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Fecal microbiota transplantation modulates jejunal host-microbiota interface in weanling piglets

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Abstract

Background Weaning-associated enteric diseases are a major concern in the swine industry. This study investigates the effects of fecal microbiota transplantation (FMT) on the jejunum of weanling piglets, a segment of bowel less studied in terms of microbiomic changes despite its primary involvement in major post-weaning enteric diseases, including postweaning diarrhea (PWD). Thirty-two 3-week-old piglets were divided equally into two groups: Control and FMT. The FMT group received fecal microbiota preparation from 3-month-old healthy pigs on the 1st and 3rd day after weaning. Half of each group was inoculated with an enterotoxigenic *E. coli* (ETEC) isolate 10 days post-FMT. Piglets were euthanized in the third week (14th and 18th days post-FMT) after weaning to collect intestinal tissues and contents for microbiomic, metabolomic, and transcriptomic analyses.

Results The jejunal microbiota showed a significant increase in alpha diversity in the third week post-FMT compared with the ileum and colon. FMT significantly enriched the jejunal microbiota composition, while multiple bacterial genera were specifically lacking in control weanling piglets. FMT was strongly associated with the enrichment of the genus *Pseudoscardovia* of the *Bifidobacteriaceae* family, which was found lacking in the jejunum of weanling control piglets and inversely associated with the abundance of the genus *Bifidobacterium* within the same family. Other genera associated with FMT included *Solobacterium*, *Shuttleworthia*, and *Pseudoraminibacter*, whereas bacteria such as *Erysipelotrichaceae* and *Acidaminococcus* were identified as most abundant in the control piglets. Metabolomic analysis revealed a significant modulatory effect of FMT on carbohydrate, amino acid, nucleotide, vitamin, and xenobiotic metabolisms, suggesting improved nutrient utilization. Transcriptomic analyses further confirmed the regulatory effects of FMT on gene expression associated with immune, metabolic, barrier, and neuroendocrine functions. Prior FMT treatment in the context of ETEC infection indicated a potential protective role, as evidenced by a significant shift in microbial diversity and metabolomic compositions and decreased diarrhea severity even though no effect on pathogen shedding was evident.

Conclusions This study underscores the promise of FMT in enhancing jejunal health. In addition, the results suggest that FMT could be considered a potential strategy to address conditions associated with small intestinal dysbiosis in swine and other monogastric species with similar gut anatomy and physiology, such as humans.

Keywords Postweaning diarrhea, Fecal microbiota transplantation, Weanling piglets

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Introduction

In commercial swine production, weaning is one of the most critical events in pig development, which involves abrupt dietary, environmental, and social changes [1]. Recent studies confirm that abrupt change in dietary and environmental conditions and weaning stress converges into a state of gut dysbiosis, which is indeed pivotal in precipitating weaning-associated diseases such as postweaning diarrhea (PWD) in piglets [2–4]. After birth, the piglet gut is quickly colonized with maternal and environmental bacterial flora, and its composition and diversity play a key role in gut health and susceptibility to enteric infections [5]. The sudden transition to a solid diet, along with environmental factors and stress at weaning, results in dramatic changes in the piglet gut microbiome, causing gut dysbiosis [6].

Alterations in the gut microbiota are closely associated with major post-weaning enteric bacterial infections [2]. Moreover, dysbiosis induces changes in intestinal metabolites, increases intestinal barrier permeability, reduces mucus production, and elevates pro-inflammatory cytokines in the gut [7, 8]. Post-weaning gut dysbiosis has been identified as the key step in enterotoxigenic *E. coli* (ETEC) colonization and progression to PWD [2]. Furthermore, recent studies indicate that weaning-associated illnesses, such as ETEC infection directly induce and further exacerbate gut dysbiosis in piglets, which subsequently mediates intestinal inflammation and diarrhea [9–11]. These studies establish gut dysbiosis as a key factor in the pathogenesis of weaning-associated swine diseases, especially PWD, and its involvement in both the initiation and manifestation of the disease. Therefore, counteracting gut dysbiosis during the weaning transition could prevent such infections and reduce the clinical manifestation of the disease at the same time.

Husbandry practices associated with dietary and environmental changes and piglet weaning age are parameters that are not likely to change significantly in commercial swine production due to practical and economic concerns. However, it is possible to control post-weaning dysbiosis based on current microbiome research knowledge. Fecal microbiota transplantation (FMT) has been widely used in human and veterinary medicine to treat a number of enteric diseases [12, 13]. These include diseases such as *Clostridioides difficile* infection (CDI), inflammatory bowel disease (IBD), ulcerative colitis, and Crohn's disease [14]. Management of CDI in humans is the most notable example of the successful use of FMT as a radical therapeutic strategy against dysbiosis and enteric infections [15]. For example, PWD in piglets has a similar pathogenesis to CDI in terms of gut-dysbiosis and subsequent pathogen colonization [16]. Thus, stabilizing the gut microbiome by transplanting mature and healthy gut microflora to weaning piglets could potentially prevent gut dysbiosis and PWD.

Intestinal changes associated with FMT have been extensively studied in humans, rodents, and, to a lesser extent, domestic animals, including pigs [12, 13, 17–35]. Most of these studies have focused on the large intestinal microbiota, particularly the colon. However, research on the impact of FMT on the small intestine—the primary target site of ETEC infection remains limited. In addition to ETEC, major swine enteric diseases, including clostridial and rotaviral infections, predominantly affect the small intestine, especially the jejunum, the primary site of nutrient absorption and the longest bowel segment [36]. The involvement of this segment, along with ileum, is also significant in other infections such as *Salmonella*, *Lawsonia,* and enteric coronaviral infections, highlighting the importance of jejunum in FMT studies [36]. Therefore, this study investigates the effects of FMT on the microbiomic, transcriptomic, and metabolomic environments of the jejunum, focusing on PWD caused by ETEC infection in piglets.

Materials and methods

Donor animals, preparation of FMT mixture, and ETEC inoculum

The fecal microbiota mixture (FMT mix) for this study was prepared from six healthy 3-month-old donor pigs as per a recently published protocol [37]. All donor pigs were screened for PEDV, PCV, and swine influenza viruses and enteric parasites at the Iowa State University – Veterinary Diagnostic Laboratory (ISU-VDL). Briefly, freshly voided fecal material was aseptically collected onto sterile pre-reduced PBS in 50 ml tubes and was transferred on ice to an anaerobic workstation for further processing. The pooled fecal material was further diluted five times with an adequate amount of pre-reduced phosphate-buffered saline (PBS) and homogenized in a mixer. The suspension was filtered through sterile sieves and administered orally to the recipients or mixed with 10% sterile glycerol to store at − 80 °C. For ETEC inoculation, an F18-positive clinical hemolytic *E. coli* isolate from ISU-VDL was used for this study. The genotype of the ETEC clinical isolate used in this study was validated using a PCR-based ETEC-virulence panel at ISU-VDL and visually demonstrating bacterial attachment to jejunal mucosa using RNAscope in situ hybridization targeting F-18 pilus (Supplementary Fig. 1). The bacterium was cultured overnight in tryptic soy broth, and the bacterial count was enumerated by dilution and plating. The final inoculum composed of 10^6 CFU/ml ETEC cells was reconstituted in 5 ml PBS and transported to the animal facility on ice for immediate inoculation.

ETEC infection experiment, sample collection, and ETEC quantification

All animal experiments and procedures were performed according to the Institutional Animal Care and Use Committee (IACUC) under protocol IACUC-22–246 and 18–342. Piglets were sourced from a known herd with confirmed genetic susceptibility to ETEC infection [38]. Thirty-two suckling piglets weaned and weighed at three weeks of age were randomly divided into four experimental groups ($n=8$ per group), which were housed in individual pens with ad-libitum access to starter diet and drinking water. The treatment groups include: Control group, FMT control group (FMT), ETEC challenge control group (EC), and FMT-EC challenge group (FMT + EC). FMT and FMT + EC groups were provided with the fecal microbiome mix (5 mL solution orally each day) on the 1st and 3rd days of the experiment (1st and 3rd day of weaning) by direct oral gavage [27]. On approximately day 10 of the study (10th-day post-weaning), animals in EC and FMT + EC groups were inoculated orally with approximately 10^6 CFU of F18-positive ETEC. Control groups were orally gavaged with PBS. Animals were weighed and observed for diarrhea for 8 days, and fecal samples were collected at different time points (0, 2, 4, and 8 dpi). Four piglets per each group were sacrificed each on the 4th and 8th-day post-infection (14th and 18th days post-FMT), and the intestinal tissue (jejunum, ileum and colon in RNA Later for RNAseq, in 10% buffered formalin for histopathology) and fecal material (from mid -jejunum, mid-ileum, and terminal colon (rectum)–fresh for immediate bacteriological analysis, flash frozen and stored at − 80 °C for 16 s RNA sequencing and metabolome analysis) were collected. The number of pigs with diarrhea was recorded daily throughout the study. The severity of diarrhea was recorded using a published scorecard by two independent personnel blinded to treatment groups [38]. Fecal hemolytic *E. coli* shedding was evaluated by plating fecal swabs onto Remel Blood Agar (TSA with 5% sheep blood) and incubating at 35 °C for 24 h. Hemolytic *E. coli* shedding was determined using a semi-quantitative method based on the growth of *E. coli* on the plates [38]. Shedding was scored on a five-point scale from 0 to 4: 0 indicated no growth, 1 represented hemolytic colonies only in the primary streak, 2 indicated compatible growth extending into the secondary streak, 3 showed growth into the tertiary streak, and 4 represented growth of hemolytic *E. coli* into the quaternary section of the agar plate. Fecal samples from infected and non-infected were routinely tested for ETEC by culture. The identity of *E. coli* suspect colonies (one hemolytic colony per sample) was confirmed using matrix-assisted laser desorption ionization time-of-flight mass spectrometry (MALDI-TOF MS) at ISU VDL [39]. Total DNA was isolated from 200 mg of feces using QIAamp Fast DNA Stool Mini Kit (QIAGEN, Carlsbad, CA) as per manufacturer's instruction. Real-time PCR was performed using CybrGreen Q-PCR Kit (New England BioLabs, Ipswich, MA) in a QuantStudio 3 PCR machine (Thermo Fisher Scientific, Waltham, MA) according to a previously published protocol and oligonucleotide primers for F18 gene [40]. Total DNA isolated from 200 µl of *E. coli* suspension of known titers was used as a standard, and the absolute quantification of F18 positive ETEC was performed by a standard curve method [40]. A graphical representation of the experimental design is provided as supplementary information (Supplementary Fig. 2).

16 s- rRNA sequencing and microbiome analysis

DNA extraction and sequencing

Total DNA was extracted from jejunal, ileal, and colon contents separately from different treatment groups (from all piglets sacrificed on 14th and 18th days post-FMT; $n=8$) using the DNeasy PowerLyzer PowerSoil Kit (Qiagen, MD, United States), and all DNA samples were stored at − 20 °C until further analysis on the Illumina MiSeq sequencing platform. Each sample's total DNA was fragmented and tagged with sequencing adapters using the Nextera XT DNA Library Preparation Kit (Illumina, San Diego, CA). The gene-specific sequence targeted the bacterial 16S V3 and V4 regions. The Illumina DNA library was prepared using calibrated Ampure beads-purified PCR products with a final loading concentration of 3 pM and 25% PhiX control, sequenced using the MiSeq V3 Kit on a MiSeq at 300 bp, 60 cycles, in paired-end mode. Metagenomic sequencing produced an average of ~ 1 million reads per sample. After sequencing, the reads underwent quality assessment, and paired ends were merged. PCR products were pooled equally based on molecular weight and concentrations.

Statistical and network analysis

The sequencing data was processed using a customized workflow of the Divisive Amplicon Denoising Algorithm (DADA2 v1.10). This process yielded a table of amplicon sequence variants (ASVs) through denoising, incorporating quality control, primer removal, and taxonomy assignments. Cutadapt trimmed forward and reverse primers, and reads were merged to generate paired-end sequences. Quality checks ensured the adequacy of read lengths and quality. The output was a BIOM table detailing sequences, ASV abundances, and taxonomy. MicrobiomeAnalyst was utilized for all analyses, serving as a comprehensive tool for statistical, visual, and meta-analysis of microbiome data [41]. It employed the MicrobiomeAnalyst R package for statistical analysis and graphical outputs. Features present in less than 20% of

samples or with minimum counts below four were filtered out, and features with low variance were removed based on interquartile range criteria. Samples were rarified to equal sequencing depth, normalized using total sum scaling (TSS), and analyzed for alpha and beta diversity, utilizing various indexes and statistical methods, including PCoA ordination with the Bray–Curtis index and PERMANOVA [42]. Core microbiome and biomarker analyses were conducted, identifying significant features and their effect sizes. The robustness of the taxonomic differences between the treatment groups was assessed using linear discriminant analysis effect size (LEfSe) analysis [43]. Heat trees were constructed using the "Metacoder" R package to visualize differentially abundant features, with significance determined by adjusted *p* values, employing a default Wilcoxon *p* value cutoff of −0.1 and a linear discriminant analysis score (LDA) score threshold of 2.0 [44]. General linear models were utilized through MaAsLin2 in MicrobiomeAnalyst to identify associations between microbial features and experimental metadata [45]. The primary metadata, such as treatment groups, was included as a "fixed effect," while time points were controlled as covariates, also as "fixed effects." This approach allowed for the control of potential confounding variables and the extraction of robust statistics for the associations between microbial features and experimental conditions. The analysis of composition of microbiomes (ANCOM) was performed in QIIME2 to identify differentially abundant taxa across experimental groups. In this analysis, microbial taxa abundance tables were input into the ANCOM plugin, and taxa with significant differences in relative abundance were identified based on W-statistics [46].

Untargeted metabolome analysis of jejunal contents

Qualitative large-scale profiling

Untargeted metabolomic analysis of the jejunal contents (from three piglets sacrificed 14th day post-FMT) was performed at the Mayo Metabolomic Core facility (Rochester, MN). Jejunal contents were deproteinized with a six-fold volume of cold acetonitrile/methanol (1:1 ratio), subjected to intermittent vortexing on ice for 30 min at 4 °C, and then centrifuged at 18,000xg. 13C6-phenylalanine (3 µl at 250 ng/µl) was added as an internal standard to each sample prior to deproteinization. The supernatants were split into two aliquots and dried down for analysis using an Agilent Technologies 6550 Q-TOF Mass Spectrometer coupled with a 1290 Infinity UHPLC. Data were acquired under both positive and negative electrospray ionization conditions across a mass range of 100–1200 m/z at a resolution of 10,000–35,000 (separate runs). Metabolite separation was achieved with a hydrophilic interaction column (HILIC, ethylene-bridged hybrid 2.1 × 150 mm, 1.7 µm; Waters) and a reversed-phase C18 column (high-strength silica 2.1 × 150 mm, 1.8 µm; Waters), each with a 20-min run time at a flow rate of 400 µl/min. Each sample underwent four runs to maximize metabolite coverage. Samples were injected in triplicate, with a quality control sample comprising a subset from the study, injected multiple times during each run. Raw data files were converted to.cef format using Masshunter DA Reprocessor software (Agilent). Data alignment and peak matrix conversion were performed with Mass Profiler Professional (Agilent). Unsupervised principal component analysis, ANOVA, 3D plots, heat maps, and partial least squares discrimination analysis (PLS-DA) were used for analysis. This process yielded a list of accurate mass molecular weights for differentially expressed components, which were then matched against the Metlin database for putative identification. Identified components were verified against reference standards. The Q-TOF method's mass accuracy was < 5 ppm, with retention time precision < 0.2%. A 1.2 × fold change was detectable with 4% precision.

Metabolome data and pathway analysis

A threshold of at least 5000 peak intensities in 75% of samples was set for eligibility in downstream analyses. The R package MetaboAnalystR facilitated data normalization, differential expression analysis, and visualization. Metabolites were normalized (SumNorm), log-transformed, and mean-centered (MeanCenter). Principal component analysis (PCoA) highlighted data heterogeneity, trends, and outliers. Hierarchical clustering analysis (HCA) elucidated clustering patterns among sample injections, group replicates, and metabolite clusters associated with clinical variables. Univariate analysis, including Student's unpaired *t*-test between Control vs. FMT and EC vs. FMT + EC, was conducted with multiple testing corrections to identify differentially expressed metabolites, requiring an FDR-adjusted *p* value ≤ 0.05 and |fold change| ≤ 1.5.

RNA-seq analysis of jejunal tissue

Tissue processing and RNA-seq analysis were performed by BGI Genomics Inc. (Cambridge, MA) as previously described [47]. Intestinal tissue samples (a section from the mid jejunum) from three piglets sacrificed on day 14th post-FMT in each group were used for further processing. Total RNA from each sample was extracted and purified using the TRIzol method. RNA sequencing was then performed on a BGISEQ500 platform (BGI Genomics). Ten samples underwent multiplexing, sequencing, differential gene expression analysis, and transcriptomic expression analysis. Quality control of raw data was conducted using SOAPnuke software, and clean reads were

obtained after removing rRNA and other contaminants. The clean reads were mapped to the *Sus scrofa* genome sequence using HISAT for mRNA and long non-coding RNA quantification and Bowtie2 for gene sequence alignment. For circRNAs quantification, clean reads were aligned to known *S. scrofa* circRNAs. Small RNAs were identified by mapping clean tags to a miRNA database and the *S. scrofa* genome sequence. Expression levels for each gene were calculated based on the mapped reads. Differential expression analysis was carried out using the DEGseq method via MA-plot software, with expression values transformed into log2 values. Student's *t*-test was used to compare gene expression levels between groups. Differentially expressed genes (DEGs) were identified based on p-values less than 0.05 and log2 fold changes (FC) in gene expression above or below 1. DEG functional classifications and pathways were assessed using Gene Ontology (GO) Elite and DAVID Bioinformatics Resources for Kyoto Encyclopedia of Genes and Genomes (KEGG) pathways analysis. All data could be manually extracted using the Dr. Tom online analysis system provided by BGI Genomics Inc.

Integrative multi-omics analyses

To elucidate the integrated metabolic and transcriptomic changes induced by FMT, a joint pathway analysis was performed using MetaboAnalyst 4.0 (https://www.metaboanalyst.ca) as described by Chong et al. (2019) [48]. For the metabolomic data, the metabolite intensities were normalized using the SumNorm method, log-transformed, and mean-centered using the R package MetaboAnalystR. For the transcriptomic data, the gene expression levels were normalized and log2-transformed for downstream analysis. Metabolomic and transcriptomic data sets were uploaded to MetaboAnalyst's Joint Pathway Analysis module, which facilitated the merging of these data sets by mapping metabolites and genes to their corresponding pathways using the KEGG database. Joint pathway analysis was performed to identify significantly impacted pathways by combining *p* values from metabolomic and transcriptomic data. The joint *p* value was calculated using Fisher's method, combining individual *p* values from metabolomic and transcriptomic analyses to assess pathway significance. Pathways with a joint *p* value < 0.05 were considered significantly enriched.

Similarly, microbiome-metabolome correlation analysis was performed using MicrobiomeAnalyst 2.0, as described previously [41]. Paired metabolome abundance lists and species abundance tables for each comparison were uploaded to the integrative microbiome metabolomics pipeline within MicrobiomeAnalyst 2.0 (https://www.microbiomeanalyst.ca). For statistical correlation analysis, the distance-based correlation method was utilized to detect both linear and non-linear correlations. The results were summarized as an interactive heatmap. To address the issue of high false positives in pairwise correlation analyses, a model-based correlation approach using over 5000 high-quality genome-scale metabolic models (GEMs) was employed. This provided a probability heatmap between microbial taxa and their metabolites. Data-driven and knowledge-driven streams of evidence were then integrated by overlaying the statistical and model-based correlation heatmaps.

Statistical analysis

Each individually housed pig was considered the experimental unit. Clinical, microbiological, and laboratory parameters from individual treatment groups and animals, including diarrhea scores and fecal shedding scores, are recorded by personnel blinded to treatment groups. The differences between the two groups were analyzed using an unpaired Student's *t*-test. Data from comparisons between two groups were analyzed statistically using the independent sample *t*-test to conduct variance analysis. Data from comparisons among four groups were analyzed by one-way analysis of variance (ANOVA) followed by Tukey's multiple-comparison test. *p* Values of ≤ 0.05 were considered statistically significant.

Results

FMT significantly altered the jejunal microbiota and enriched specific bacterial populations

To investigate the effects of FMT on the gut microbial landscape in weanling piglets, we utilized 16 s rRNA sequencing and comprehensive microbiome analysis. Piglets were given two doses of FMT mixture orally on the first week of weaning and euthanized for collecting samples on the second week of weaning. Four animals were euthanized on the 14th and 18th days, each post-FMT, and samples were collected from the jejunum, ileum, and colon for 16 s rRNA sequencing. Microbiome analysis revealed profound changes in composition (Fig. 1A), bacterial alpha diversity (Fig. 1D (Shannon index) & Supplementary Fig. 3 (Chao1 index)), and beta diversity (Fig. 1E) of the jejunal microbiota. Although some comparable compositional and beta-diversity changes were observed in the ileum and colon, alterations in the microbial diversity appeared to level up by the second week of FMT with no difference in alpha diversity indices, unlike in the jejunum. Compared with the jejunum, fewer bacterial communities were compositionally affected by FMT treatment in these segments (Supplementary Figs. 4 & 5). Therefore, from here on, we will describe only the jejunal changes due to the extent of microbiomic shifts observed in this segment, which is the focal point of our study.

FMT significantly enriched the alpha diversity of the jejunal microbiota, irrespective of sampling days ($p=0.03$) (Fig. 1D). Similarly, beta diversity analyses, as demonstrated through principal coordinates analysis (PCoA) plots, showed distinct clustering between control and FMT groups, signifying substantial differences in microbial community composition between these groups at both examined time points ($p=0.002$) (Fig. 1E). No significant differences in alpha and beta diversities were observed between samples collected from animals euthanized on the 10th and 14th days post-FMT. Comparative analysis of control and FMT groups revealed significant alterations in bacterial populations, irrespective of euthanasia and sample collection days (14 vs. 18 days post-FMT), evident from the abundance heatmaps (Fig. 1B) and phylogenetic tree abundance maps (Fig. 1C). FMT markedly increased the abundance of the class Clostridia, particularly spotlighting the orders Eubacteriales, with a pronounced increase in the families *Eubacteriaceae* and *Lachnospiraceae*. Within the *Lachnospiraceae* family, genera such as *Acetitomaculum*, *Lachnospiraceae* NK3A20 group, and *Shuttleworthia* had significant enrichment, while the *Ruminococcus gauvreaui* group showed a reduction. Additionally, there was a substantial rise in the class Gammaproteobacteria, notably within the Burkholderiales order and family, including the *Burkholderia-Caballeronia-Paraburkholderia* group. Conversely, FMT led to a significant decrease in the abundance of the class Coriobacteria and the order Coriobacteriales, particularly affecting the family *Atopobiaceae* and the genus *Olsenella*. The order Acidaminococcaceae and its corresponding family and genus, *Acidaminococcus*, were also enriched following FMT. At the family level, an increase was observed in *Erysipelotrichaceae* and the genus *Solobacterium*. Moreover, FMT significantly augmented the abundance of the genus *Pseudoscardovia* while diminishing the presence of the *Eubacterium nodatum* group (Fig. 1B, C).

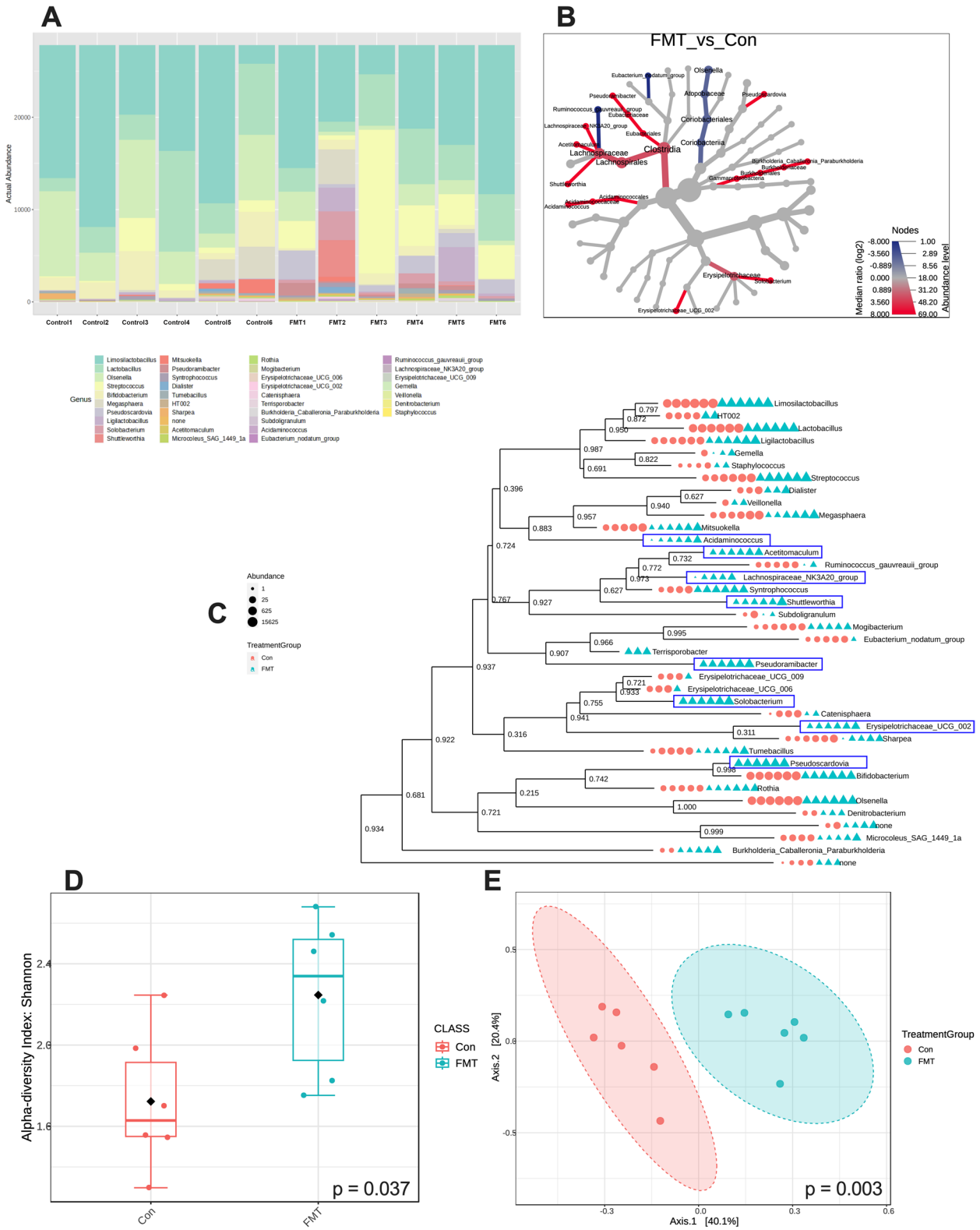
Linear discriminant analysis effect size (LEfSe) identified signature genera associated with FMT treatment, which include *Pseudoscradovia*, *Solobacterium*, *Shuttleworthia*, and *Pseudoraminibacter*, whereas bacteria such as *Erysipelotrichaceae* and *Acidaminococcus* were identified most abundant in control piglets (Fig. 2C). These differences were evident in the differences in the core microbiome compositions in the control and FMT groups. The core microbiome for each group was identified based on a sample prevalence of > 20% and at a relative abundance frequency of > 0.01% at the genus level. Although weaning piglets harbor *Bifidobacterium* in their jejunum, it appears to be replaced by *Pseudoscardovia* in the FMT group, which originates from young adult donor pigs aged three months (Fig. 2A, B, E). This is evidenced by the inverse association between these two genera (*Bifidobacterium* vs. *Pseudoscardovia*) (Fig. 2E) and the composition of the core microbiome (Fig. 2A, B) of control and FMT groups. Multifactor analysis employing multiple linear regression with a general linear model revealed significant associations between FMT and various microbial genera, with *Pseudoscardovia* ($p=1.72E-4$) and *Acetitomaculum* ($p=8.27E-4$) standing out (Fig. 2D). This observation was further corroborated by ANCOM analysis, which highlighted a significant association between FMT and *Pseudoscardovia*. Pattern analysis (Fig. 2E) delineated specific bacterial genera associated with *Pseudoscardovia* in the FMT group, such as *Pseudoramibacter* and *Burkholderia*, and inversely correlated with bacteria prevalent in the control group, including *Bifidobacterium*, *Olsenella*, and *Eubacterium nodatum*. Other significant bacterial genera significantly and positively correlated with FMT were *Erysipelotrichaceae*_UCG_002 ($p=0.00102$), *Pseudoramibacter* ($p=0.00102$), *Acidaminococcus* ($p=0.0035$), *Solobacterium* ($p=0.00371$), and *Lachnospiraceae*_NK3A20_group ($p=0.00789$). Meanwhile, *Eubacterium*_*nodatum* group ($p=0.00597$) and *Olsenella* ($p=0.00896$) were significantly and negatively correlated with FMT (Figs. 2E & 3).

FMT reprograms metabolic pathways critical for nutrient and xenobiotic metabolisms, and gut homeostasis in the jejunal microenvironment of weanling piglets

In the comprehensive metabolome analysis of jejunal content from weanling piglets undergoing FMT, a wide

(See figure on next page.)

Fig. 1 The effect of FMT on the jejunal microbiome composition and diversity of weanling piglets. **A** Stacked bar graph illustrating the actual abundance of jejunal gut microbiota at the genus level in control and FMT groups. **B** Heat tree depicting the hierarchical taxonomical distribution and relative abundance of significantly different microbial genera in control and FMT groups. The colors on the heat tree represent the median ratio of the log2-transformed abundance levels between the FMT and Control groups, with positive values (shades of red) indicating higher abundance in the FMT group and negative values (shades of blue) indicating higher abundance in the Control group. **C** Phylogenetic tree abundance map of microbial communities depicting comparative genetic diversity and the population density of the microbial genera in control and FMT groups. The blue square indicates selected genera that are significantly lacking in control weanling pigs but abundant in piglets that received FMT. The numbers surrounding the tree represent bootstrap values, indicating the confidence level of the branches. **D** Boxplot illustrating the Shannon index of alpha diversity within jejunal microbial communities of control and FMT groups; **E** Principal component analysis (PCoA) scatter plot demonstrating beta diversity among the jejunal microbiota of control and FMT groups



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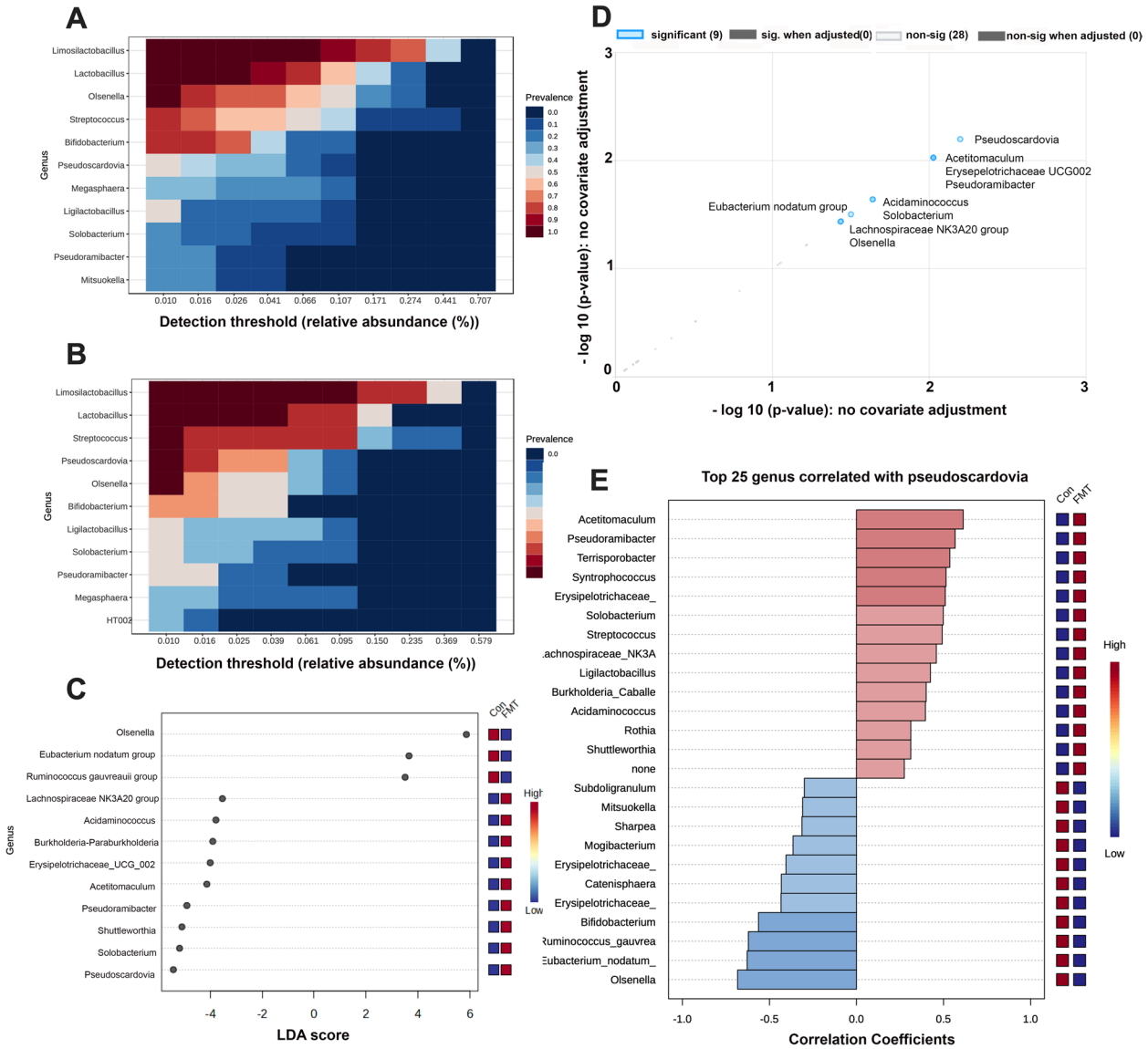


Fig. 2 The effect of FMT on the compositional patterns of jejunal microbiota of weanling piglets. **A** The heatmap illustrating the core microbiome composition of control weanling piglets. The core microbiome for each group was identified based on a sample prevalence of > 20% and at a relative abundance frequency of > 0.01% at the genus level. The *X*-axis represents the detection threshold, indicating the relative abundance percentages. The color gradient represents the prevalence of each genus across the samples, with the scale ranging from 0.0 (blue) to 1.0 (red). Higher prevalence is indicated by warmer colors (closer to red), while lower prevalence is shown by cooler colors (closer to blue). **B** Heatmap illustrating the core microbiome composition of control weanling piglets received FMT. **C** Linear discriminant analysis (LDA) effect size (LEfSe) chart displaying differentially abundant taxa between control and FMT groups. **D** Multiple regression analysis of bacterial genera associated with control and FMT piglets with representation of significance and covariate influence. **E** Correlation between the abundance of various bacterial genera in control and FMT groups and the genus *Pseudoscardovia* in the jejunum

spectrum of metabolic pathways has been significantly altered, illustrating the intricate interaction between gut bacterial metabolism and intestinal epithelial cell metabolism. In the heatmap visualizations, distinct patterns were observed between the FMT and control groups across all analytic modes (nc, pc, nhilic, and philic modes), suggesting that FMT substantially impacts metabolite levels within the jejunal content (Fig. 4A (nc mode), Supplementary Figs. 6 &7 (all other modes)). The purpose of using different analytical methods for heatmap visualization is to capture the broad spectrum of metabolites present in the jejunal content, which vary in their chemical properties and behaviors during analysis. Each mode—nc (normal phase chromatography),

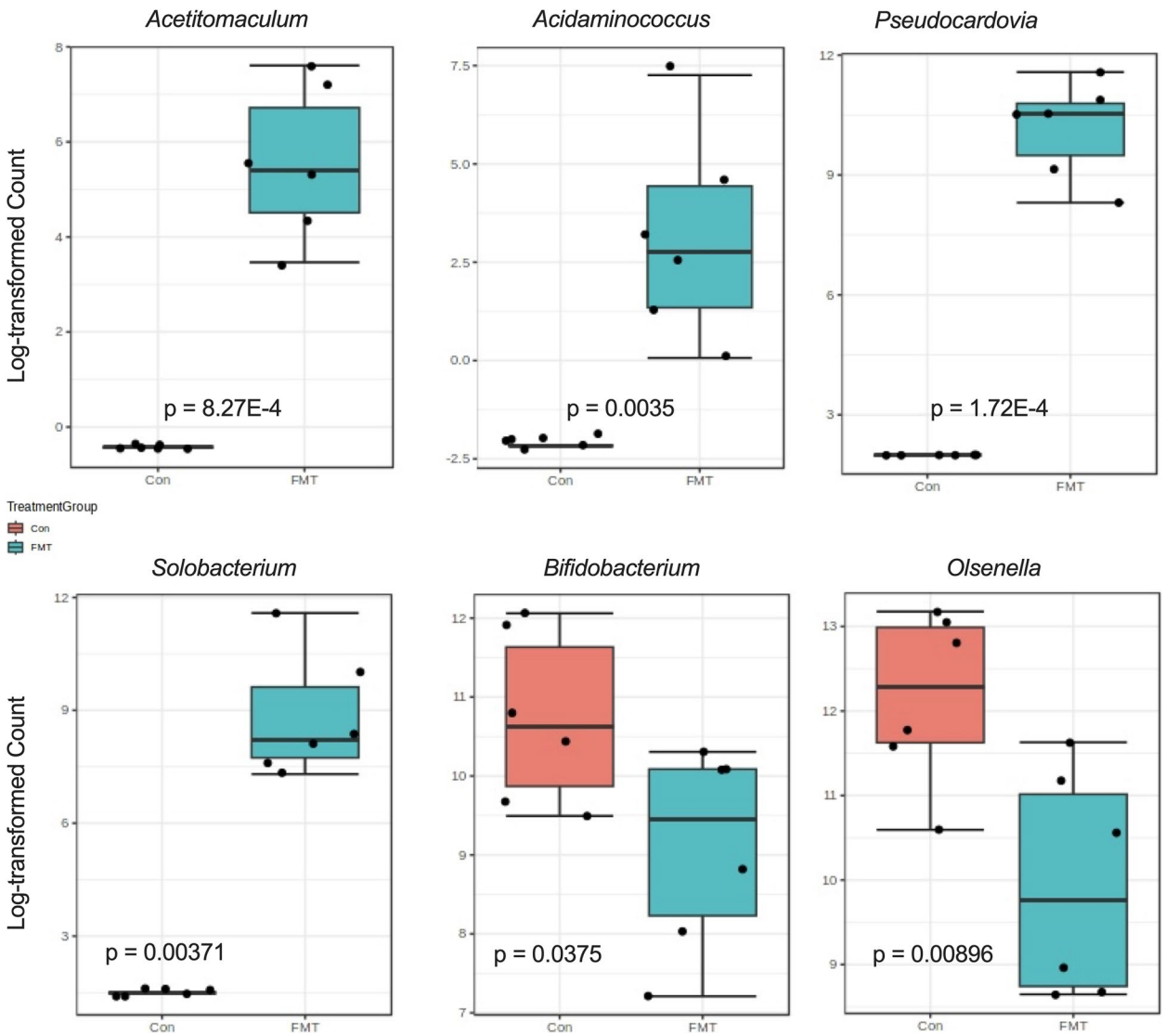


Fig. 3 Major bacterial genera differentially abundant in control and FMT groups. Boxplots depicting the difference in the abundance of the genera *Acetitomaculum*, *Acidaminococcus*, *Olsenella*, *Bifidobacterium*, and *Solobacterium* between the control FMT groups

pc (polar compound chromatography), nhilic (normal hydrophilic interaction chromatography), and philic (polar hydrophilic interaction chromatography)—targets different types of metabolites based on their polarity, solubility, and interaction with the chromatography medium. By employing these varied analytical modes, we are able to achieve a comprehensive profiling of the metabolome, ensuring that a wide range of metabolites—from non-polar to highly polar—are captured and analyzed. The principal component analysis (PCoA) plots further illustrate the separation between the FMT and control groups (Fig. 4B). The PCoA plots show tight clustering within the FMT group compared with the control, indicating a more homogenous metabolic response among the FMT-treated piglets (Fig. 4B). This homogeneity suggests that FMT leads to a convergent metabolic state, possibly reflecting the establishment of a new, stable microbiome environment in the jejunum.

Pathway analysis revealed significant changes in the metabolic pathways in jejunal contents following FMT (Fig. 4C (nc mode), Supplementary Fig. 7 (all other modes)). The drug metabolism pathway involving cytochrome P450 showed significant changes (nc: $p = 3.36e\text{-}09$), highlighting FMT's potential to modulate xenobiotic processing. For example, relative levels of the metabolite 6-methylmercaptopurine, a metabolite of azathioprine, was significantly increased in FMT group compared with control indicating enhanced drug metabolism

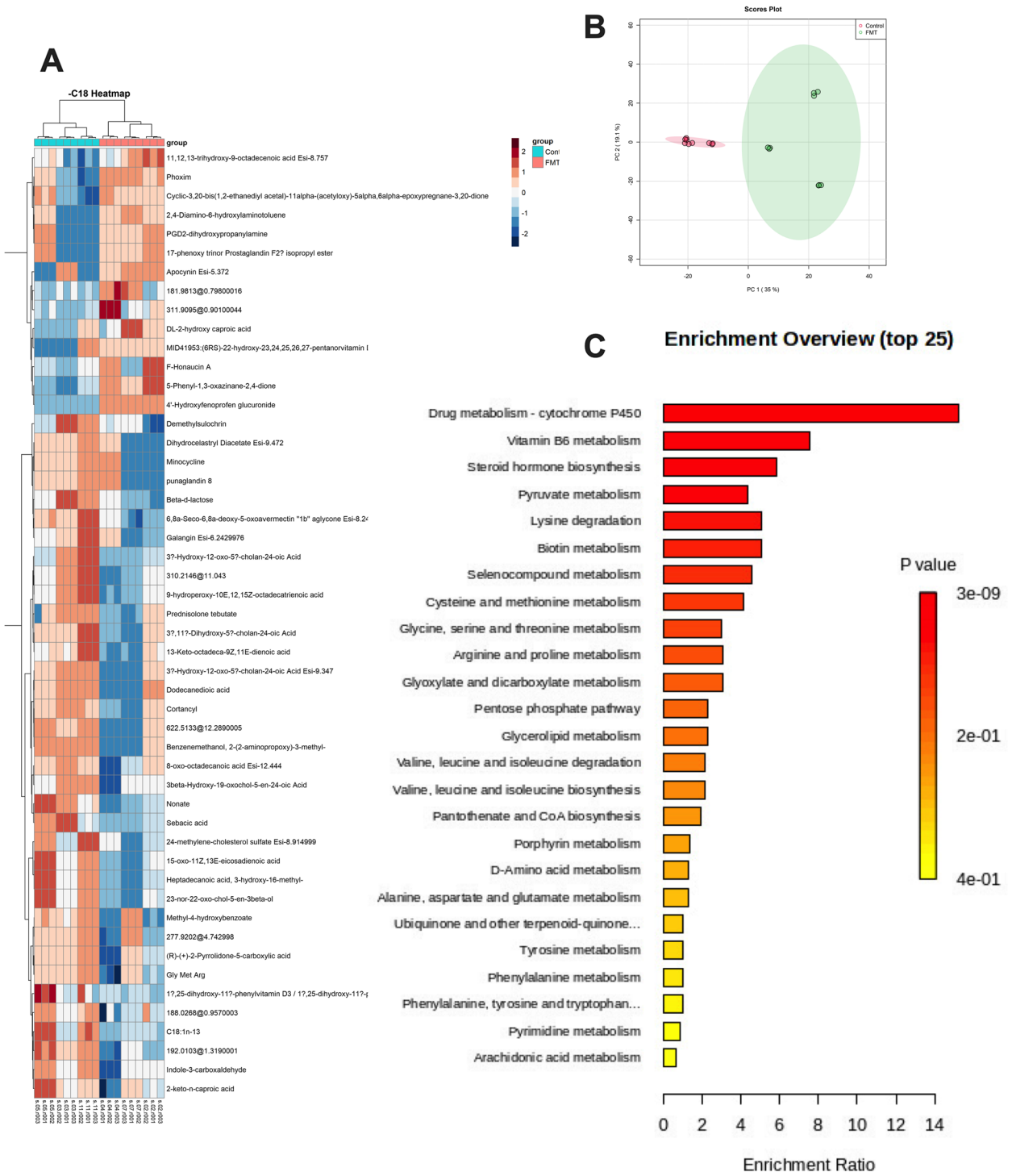


Fig. 4 Effect of FMT on the jejunal metabolome of weanling piglets. **A** Representative metabolomic heatmap illustrating differential metabolite enrichment in control and FMT groups (nc mode analysis). Results for other modes (pc, nhilic, and philic modes) are provided in Supplementary Fig. 8. **B** This principal coordinates analysis (PCoA) plot depicts the variance in metabolomic profiles between control and FMT groups. PCoA results for other modes (pc, nhilic, and philic modes) are provided in Supplementary Fig. 9. **C** Bar chart representing enrichment analysis of metabolic pathways. The figure represents an overview of the top 25 enriched metabolic pathways following FMT from nc mode metabolomic data. Enrichment results for other modes (pc, nhilic, and philic modes) are provided in Supplementary Fig. 10

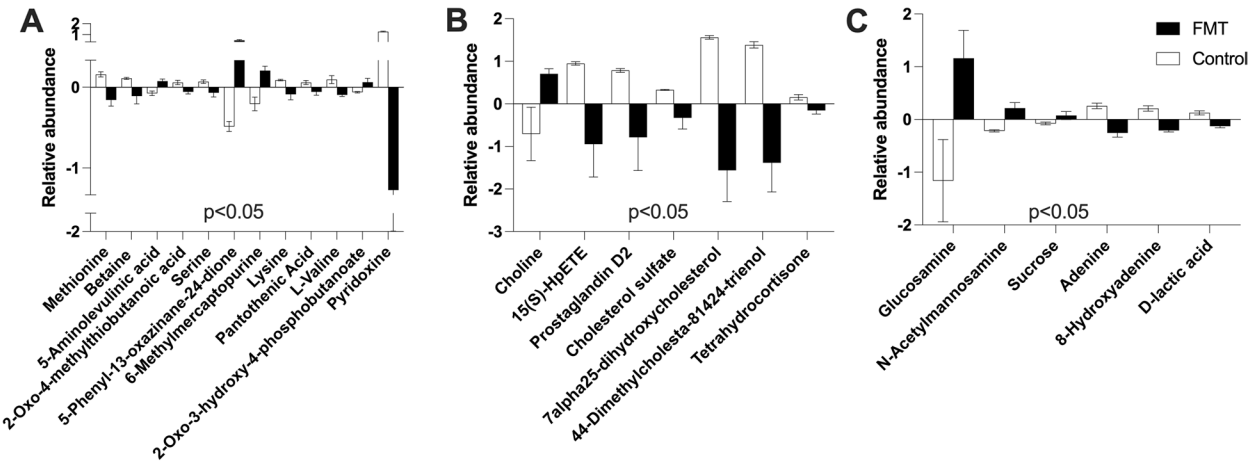


Fig. 5 Effect of FMT on the relative abundance of major differentially enriched jejunal metabolites in weanling piglets. **A** The relative abundance of selected jejunal metabolites associated with drug, vitamin, and amino acid metabolisms between control and FMT groups. **B** The relative abundance of selected jejunal metabolites associated with lipid metabolism between control and FMT groups. **C** The relative abundance of selected jejunal metabolites associated with carbohydrate metabolisms between control and FMT groups

in FMT group (Fig. 5A). Vitamin B6 metabolism, essential for amino acid, glucose, and lipid metabolism, was significantly altered (nc: $p=2.54e-03$), suggesting FMT's impact on critical nutritional pathways (Figs. 4C & 5C). Jejunal levels of both vitamin B5 (pantothenic acid) and B6 (pyridoxine) were significantly reduced in the FMT group compared to control, whereas the EC group exhibited higher levels of vitamin B5 (Fig. 5A). Simultaneously, levels of 2-Oxo-3-hydroxy-4-phosphobutanoic acid, a vitamin B6 metabolite, increased in the jejunum, suggesting enhanced metabolism of such vitamins in FMT-treated piglets (nc: $p=0.00417$) (Fig. 5A). The pathways of lysine degradation and biotin metabolism also underwent significant adjustments (nc: $p=1.93e-02$), emphasizing the effect of FMT on amino acid and nutrient processing (Figs. 4C & 5A). Relative jejunal levels of free lysine, serine, valine, methionine, betaine (trimethyl derivate of glycine), and amino acid metabolites such as 2-Oxo-4-methylthiobutanoic acid were significantly reduced in the FMT group compared to control suggesting enhanced absorption or downstream metabolism (Fig. 5A).

Lipid metabolism and the biosynthesis of unsaturated fatty acids, pivotal for maintaining lipid homeostasis and membrane composition, were notably affected, demonstrating FMT's regulatory role on lipid-related pathways (Figs. 4C and 5B). Alterations in steroid hormone biosynthesis (nc: $p=1.04e-02$) indicate a significant role in regulating growth and metabolism (Figs. 4C & 5B). For example, ETEC infection significantly increased cholesterol sulfate levels in jejunum compared with uninfected control, whereas FMT significantly reduced free jejunal cholesterol sulfate in both ETEC-infected and non-infected groups, suggesting increased absorption of downstream metabolism (Fig. 5B). In addition, compared with control and ETEC groups, FMT significantly reduced the jejunal levels of tetrahydrocortisone, a stress-associated steroid metabolized by gut microbiota, suggestive of enhanced bacterial metabolism of this compound (Fig. 5B). Similarly, jejunal levels of 7-a,25-dihydroxycholesterol, and 4.4-dimethylcholesta-8.14.24-trienol were reduced in the FMT group compared with the control, signifying the impact of FMT in cholesterol and bile acid metabolism (Fig. 5B). The alteration in primary bile acid metabolism suggests a substantial impact of FMT on fat digestion and absorption, cholesterol homeostasis, and the regulation of the gut microbiome composition. Moreover, arachidonic acid metabolism, which is critical in inflammation and the innate immune response, was significantly altered by FMT (Figs. 4C & 5B). This was evidenced by the substantially reduced levels of inflammatory mediators such as prostaglandin D2, 11,12-epoxyeicosatrienoic acid, and 15-hydroxyeicosatetraenoic acid (15(S)-HETE) compared with the control. Similarly, the relative concentration of choline was significantly increased in the FMT group (pc: $p=0.042$) (Fig. 5B).

Carbohydrate metabolism, including pathways like glycolysis and gluconeogenesis, was significantly altered, underlining the metabolic flexibility and energy management adaptations driven by FMT (Fig. 4C). Shifts in pyruvate metabolism (nc: $p=1.41e-02$) point to changes in the central pathways of energy production (Fig. 4C). Another notable finding is the increased level of D-lactose in the jejunal content following FMT. D-lactose is an isomer of lactose that is primarily produced by bacteria

(Fig. 5C). This suggests that the microbiota from the donor young adult pigs likely contains a higher proportion of lactate-producing bacteria, which are adapted to a solid diet, as opposed to the milk-based, pre-weaning diet of piglets. Another possibility is reduced metabolism or absorption of D-lactate in the FMT group. However, further studies are required to confirm this. In addition, the FMT group showed a notable increase in amino sugars such as glucosamine and n-acetyl mannosamine, key for synthesizing glycosaminoglycans compared with both non-challenged controls (Fig. 5C). Similarly, relative levels of sucrose were significantly increased in the FMT group compared with control with significance unclear. Changes in nucleotide metabolism indicate significant shifts in nucleotide biosynthesis and degradation, which are crucial for energy transfer, genetic expression, and cellular signaling (Figs. 4C & 5C). The FMT group had significantly lower levels of free nucleic acids such as adenine and hydroxyadenine in the jejunal content (Fig. 5C).

The microbiome-metabolome correlation analysis of the jejunal content from weanling piglets identified significant correlations between differentially enriched bacterial genera and differentially enriched gut metabolites in the FMT group compared with the control. The prediction heatmap (Fig. 6) illustrates these correlations, where red indicates strong positive correlations, while blue indicates no correlation (correlation score = 0). The major metabolites differentially enriched in the FMT group that correlate with differentially enriched bacterial genera include phosphoserine, L-histidine and its metabolite 3-(Imidazol-4-yl)−2-isopropyl phosphate, branched-chain amino acids such as valine and isoleucine; L-arogenate, a metabolite of tyrosine and phenylalanine; gamma-glutamyl-beta-cyanoalanine, a metabolite associated with detoxification; 5-(2-hydroxyethyl)−4-methyl thiazole, a metabolite involved in thiamine metabolism; sugars including D-lactic acid and D-glucose; and N-acetylmannosamine, the building block of sialic acid integral for the gut mucus barrier. Interestingly, strong correlations were observed with alterations in several of these metabolites, which strongly correlate with the differential abundance of Bifidobacteria, significantly reduced in abundance in the FMT group compared to the control. A limitation of this analysis is that the metabolic correlation information on Pseudoscardovia—the genus most significantly enriched in the FMT group and inversely correlated with the abundance of Bifidobacteria—is absent in these results due to a lack of metabolic pathway database on this newly identified bacterium.

Overall, these findings collectively demonstrate the potential of FMT treatment to induce broad metabolic reprogramming in the jejunal environment of weanling piglets, enhancing nutrient absorption, metabolic efficiency, and overall gut health.

FMT modulates the jejunal gene expression associated with immune, metabolic, barrier, and neuroendocrine functions in weanling piglets

Our comprehensive transcriptomic analysis of jejunal tissue from weaning piglets revealed significant alterations in gene expression following FMT, underscoring the profound impact of microbial communities on the jejunal environment and its biological processes. Several genes were significantly upregulated in FMT-treated piglets, indicating a multifaceted response encompassing immune regulation, antimicrobial defense, metabolic processes, and cellular signaling pathways (Fig. 7A, B).

Among the upregulated genes, *LYZL4* (Lysozyme A) stands out for its potential role in enhancing antimicrobial activity, suggesting that FMT may support mucosal defense mechanisms against pathogenic bacteria. Similarly, *PGLYRP2* (peptidoglycan recognition protein 2) another gene associated with antibacterial immune responses, was elevated, further supporting the notion that FMT enhances gut immunity. The immune response was further highlighted by the upregulation of *KLRB1* (Killer Cell Lectin Like Receptor B1), involved in immune surveillance, and *TNFRSF11B* (TNF Receptor Superfamily Member 11b), a key regulator of inflammation and immune responses. These changes imply a nuanced modulation of the immune system, possibly reflecting a more tolerant and regulated environment conducive to beneficial microbiota.

In terms of metabolic implications, *UGDH* (UDP-glucose 6-dehydrogenase), a gene involved in carbohydrate metabolism, was upregulated, suggesting alterations in mucosal carbohydrate processing, and potentially affecting mucus production and the mucosal barrier. Additionally, *PNP* (purine nucleoside phosphorylase), critical for purine metabolism, indicates a broader impact of FMT on nucleotide turnover and, indirectly, on amino acid metabolism and protein synthesis. The analysis also highlighted genes such as *ALOX12B* (arachidonate 12-lipoxygenase, 12R type) and *HAVCR1* (hepatitis A virus cellular receptor 1), which are implicated in lipid metabolism and immune modulation, respectively. The upregulation of these genes might reflect changes in lipid signaling pathways and immune cell interactions within the gut, further emphasizing the extensive influence of FMT on host physiology.

Several genes with roles in cellular structure and signaling were also upregulated, including *ADGRF1* (adhesion G protein-coupled receptor F1) and *UMODL1* (uromodulin like 1), suggesting that FMT may influence cell adhesion, tissue integrity, and signaling pathways

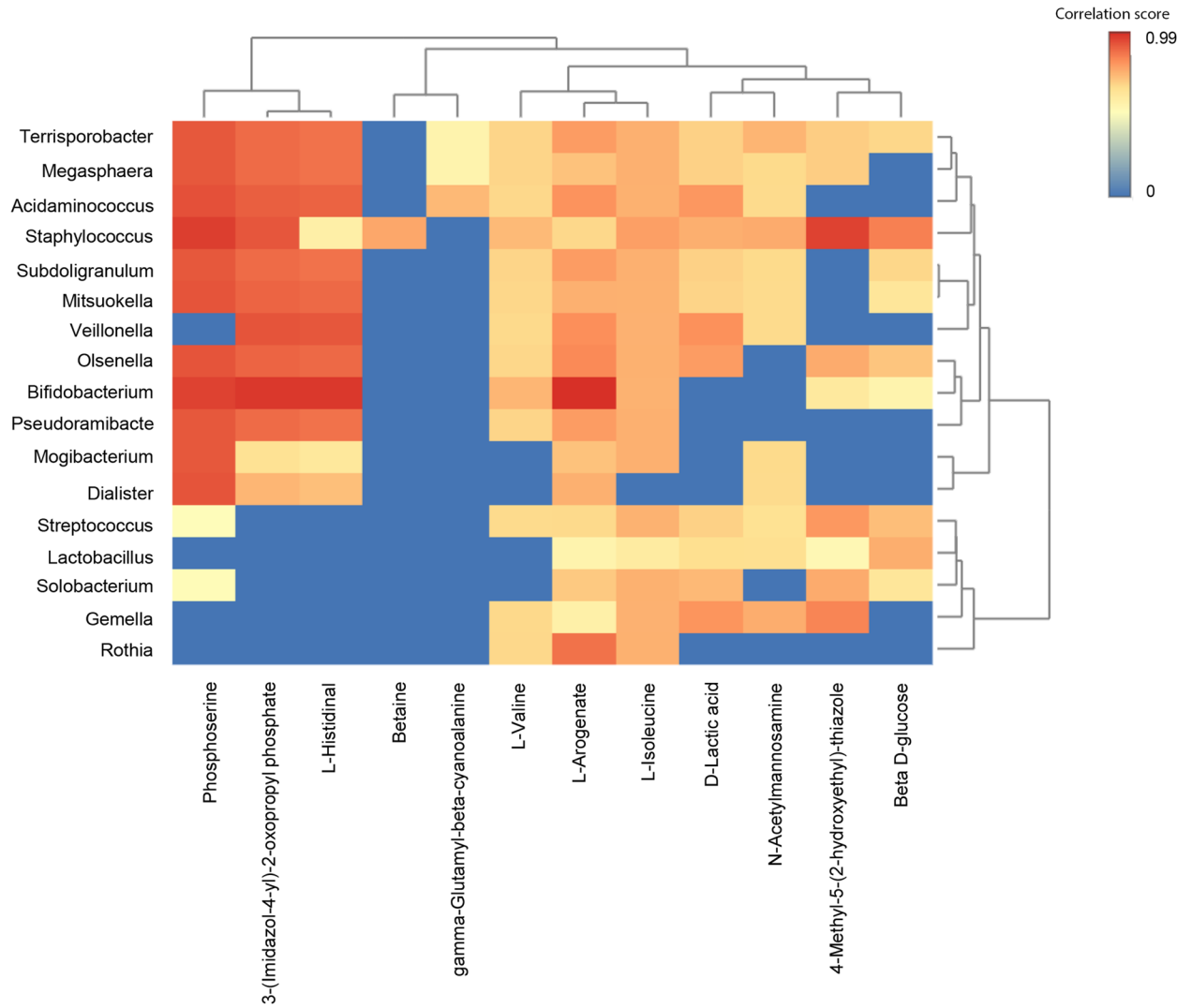


Fig. 6 Jejunal microbiome-metabolome correlation at genus level in weanling piglets (FMT vs. Control). This heatmap illustrates the correlations between various bacterial genera enriched in the jejunum of piglets that received FMT and key differentially enriched jejunal metabolites in the FMT group compared with controls. The heatmap's color gradients illustrate the correlation coefficient between each bacterial genus and metabolites

critical for maintaining gut homeostasis. Moreover, genes such as *MUC19* (Mucin 19), involved in mucus production, and *KLHL14* (Kelch like family member 14), associated with protein ubiquitination, underscore the complexity of the gut's response to FMT, impacting everything from the physical barrier against pathogens to the regulation of protein turnover and signaling within intestinal cells.

Transcriptomic analysis not only highlighted upregulated genes but also identified a significant number of genes that were downregulated in the FMT group (Fig. 9). Among these, *CHGA* (chromogranin A), involved in the regulation of neuroendocrine and stress responses, and *C5AR1* (complement component 5a receptor 1), a critical mediator of inflammation and immune response, was significantly decreased. This downregulation may indicate a dampening of stress responses and inflammatory signaling, aligning with the potential anti-inflammatory effects of FMT. *FXYD6* (FXYD domain containing ion transport regulator 6), associated with sodium–potassium ATPase regulation and thus electrolyte balance, also showed reduced expression. Similarly, genes like *CSF1R* (colony stimulating factor 1 receptor), crucial for the development and function of macrophages, and *CLEC3B* (C-type lectin domain family 3 member B), involved in coagulation and innate immunity, were downregulated,

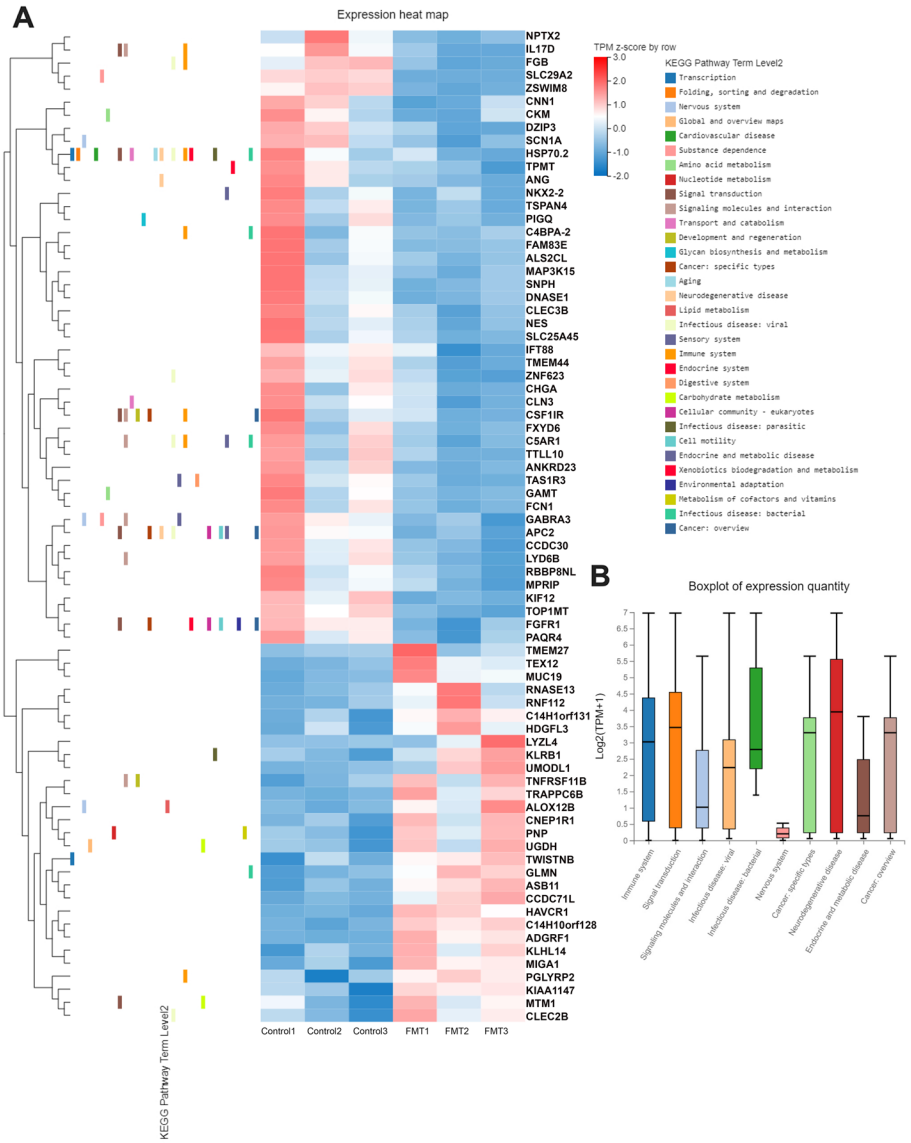


Fig. 7 Effect of FMT on the jejunal gene expression in weanling piglets. **A** RNAseq results demonstrating the relative expression of selected differentially expressed genes (DEGs) and associated metabolic pathways in control and FMT groups. **B** Difference analysis boxplot showing expression quantity of genes associated with different pathways and systems

suggesting alterations in immune cell regulation and potentially a shift toward a more regulated, less pro-inflammatory state.

Neurogenesis and neuronal function seem to be influenced as well, as indicated by the downregulation of *NES* (Nestin), which is involved in neural stem cell growth, and *GABRA3* (gamma-aminobutyric acid type A receptor alpha3 subunit), highlighting potential changes in gut-brain axis communication post-FMT. Metabolic pathways were also affected, with genes like *GAMT* (guanidinoacetate N-methyltransferase), essential for creatine synthesis, and *SLC25A45* (solute carrier family 25 member 45), part of the mitochondrial transport system, showing decreased expression. This might reflect metabolic adjustments to optimize energy utilization and mitochondrial function in response to the altered gut microbiome. Similarly, *DNASE1* (deoxyribonuclease I), involved in DNA degradation, and PIGQ (phosphatidylinositol glycan anchor biosynthesis class Q), important for glycosylphosphatidylinositol (GPI) anchor biosynthesis, were reduced, indicating modifications in cellular turnover and membrane dynamics. Structural components and cellular assembly processes also appear to be modulated, as seen in the downregulation of *KIF12*

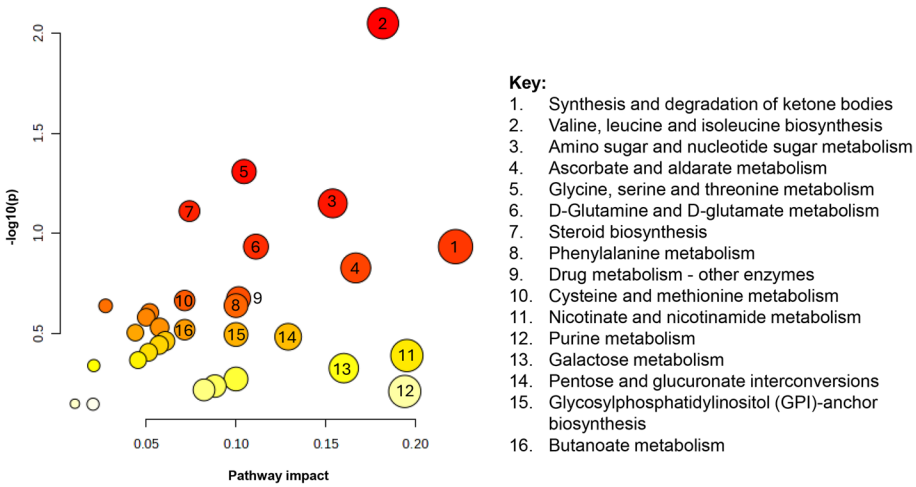


Fig. 8 Jejunal transcriptome-metabolome joint pathway analysis of weanling piglets (FMT vs. Control). Jejunal transcriptome-metabolome joint pathway analysis of FMT group and control group. The bubble plot depicts the impact and significance of various pathways, with the size of the bubble indicating pathway impact and the color representing the -log10(p) value, reflecting the significance level of the pathways

(kinesin family member 12) and *IFT88* (intraflagellar transport 88), which are involved in cytoskeletal organization and ciliogenesis, respectively. This could imply changes in cell morphology and motility in response to FMT.

Furthermore, the downregulation of *FGFR1* (fibroblast growth factor receptor 1), a key player in cell growth, and *ANG* (Angiogenin), involved in angiogenesis, suggests a potential recalibration of tissue growth and repair mechanisms, possibly optimizing them for the new microbial milieu. The observed downregulation extends to genes with specific localized functions, such as *FGB* (fibrinogen beta chain), involved in blood clotting, and *IL17D* (interleukin 17D), which plays a role in immune defense mechanisms. This broad spectrum of downregulated genes underscores the complex interaction between the gut microbiome and host gene expression, highlighting the profound impact of FMT on reducing pro-inflammatory signaling, modulating stress responses, and adjusting metabolic and immune functions. However, further confirmation using individual gene expression studies is required to further validate these results.

The joint pathway analysis (Fig. 8) not only validated the findings from the individual transcriptomic and metabolomic analyses but also provided a comprehensive overview of the interconnected metabolic and gene expression changes induced by FMT in weanling piglets**.** This integrated approach brought to light several significantly impacted pathways, reflecting the broad spectrum of metabolic and gene expression changes triggered by FMT in the jejunal environment of weanling piglets. The significant enrichment of pathways related to amino acid, carbohydrate, and lipid metabolism underscores FMT's role in enhancing the metabolic flexibility and nutrient utilization capabilities of the jejunum.

The pathway most highly impacted was valine, leucine, and isoleucine biosynthesis, indicating substantial changes in the metabolism of these essential branched-chain amino acids. Purine metabolism also showed significant impact, reflecting enhanced nucleotide biosynthesis and turnover. Another highly affected pathway was glycine, serine, and threonine metabolism, underscoring the alterations in amino-acid turnover in the jejunum following FMT. The steroid biosynthesis pathway was also significantly impacted, suggesting changes in lipid metabolism Synthesis and degradation of ketone bodies was another pathway with a notable impact. Additionally, ascorbate and aldarate metabolism was significantly altered, indicating changes in antioxidant processes and vitamin C metabolism.

The analysis also highlighted significant changes in amino sugar and nucleotide sugar metabolism, reflecting increased glycan biosynthesis and nucleotide turnover. D-glutamine and D-glutamate metabolism was notably altered. Cysteine and methionine metabolism was enriched, indicating changes in sulfur-containing compound production. Other impacted pathways included drug metabolism and nicotinate and nicotinamide metabolism, which indicated increased NAD + production. Galactose metabolism showed significant enrichment, reflecting changes in carbohydrate metabolism. The pentose and glucuronate interconversion pathway was also notably involved, emphasizing its role in carbohydrate metabolism and detoxification.

Finally, glycosylphosphatidylinositol (GPI)-anchor biosynthesis and butanoate metabolism pathways were

significantly impacted, with the former suggesting changes in cellular signaling and membrane anchoring processes, and the latter indicating enhanced production of short-chain fatty acids. These findings collectively provide a comprehensive picture of the metabolic shifts induced by FMT, highlighting its broad impact on various biological processes critical for the health and development of weanling piglets.

Prior FMT treatment differentially impacts ETEC-associated changes and outcomes in the jejunum of weanling piglets

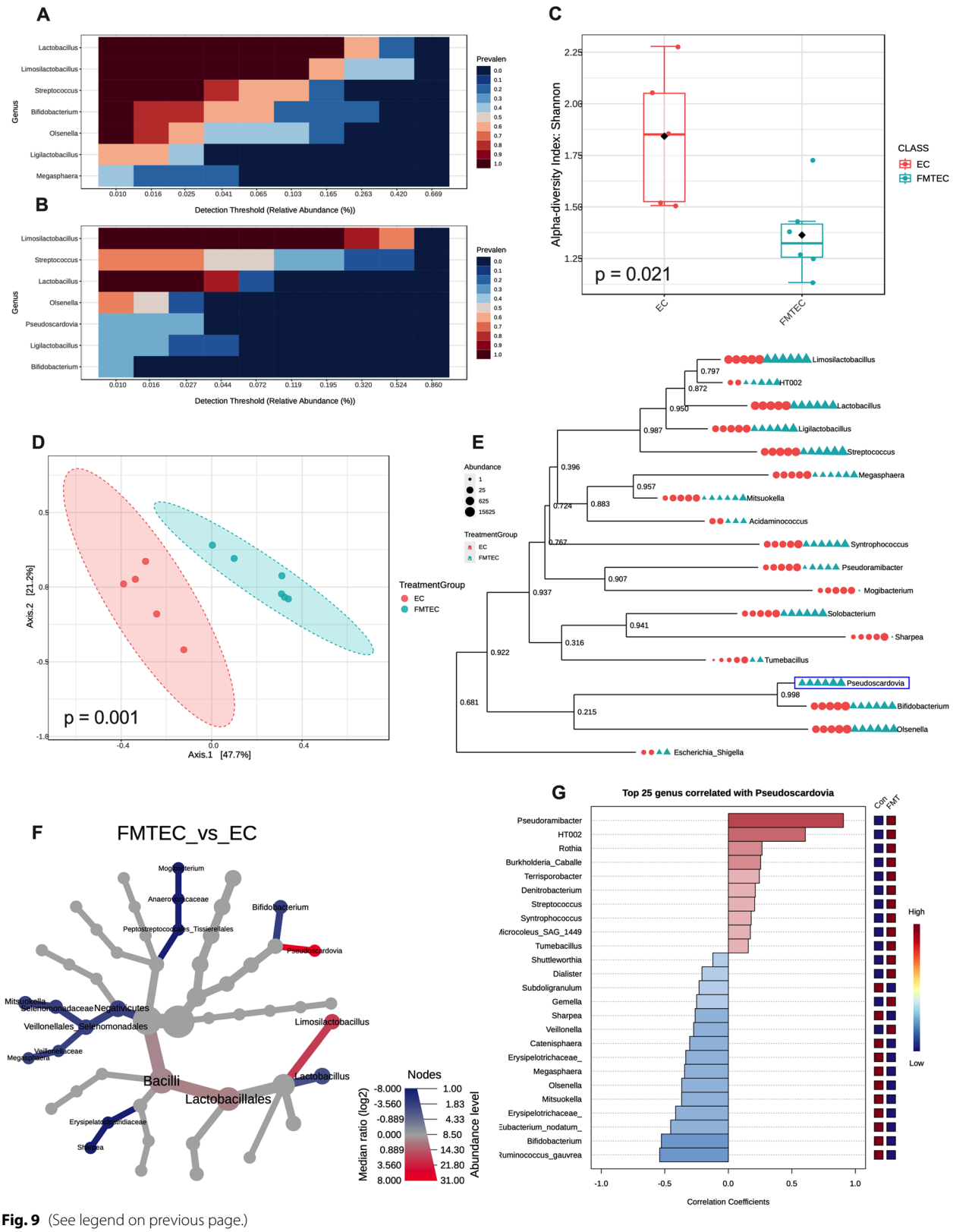
ETEC infection was confirmed in all the pigs in the challenged group sampled at both 4th and 8th dpi by PCR and genotyping of fecal samples from challenged animals. No F-18 + ve ETEC was detected in unchallenged controls. Microbiome analysis of the Control and ETEC groups revealed an interesting finding: No significant differences were observed in alpha diversity, beta diversity, and the abundance of specific taxa between the jejunal contents of these two groups (Supplementary Figs. 9A, B & C respectively). Two-factor analysis further highlighted this observation by revealing no significant associations with treatment groups for any specific taxa, except for a positive association with the class Eubacteriales in the ETEC-infected group (data not shown). This result suggests that the gut microbiota of weanling piglets, regardless of ETEC infection, are strikingly similar. It indicates a de facto dysbiotic state of gut dysbiosis in control weanling piglets comparable to those with ETEC infection. However, metabolome analysis of ETEC and control groups revealed significant differences in metabolite levels in ETEC infection with the enrichment of specific metabolic pathways post-ETEC infection (Supplementary Figs. 10A, B & C). In addition, ETEC infection significantly altered jejunal gene expression compared to control, as seen in the RNA-seq results reflecting molecular changes associated with the infection process (Supplementary Figs. 11A, B). However, inoculation of our ETEC strain at a dose of 10^6 CFU/ml induced mild to moderate diarrhea in the challenged group, peaking at 4-day post-challenge; however, the infection did not cause severe clinical signs or weight loss in our experiment post-challenge. Histological changes in jejunum were minimal to mild in the infected animals with scattered adherent bacterial cells, mild submucosal edema and mixed inflammatory infiltration, and rare crypt abscesses (Fig. 12B). The low number of visible ETEC in DPI 8 samples is not unexpected as ETEC attachment is associated with clinical diarrhea, which peaked at DPI 4.

Microbiome analysis revealed significant differences in microbiome composition and diversity between ETEC and FMT-EC groups. Significant differences were observed in beta diversity between FMT-EC and ETEC groups, as demonstrated by distinct clustering of each group on the PCoA plot ($p = 0.02$) (Fig. 9D). Interestingly, there was a significant reduction in alpha diversity in ETEC-infected piglets that received FMT (FMT-EC group) (Fig. 9C (Shannon index) & Supplementary Fig. 12 (Chao1 index)). The comparison of the ETEC and FMT-EC groups revealed a positive association between the abundance of *Pseudoscardovia* ($p = 0.005$) and prior FMT treatment in ETEC-infected animals, which was also reflected in the core microbiome composition of the FMT-EC group (Fig. 9A, B). Conversely, the absence of FMT treatment in ETEC-infected groups was associated with a decreased abundance of genera such as *Bifidobacterium*, *Mogibacterium*, *Lachnospiraceae*_NK3A20 group, and Sharpea (Fig. 9F, E). The most notable difference between these groups was an increased abundance of the genus *Pseudoscardovia* in the FMT-EC group compared with the ETEC group, and a lower abundance of the genus *Bifidobacterium* as seen in FMT vs. Control groups as described previously (Figs. 9F, E & 10). Furthermore, ETEC-infected piglets that received FMT exhibited a decreased abundance of a few bacterial genera, including *Sharpea*, *Mogibacterium*, *Lachnospiraceae*, and *Acidaminococcus* (Figs. 9F, E & 10). These findings underscore the significant impact of FMT on modulating the gut microbiota composition in the context of ETEC infection, particularly highlighting the role of FMT in enhancing the abundance of beneficial genera such as *Pseudoscardovia*.

Metabolomic analysis revealed significant differences in the enrichment of various pathways and individual

(See figure on next page.)

Fig. 9 The effect of prior FMT on the jejunal microbiome compositional patterns and diversity of ETEC-infected weanling piglets. **A** Heatmap illustrating the core microbiome composition of ETEC-infected piglets. **B** Heatmap illustrating the core microbiome composition of ETEC-infected piglets that received prior FMT treatment. **C** Boxplot illustrating the Shannon index of alpha diversity within jejunal microbial communities of ETEC-infected control piglets (EC) and ETEC-infected piglets that received prior FMT treatment (FMT-EC). **D** Principal component analysis (PCoA) scatter plot demonstrating beta diversity among the jejunal microbiota of EC and FMT + EC groups. **E** Phylogenetic tree abundance map of microbial communities depicting comparative genetic diversity and the population density of the microbial genera in EC and FMT + EC groups. **F** Heat tree depicting the hierarchical taxonomical distribution and relative abundance of significantly different microbial genera in EC and FMT + EC groups; **G** Correlation between the abundance of various bacterial genera in EC and FMT + EC groups and the genus *Pseudoscardovia* in the jejunum



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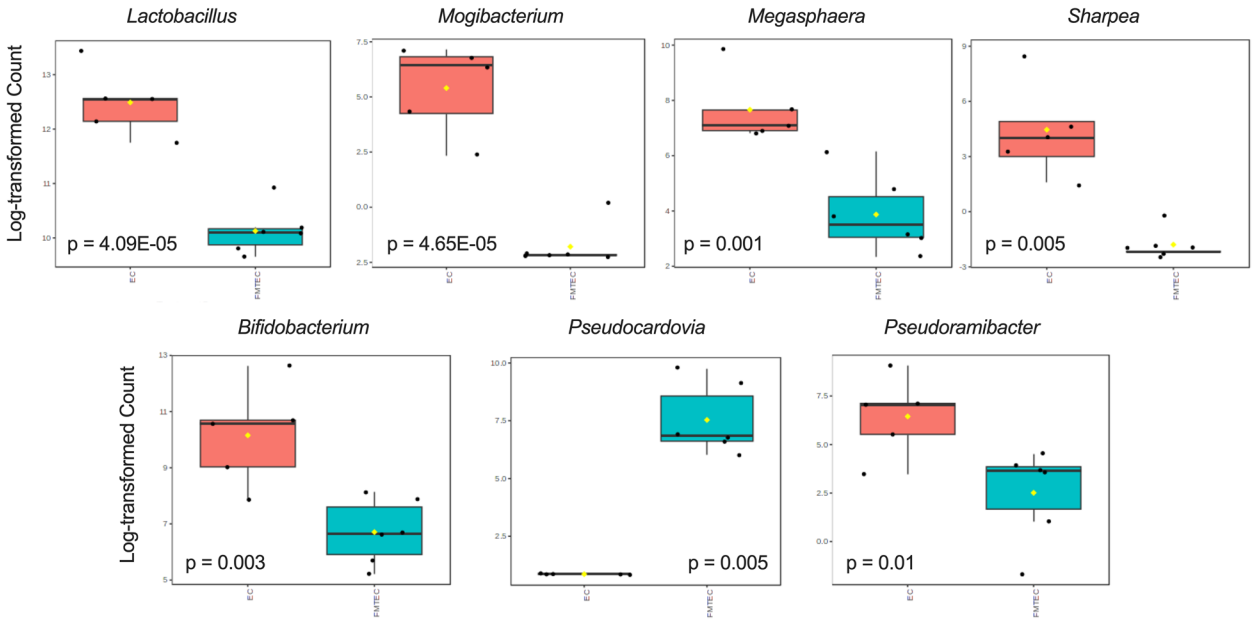

Fig. 10 Major bacterial genera differentially abundant in EC and FMT + EC groups. Boxplots depicting the difference in the abundance of the genera *Lactobacillus*, *Mogibacterium*, *Megasphaera*, *Sharpea*, *Bifidobacterium*, *Pseudoscardovia* and *Paeudoramibacter* between the EC and FMT + EC groups

abundance of different metabolites in the jejunal content of ETEC and FMT-EC groups, confirming ETEC-induced metabolomic alterations in the jejunal lumen (Fig. 11A, B & C). These changes were similar to those of the Control vs FMT groups to an extent, including enrichment of carbohydrate, amino acid, and purine metabolism following FMT treatment (Fig. 11C). However, fecal microbiota-associated changes in the jejunum of weanling piglets challenged with ETEC were not always favorable. Increased levels of histamine and its metabolite imidazole acetic acid were observed in the FMT-EC group compared with the ETEC challenge alone (Fig. 11D). This suggests that FMT might trigger a local allergic or immune response in the ETEC-challenged group, potentially leading to inflammation or an intestinal permeability response in these animals.

In addition to the shifts observed in the jejunal microbiomic and metabolomic signatures, significant differences were observed in the diarrhea scores between ETEC and FMT-EC groups to an extent (Fig. 12A). FMT-treated animals showed significantly lower diarrhea scores at four days post-infection compared to ETEC controls. Diarrhea gradually subsided after 4 days in all groups, and no significant differences in diarrhea scores were observed after four days post-infection. This is not surprising as ETEC-induced diarrhea is more of a functional diarrhea rather than structural in piglet intestine. Moreover, the ETEC challenge induced only mild clinical signs in our experiment. TaqMan qPCR targeting F-18 pilus on fecal samples from 4 days post-challenge (peak diarrhea score) revealed no significant differences in fecal ETEC load between ETEC and FMT-EC groups (Fig. 12C). No significant differences were observed in the overall *E. coli* shedding score between the treatment groups (Supplementary Fig. 13).

Discussion

The integration of FMT into the management of PWD in piglets presents a novel approach to mitigating the adverse effects of gut dysbiosis that typically follows weaning. Our findings corroborate and extend existing knowledge on the multifaceted benefits of FMT in weanling piglets, encompassing microbiomic, metabolomic, and transcriptomic dimensions, which collectively contribute to a healthier gut environment in weanling piglets [25–27, 29–31, 37, 49, 50].

For example, Tang et al. (2020) investigated the effect of FMT on gut bacterial community, immune response, and barrier function in weanling piglets, finding marked improvements in growth performance, colon length, and a significant modulation of gut microbiota composition. This approach also ameliorated diarrhea, demonstrating FMT's role in enhancing intestinal health and disease resistance [29]. A similar study focused on the practical application of FMT, examining the effects of different delivery methods on piglet growth and gut microbiome composition. While all FMT treatments improved body weight and daily gain compared with controls, the study

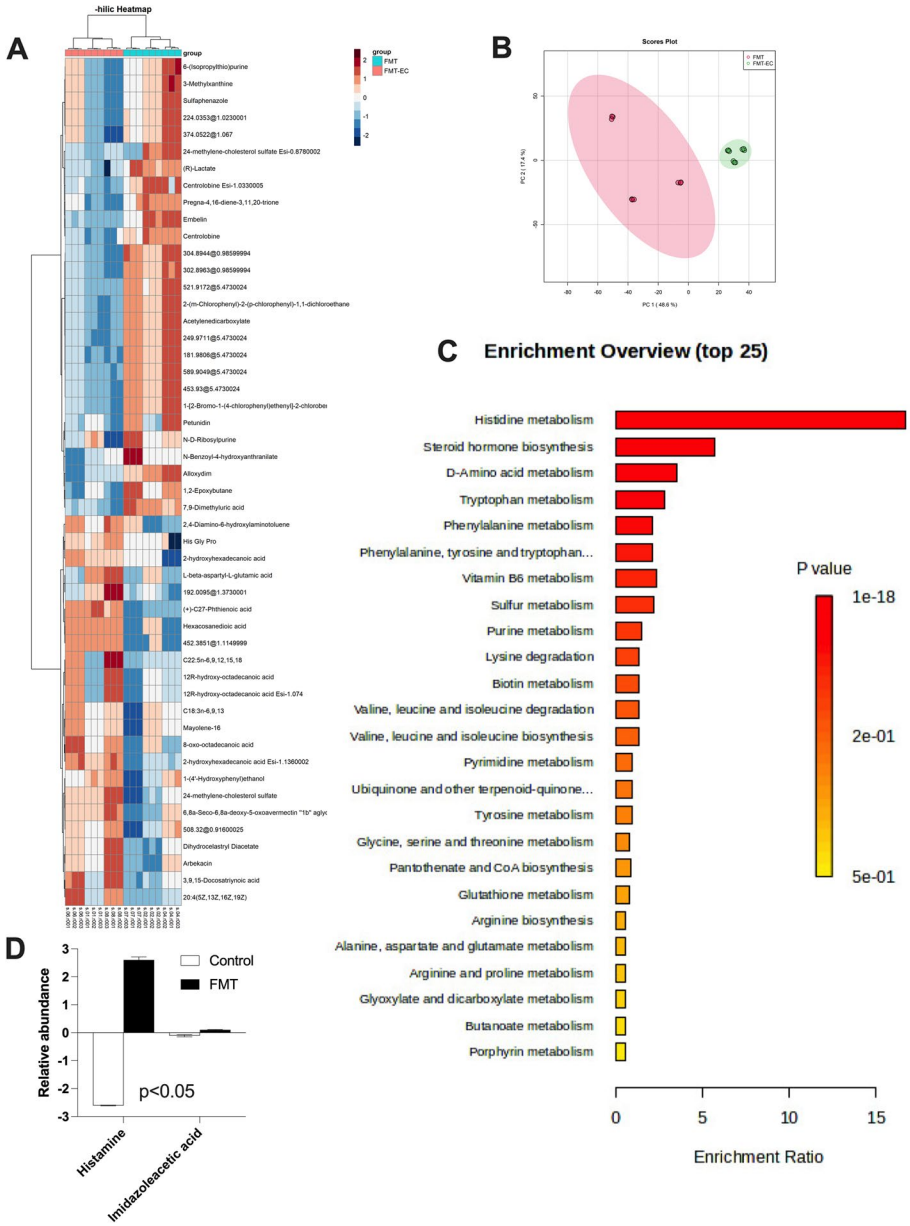

Fig. 11 Effect of prior-FMT on the jejunal metabolome of ETEC-infected weanling piglets. **A** Representative metabolomic heatmap illustrating differential metabolite enrichment in control and FMT groups (nc mode analysis). **B** Principal coordinates analysis (PCoA) plot depicting the variance in metabolomic profiles between EC and FMT + EC groups. **C** Bar chart representing enrichment analysis of metabolic pathways. The figure represents an overview of the top 25 enriched metabolic pathways in EC vs. FMT + EC groups derived from "nc mode" metabolomic data. **D** Effect of FMT on the relative abundance of jejunal histamine and histamine metabolite imidazole acetic acid in ETEC-infected weanling piglets

highlighted that lyophilized in-feed FMT notably altered the gut microbial community structure, indicating a potential path for easier FMT administration in swine management [51].

Several studies demonstrated the effect of FMT on gut immune response and barrier functions. Xiang et al. (2020) demonstrated an early-life intervention combining FMT with probiotics, significantly reduced inflammation, improved intestinal barrier function, and alleviated weaning stress [32]. Similarly, Ma et al. (2021) also demonstrated the ability of FMT to enrich beneficial bacteria, enhance tight junction proteins, and downregulate inflammatory cytokines, thereby fostering a healthier gut environment [24]. Rahman et al. (2023) demonstrated enrichment of specific beneficial bacterial genera and increase in propionate production in the

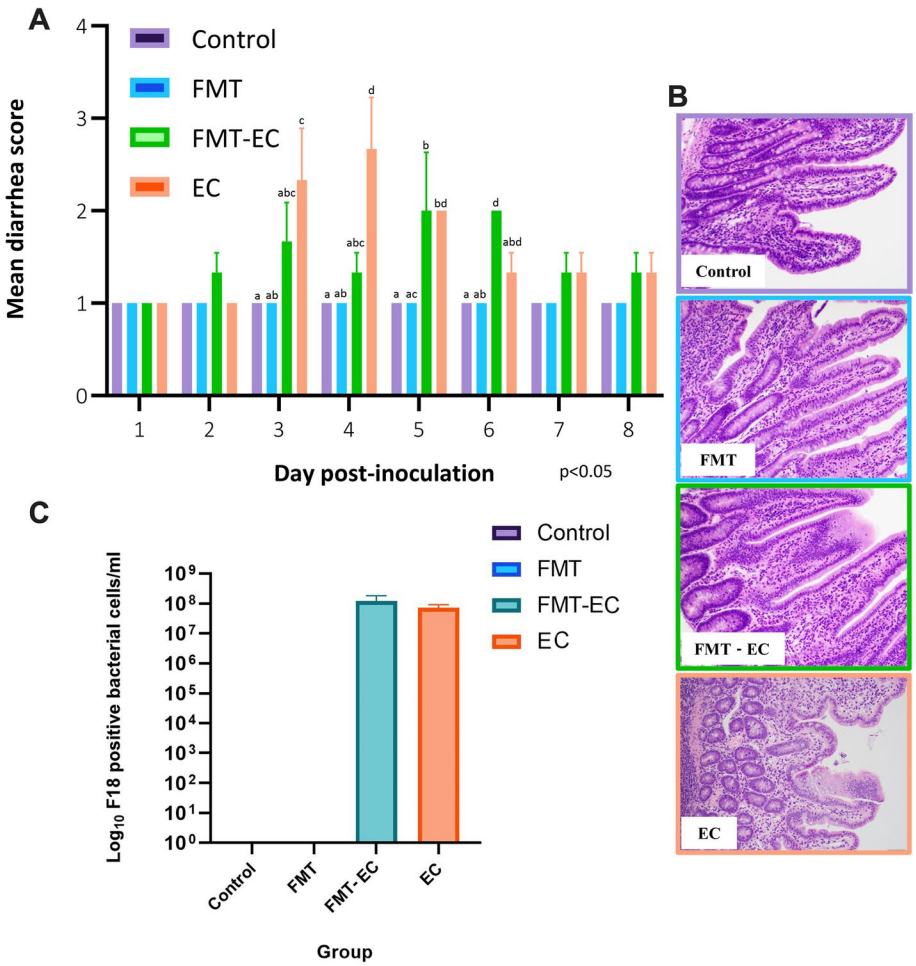

Fig. 12 Effect of FMT on diarrhea score, jejunal histology, and fecal ETEC shedding in weanling piglets. **A** Temporal diarrhea score assessment in different treatment groups at different time points. **B** Jejunal histology of different treatment groups at peak diarrhea (4th-day post-ETEC challenge). **C** Fecal F-18 + ETEC bacterial load at 4th-day post-ETEC challenge in different treatment groups

cecum, contributing to a modest alteration in microbial composition and enhanced metabolite production related to amino acid metabolism in FMT treated weanling pigs [27]. In addition, Liu et al. (2024) demonstrated that FMT reduces the levels of gut microbiota-derived lipopolysaccharides (LPS), mitigates oxidative stress, and maintains intestinal barrier integrity, ultimately reducing diarrhea caused by early weaning-induced colon microbiota dysbiosis [52]. Cheng et al. (2018) demonstrated that FMT increased beneficial gut bacteria, enhanced protective autophagy, and improved metabolic profiles, leading to better intestinal morphology and reduced permeability [53]. On the other hand, Nowland et al. (2020) observed an increase in diversity and the presence of potentially pathogenic and beneficial bacteria. This study emphasizes the capability of FMT to alleviate enteric dysbiosis, despite the increase in *Escherichia coli*, by also enriching beneficial microbes such as *Lactobacillus mucosae* [25].

Unlike previous studies that explored FMT-associated changes in fecal samples or colon contents, our study specifically focused on the jejunum, the longest segment of the swine intestine, which is the primary site of nutrient absorption and ETEC colonization. Therefore, we examined the metabolomic and microbiomic changes of the jejunal contents and gene expression changes in the jejunal tissue following FMT in weanling piglets. The results demonstrate that FMT significantly enhances jejunal microbial diversity and alters the composition of the jejunal microbiota in weanling piglets, specifically enriching small intestinal bacterial populations such as *Pseudoscardovia*. Notably, the increase in microbial diversity and specific taxa adjustments mimic those seen in a mature and healthy gut, suggesting that FMT accelerates the maturation of the piglet's gut microbiota postweaning. This is particularly relevant given the disruptive impact of weaning on the gut. Unlike the ileum and colon, the

jejunum showed an increase in alpha diversity in the third week post-FMT, indicating the long-lasting impact of FMT in this under-explored but important intestinal segment. Unlike previous studies, changes in microbial diversity in distal intestinal segments (ileum and colon) following FMT were minimal in our experiment. This might be attributed to the variability in study designs, differences in FMT inoculum and dose, frequency of administration, and the timing of post-FMT intestinal content collection. In our study, microbiome changes were analyzed from samples collected during the third week of FMT administration after two doses. The robust changes observed in microbial diversity indices in the jejunum are likely due to its comparatively lower baseline microbial diversity relative to the distal segments, as well as physiological factors and its unique microenvironment. These factors may have contributed to a prolonged effect in the jejunum, whereas compositional stabilization in the distal segments might occur more rapidly due to their inherently higher microbial diversity.

Pseudoscardovia, a genus closely related to the more widely known genus of *Bifidobacteria* within the family *Bifidobacteriaceae*, represents a relatively understudied microbial member within the small intestinal microbiota [54, 55]. Results indicate the lack of this bacterium in the jejunum of weanling piglets as seen in the abundance data from the control animals. Interestingly, a marked increase in the abundance of *Pseudoscardovia* in the jejunum, inversely associated with the abundance of *Bifidobacterium* marks a notable shift in microbial populations post-FMT within the same bacterial family. To the best of our knowledge, this is the first report on the lack and abundance of this specific, and recently discovered small intestinal bacterium, in weanlings and FMT treated piglets. *Bifidobacterium* populations are predominantly associated with milk feeding in piglets due to their capability to ferment lactose and other simple sugars present in milk [56, 57]. As piglets transition from a milk-based to a solid-food diet post-weaning, the gut microbiome undergoes significant compositional changes to adapt to the new dietary substrates. The increased abundance of jejunal *Pseudoscardovia* in FMT-treated weaning pigs, as observed in our study, suggests a notable adaptation of the gut microbiome to solid diets. This transition identified as one of the most statistically significant compositional changes in FMT-treated animals may imply a beneficial adaptation mechanism. A recent study on the gut microbiome of conventionally vs. pasture-raised pigs revealed that *Pseudoscardovia radai*, among other bacteria, was more abundant in pasture-raised pigs, suggesting its association with a diet rich in complex plant polysaccharides found in pasture environments [58]. This finding aligns with the observation of increased abundance of this bacterium post-FMT in weaning pigs transitioning to solid feeds, indicating its potential role in efficiently metabolizing complex plant polysaccharides and fibers, which are prevalent in solid diets as opposed to the lactose-dominant milk diet that favors *Bifidobacterium* growth. Similarly, another study on the temporal dynamics of rumen microbiota in early weaned lambs, revealing that dietary changes, such as early weaning, can have a profound impact on the microbiota composition, including fluctuations in *Pseudoscardovia* abundance [59]. Thus, the observed increase in *Pseudoscardovia* post-FMT could reflect an adaptive mechanism to dietary changes, contributing to a healthier and more resilient gut microbiome in weaning pigs.

In addition to *Pseudoscardovia*, other notable genera enriched by FMT include *Pseudoramibacter* and *Solobacterium*. *Pseudoramibacter* is associated with adaptation to complex solid diet, as they degrade complex polysaccharides and fibers into medium-chain fatty acids (MCFAs), which have multifactorial effects in promoting gut health and growth performance in pigs [60–63]. The abundance of *Solobacterium* is often associated with healthy suckling to weaning microbiome transition, microbiota adaptation to solid diet, especially utilization of fibers and tannins, and found to be associated with better fat deposition and disease resistance in pigs [61, 64–67]. The collective presence of these bacteria in FMT-treated piglets underscores the potential of FMT to enhance growth performance, improve feed efficiency, and reduce the incidence of gastrointestinal diseases by establishing a balanced gut microbiota.

Significant shifts in metabolic pathways in the jejunal contents observed post-FMT, including those involved in energy production, nutrient metabolism, and xenobiotic processing, underline the profound impact of microbial communities on the host gut metabolome . These changes, further validated by microbiome and transcriptome co-analyses, not only reflect a potential direct microbial contribution to metabolic functions but also suggest a reprogramming of the host's jejunal epithelial cells toward improved nutrient turnover and utilization. These findings are in line with previous reports of increased colonic metabolites, including lactic acid, vitamins, fatty acids, and lipids in FMT treated weanling and newborn piglets [26, 33, 53, 68]. The alterations in jejunal metabolites involved in vital processes such as vitamin B6 metabolism and lipid biosynthesis are particularly noteworthy, as they hint at enhanced nutritional status and potentially better growth performance in FMT-treated piglets.

Concurring with previous findings, our transcriptomic analysis provided insights into the regulatory impact of FMT on gene expression within the jejunal tissue,

highlighting significant modulation in genes involved in gut immune response, metabolism, gut barrier, and homeostasis in weaning piglets [24, 29, 32, 52]. Upregulation of genes related to antimicrobial defense and immune modulation suggests that FMT not only shapes the microbial landscape of the jejunum but also primes the mucosal immune system for enhanced resilience against pathogens. Concurrently, the downregulation of genes associated with stress responses and pro-inflammatory signaling indicates a more homeostatic and less inflammatory gut environment post-FMT, aligning with observations of reduced severity in diarrhea scores among FMT-treated animals facing ETEC challenges [28, 29, 52]. Moreover, the transcriptome-metabolome joint pathway analysis further validated the metabolite shifts induced by FMT, highlighting significant changes in key pathways, including those involved in energy, lipid, amino acid, nucleotide, and xenobiotic metabolism. Moreover, key differentially expressed gut metabolites correlated with differentially enriched jejunal bacterial genera in weanling piglets following FMT. This information could be used to develop precision microbiota and metabolite-based strategies to enhance the gut health of weanling piglets.

While ETEC infection induced significant disruptions in both microbiomic and metabolomic profiles, prior FMT treatment provided a degree of resilience against such alterations. The differences in diarrhea scores and microbiome composition between the ETEC and FMT-EC groups underscore the protective effect of FMT against enteric infections. These results corroborate previous observations on the protective effect of FMT against post-weaning diarrhea [28, 29, 52]. This protective mechanism likely involves a combination of enhanced microbial diversity, competitive exclusion of pathogens, and modulation of host immune responses, ultimately reducing the severity of infection-induced dysbiosis and its clinical manifestations.

Conclusion

In summary, this study highlights the potential of FMT in counteracting the detrimental effects of weaning-induced gut dysbiosis in the piglet jejunum. The results indicate that FMT is a promising strategy for mitigating post-weaning diseases associated with this bowel segment. By fostering a more resilient and health-conducive gut microbiome, enhancing metabolic efficiency, and modulating immune and stress-related gene expression, FMT emerges as a potential multifaceted intervention that addresses the complex pathogenesis of PWD. Additionally, the results from this pig study could be extended to other species with similar gastrointestinal anatomy and physiology, such as humans with small intestinal dysbiosis and associated illnesses like small intestinal bacterial overgrowth (SIBO). Future research opportunities include optimizing FMT protocols for swine production (e.g., using a more defined, precision microbiota consortium rather than crude intestinal contents) and exploring the long-term impacts on growth performance and health. In addition, FMT can be considered a potential strategy to address conditions associated with small intestinal dysbiosis in other species, including humans.

Supplementary Information

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Supplementary Material 1
Supplementary Material 2
Supplementary Material 3
Supplementary Material 4

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Authors' contributions
SM conceived the idea, developed the project, supervised and conducted the experiments, analyzed the data, and prepared the manuscript. AR co-developed the project, supervised and conducted the animal experiments, analyzed the data, and reviewed the manuscript. JS co-developed the project, supervised the microbiome part of the experiment, and reviewed the results and manuscript. MSM performed the microbiome analysis, analyzed and visualized the results, and co-wrote and reviewed the manuscript. FY conducted the metabolome and transcriptome analysis, visualized the results, compiled the omics data, and reviewed the manuscript. BS co-supervised the multi-omics analysis, sample processing, and manuscript writing. EB co-supervised the infection and pathology component of the project and reviewed the manuscript. OS supervised and conducted the microbiology and infection component of the study and reviewed the manuscript.GL supervised and conducted the microbiome sample processing.

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Data availability
Sequence data that support the findings of this study have been deposited in NCBI Genbank with primary accession PRJNA1109784. Metabolome and gene expression data sets are provided as supplementary information files.

Declarations

Ethics approval and consent to participate
All animal experiments and procedures were performed according to the Institutional Animal Care and Use Committee (IACUC) under protocols IACUC-22–246 and 18–342.

Consent for publication
This manuscript does not contain any individual person's data in any form. All of the material is owned by the authors, and no permissions are required.

Competing interests
The authors declare no competing interests.

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