbiota of pigs at different ages affect their susceptibility to PDCoV. Multi-omics analysis revealed that FMT reduces susceptibility to PDCoV infection in pigs by maintaining intestinal health and modulating arginine biosynthesis in the gut microbial community. Moreover, FMT decreases inflammatory response by inhibiting the TLR4/MyD88/NF-κB signaling pathway (Fig. 11). These findings provide valuable insights for the prevention and control of enteropathogenic coronaviruses.

Supplementary Information

The online version contains supplementary material available at https://doi.org/10.1186/s40168-025-02092-z.

Additional file 1. Supplementary information.

Additional file 2. Tables S3–S5.

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Authors' contributions

Y.-F.Z., wrote the paper and organized and typeset the images; L.-L. S., and X.-L. S. d analyzed the data.; C.-R. Q. and X.-H. W helped raise the animals; H.-Y.L., S.-J. M. and X.-H. J. helped revise the manuscript; Z.-Y. W. and H. H. designed the experiments and revised the manuscript.

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Data availability

No datasets were generated or analysed during the current study.

Declarations

Ethics approval and consent to participate

The research protocols for animal experiments were approved by the Animal Care and Use Committee of Henan Agricultural University (Zhengzhou, China) and were performed in accordance with the "Guidelines for Experimental Animals" of the Ministry of Science and Technology (Beijing, China).

Consent for publication

Not applicable.

Competing interests

The authors declare no competing interests

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