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Intestinal Microbiota Is a Key Target for Load Swimming to Improve Anxiety Behavior and Muscle Strength in *Shank 3*$^{-/-}$ Rats

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Abstract

Autism spectrum disorder (ASD) is a neurodevelopmental disorder characterized by social disorder and stereotypical behavior, and its incidence rate is increasing yearly. It is considered that acritical period for the prognosis of young children with ASD exists, thus early treatment is crucial. Swimming, due to its comforting effect, is often used to induce enthusiasm in young children for completing activities and has a good effect in the treatment of ASD, but the effective path of swimming has yet to be reported. The intestinal microbiota of ASD patients and animal models has been reported to be different from that of healthy controls, and these changes may affect the brain environment. Therefore, whether the intestinal microbiota is involved in the treatment of ASD by early swimming is our concern. In this study, we used 8-day old *Shank3* gene knockout rats with 8 weeks of early load swimming training and conducted behavioral, small intestine morphology, and intestinal content sequencing after training. The results showed that early load swimming significantly reduced the stereotyped and anxious behaviors of *Shank3*$^{-/-}$ rats, increased their muscle strength, increased the length of intestinal villi and the width of the muscular layer after *Shank3* knockout, and affected the abundance of intestinal microorganisms. The abundances with statistical significance were *Lactobacillus*, *Lachnospiraceae*, and *Alloprevotella*. To further confirm the role of intestinal microorganisms in it, we designed a 14-day intestinal stool transplantation experiment. Fecal microbiota transplantation demonstrated that load swimming can significantly reduce the anxiety behavior of *Shank3* rats, increase their muscle strength, change the structure of the small intestine, and affect the abundance of intestinal contents. The abundance of *Epsilonbateraeota, Prevotella*, and *Bacteroides* significantly changed after transplantation. Our findings confirm the possibility of early load swimming therapy for individuals with ASD and explain that the intestinal microbiota is a key pathway for early exercise therapy for patients with ASD.

Keywords Autism spectrum disorder · *Shank3* · Fecal microbiota transplantation · Loading swimming

Introduction

Adolescence or early adulthood is an important period for brain development [1]. Significant changes in the structure and function of neurons also occur during puberty. Many mental diseases can manifest in this fragile period [2, 3]. Researchers have found that early intervention can optimize the behavior of children with ASD [4]. The microbiota is critical to host physiology and brain development and function [5]. The change in microbial flora will not only affect the function of the immune system and anti-infection ability [6, 7]. A change in microbial flora also interferes with normal brain function and behavior, including anxiety [8, 9], social skills [10], and memory [11]. The special nature, relative abundance, and diversity of microbiota in depression [10], irritable bowel syndrome [12], autism [13, 14], and other diseases further confirm the importance of intestinal microbial environment balance. Microbial interventions such as fecal microbiota transplant have been found to have therapeutic potential for humans [15]. In an uncontrolled study of 60 patients with chronic fatigue syndrome and gastrointestinal dysfunction treated with fecal microbiota transplant, 50% had resolved sleep deprivation, lethargy, or fatigue [16]. Differences in gut

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microbiota between individuals with ASD and controls were found in both human and animal populations [17, 18].Transplanting the gut flora of human donors with ASD into sterile mice showed that colonization of ASD flora can induce typical autistic behavior [19]. Therefore, it is worthwhile to explore the effects of intestinal microorganisms and fecal microbiota transplantation on ASD in the context of early intervention.

Sports meets can promote the physical and mental health and exercise ability of children with ASD and reduce stereotyped repetition and self-injuring behaviors [20]. Exercise has been indicated to play an important role in preventing and recovery from intestinal disorders in patients with inflammatory bowel disease [21]. Exercise during in early life increases *probiotic lactobacilli* and *butyric acid*-producing bacteria, which help promote brain plasticity, emotion/behavior, and skeletal muscle continuous exercise induced adaptation by influencing various aspects of host function [22]. Weight-bearing aerobic exercise has been used in the treatment of muscle, bone, and cardiovascular diseases [23]. Karen [24] found that rhythmic weight-bearing exercise can effectively prevent spinal cord injury (SCI)-induced disabling neuropathic pain. However, there are few reports on whether it can regulate the imbalance of ASD intestinal microorganisms.

Shank3 is a key postsynaptic density (PSD) molecule of glutamate synapses that is critical to normal synaptic function [25]. *Shank3* haplotype defects caused by deletion or mutation will lead to developmental disorders, accounting for 0.69% of autistic patients [26–29]. ASD behavior caused by *Shank3* gene defects mainly shows impaired social behavior, increased repetitive behavior, anxiety behavior, learning and memory deficits, and impaired motor coordination [30]. The *Shank* family (*SHANK1, SHANK2, SHANK3*) has become the most powerful gene family for animal ASD modeling [31–37]. Studies have shown that the gut microbiome of Shank3 knockout mice is altered, with reduced diversity and diminished populations of certain species of bacteria such as *Lactobacillus reuteri*, *Lactobacillus brevis*, and *Lactobacillus ruminis* [38]. Another study identified dysregulation of several genera and species of bacteria in the gut and colon of both male and female Shank3 KO mice [39]. In addition, there is evidence of altered microbiota composition in feces of Shank3 knockout mice that may contribute to inflammatory responses affecting brain development [38, 40]. However, I could not find any studies specifically investigating the effect of the gut microbiome on Shank3 knockout rats. To date, only one *Shank3* deficient rat model has been reported [41]. Therefore, the establishment of more *Shank3* transgenic rat models carrying different mutations and the identification of their characteristics will contribute to a more comprehensive understanding of *Shank3*'s function, species specificity, and the ASD mechanism.

In this study, we used 8-day old Shank3 knockout rat load swimming experiment and observed changes in its behavior, small intestine morphology, and fecal microbiota 16SrRNA. We also designed fecal microbiota transplant from load swimming *Shank3* knockout rats to non swimming Shank3 knockout rats, and observed the behavior and fecal microorganism recovery to explore the potential mechanisms of beneficial effects of load swimming and FMT.

Methods and Materials

Experimental Animals and Fecal Microbiota Transplantation (FMT)

All the rat models used in this experiment were female *Shank3*$^{-/-}$ homozygous rats constructed in cooperation with the Neuroscience Research Institute, Peking University. Two sgRNAs were designed to target the upstream region of Shank3 Exon 11 or the downstream region of Exon 21. For each target site, candidate guide RNAs were designed using CRISPR design tool 1 and targeted activity was screened. Transcript Cas9mRNA and sgRNA in vitro using the MEGASortscript T7 transcription kit (AM1354, Invitrogen), and purify using the MEGAclear transcription cleaning kit (AM 1908, Invitrogen). The Sprague Dawley (SD) rat strain was used as an embryo donor. Different concentrations of Cas9 mRNA and sgRNA were mixed and co injected into the cytoplasm of single-cell stage zygote. The surviving zygote is transferred to the oviduct of SD female to produce chimera. Finally, the positive SD originator rats mated to produce F1 heterozygous breeding pairs, carrying about 26 kb deletion, and removing Shank3 Exon 11-21. The generation of *Shank3* knockout rats was conducted by Beijing Biocytogen Co. Ltd., Beijing, China.

Altered behaviors and impaired synaptic function were observed in the novel rat model with complete *Shank3*$^{-/-}$ [42]. Genotypes were determined by PCR of rat tail DNA using the primers F1 (CTGTTGGCTGAGCCTGGCATAGAG) and R1 (GCTGGAAAGAAACAACGAGAGCCAG) for the WT allele (559 base pairs) and the primers F2 (TTGTGCAC-TGC CTATGTTGACCACT) and R2 (TAGGCGAGAGAAGAT GGTGTGATTTCC) for the mutant allele (688 base pairs).

Shank3$^{-/-}$ homozygous and littermate wild-type rats were divided into three groups: wild-type control group (WT, n = 12), *Shank3*$^{-/-}$ control group (KO, n =12), and *Shank3*$^{-/-}$ loading swimming group (KLS, n =12).

Fresh fecal pellets were collected from 2-month-old KO (n = 4) and KLS (n = 4) female rats. Next, the feces of four rats collected from each group were mixed (0.1 g/ml PBS), steeped, shaken in sterile PBS (pH 7.4, 10 mL), and then filtered through a 100-μm pore mesh. The final fecal microbial

suspension was stored at −80 °C. Recipient rats (FMT-KLS and FMT-KO) were administered 500 μL of fecal microbiota for seven interval times (total 14 days) via oral gavage.

All animals were housed with free access to a standard laboratory diet and water with a 12-h light–dark cycle under standard conditions (indoor temperature 22 ± 1 °C, humidity 65–70%). This study was carried out following the USA National Institutes of Health Guide for the Care and Use of Laboratory Animals. The protocol was approved by the Peking University Animal Care and Use Committee (ethics approval ID, LA2015204).

Loading Swimming Exercise Protocol

The age of 8-day rat corresponds to 1 year for young children. In this study, a well-established early loading swimming protocol for 8day-old SD rats was used. The young rats received swimming training in the first week, gradually reaching 40 min/day, and then the pups underwent loading swimming with 2% self-weight for the second week. After the adaptation period, the young rats underwent loading swimming training with 4% self-weight consisting of 5 days/week, 40 min/session, and a water temperature of 32 °C until 8 weeks [43]. The training period was performed on Monday to Friday afternoons, and the rats rested on weekends.

Behavior tests

Three-Chamber Test

Experimental setup: A 40 × 34 × 24-cm Plexiglas box was divided into three chambers, A, B, and C, and the corridor size was 10 cm × 10 cm × 15 cm. Chambers A and B were located at both ends of the box, chamber C was in the middle, and the activity of the rats in the three chambers was automatically monitored by a computer. During the acclimation phase, the tested rats were allowed to explore the entire apparatus freely for 5 min. During phase 1, the social preference test phase, the tested rats were removed from the three chambers. Model rat A (sex- and age-matched wild-type model rat) was placed in a metal cage in the A side box, and this was social stimulus 1 (model A); an empty metal cage was placed in the B side box as a non-social stimulus (empty). Subsequently, the test rats were placed in the middle box and allowed to explore freely for 10 min. During phase 2, model rat A was still placed ipsilateral to stage 1, model rat B was placed on the empty cage side of stage 1 (model B), and the experimental rat was placed in the middle zone and recorded for 10 min. The floor plate and standing plate were wiped clean using 75% alcohol after the end of each stage. Statistical analysis was performed by two fellow students blinded to the genotype, timed back-to-back double-blind, and averaged for the calculation.

Marble-Burying Test

The marble-burying test was performed in a squirrel cage (26.6 cm× 15.3 cm× 12.5 cm) containing 5 cm of fresh litter, and 15 glass beads (diameter 2.5 cm) were evenly spaced on the surface of the litter. During the test, the mouse was placed in one corner and allowed to move freely for 30 min, and the camera recorded the mouse activity. Mice will dig up the bedding with forelimbs, hindlimbs, or the tip of the nose to bury the glass beads, which can be used to measure the anxiety level of mice by burying the incubation period and the number of buried beads. After the test was completed, the incubation period and the number of beads buried by the mice were counted. The evaluation criteria were that the time when mice first started digging for litter was recorded as the buried incubation period, and that beads, which were more than 2/3 covered by litter were recorded as buried glass beads.

Self-Grooming

Self-grooming experiments are used to describe stereotyped, repetitive behavior in rats. Experimental rats were placed inside a rectangular box (40×34×40 cm), and self-grooming behavior in the dark was recorded using an infrared camera. Rats were placed in the box for acclimatization for 10 min and recorded for 10 min. Self-grooming behaviors including rats licking their bodies and hair, wiping their faces with their front paws, and scratching their torso.

Open Field Test

The open field test mainly detected the free movement and anxiety of rats. Each group of rats was placed in a rectangular empty box (90×90×50 cm) to adapt to the environment; the experimental area was soundproof, the light was uniform, and the ambient temperature was maintained at (24.0±0.5) °C. During the experiment, the rat was gently moved into the center of the open field with cardboard, data acquisition was performed at the same time, and the video was recorded for 10 min. After each rat was tested, feces, urine, and odor elimination with 75% alcohol occurred, and the next experiment was performed after the open field was completely dry. Each rat was placed in a new cage, which was prepared in advance after testing. Keep the experimenter quiet and away from the test rats throughout the process. The experimental video was collected by the video acquisition system, and the motion trajectory and speed of the rats were analyzed using smart 3.0 software.

Maximum Grip Experiment

The grip test mainly tests the strength of the limbs of rats. Experiments were conducted using the maximum grip tester (BIO-GS3 Grip strength, USA). The first 2 days are the acclimation phase, and the third day is the formal test. Grasp the rat's limbs on the metal mesh on the grip tester, drag the rat's tail back at a constant speed, record the highest value when the rat's forelimbs are released, and test three times, each time with an interval of 10 min. The maximum force value was recorded to on the instrument.

Hematoxylin-Eosin Staining

Rat small intestine tissue hematoxylin-eosin (HE) staining observation of small intestine 1 cm, rinsed clean, and fixed in 4% paraformaldehyde for 24 h, according to the conventional method to prepare paraffin blocks, cut 4-μm thin slices and HE staining, under light microscope to observe the pathological changes of intestinal tissue.

Pyrosequencing Analysis

Fecal DNA was extracted using the Omega E.Z.N.A. Soil DNA Kit (D5625,Omega, Shanghai Baipu Biotechnology Co., Ltd) according to the manufacturer's protocol. Each sequenced sample was prepared according to Illumina 16S Metagenomic Sequencing Library protocols.

The quantity and quality of DNA were measured by a Quant-iT PicoGreen dsDNA Assay Kit. The 16S rDNA genes were amplified using 16S V3-V4 primers (forward 5-CCTACG GGNGGCWG-CAG-3, reverse 5-GACTACHVGGGTATCTA ATCC-3). Input gDNA was amplified using 16S V3-V4 primers, and a subsequent limited-cycle amplification step was performed to add multiplexing indices and MiSeq sequencing adapters. The final products were normalized and pooled, and the size of the libraries was verified by using performed by Illumina NovaSeq.

Statistical Analysis

SPSS 20.0 (SPSS Inc.) software was used for statistical analysis of all data. The results are expressed as the mean ± SEM. The results were plotted by GraphPad Prism 8 (GraphPad Software Inc.) software. The Shapiro–Wilk normality test was used to determine whether the data conformed to a normal distribution. For the comparisons, parametric tests including *t* tests and one-way analysis of variance (ANOVA) were used if the data were normally distributed (distribution tested by the Shapiro–Wilk normality test), and nonparametric approaches, including the Wilcoxon test and Kruskal–Wallis test, were used for data with a nonnormal distribution. $p < 0.05$ was considered statistically significant.

Result

The Effects of Load Swimming and FMT on Social and Stereotyped Behavior in Shank3$^{-/-}$ Rats

We conducted three box and self-combing tests, which are widely used methods for assessing social disorders and stereotyped behaviors in rats. The results of the first stage showed that there was a significant difference in the time spent in the social of companion1 (SOC1) box between WT and FMT-KLS groups ($p<0.05$, Fig. 1A). The time spent in the None SOC box in the FMT-KLS group was longer than that in the other group ($p<0.01$) (Fig. 1B). The results of the second stage showed that the KO and FMT-KO group spent significantly more time in the social of company 1 (SOC1) box than the WT group ($p<0.001$, Fig. 1C). FMT-KO group in the SOC1 greater than KLS ($p<0.05$). FMT-KLS has a longer duration in SOC1 than in WT group. When social of company 2 (SOC2) was placed in the rat cage on the right side of the box, there was no significant difference in the residence time of the KO group or FMT-KO group on that side, but FMT-KLS group in SOC2 is shorter than KO and WT group ($p<0.05$, Fig. 1D). Figure 1 E showed that the hair conditioning time in the KO and FMT-KO group was significantly increased compared to that in the WT group ($p<0.01$, $p<0.01$). KLS group and FMT-KLS group has significance difference from WT ($p<0.01$, $p<0.05$,). The hair conditioning time in the self-grooming water FMT-KO group was significantly increased compared to that in the WT and FMT-KO group ($p<0.01$, $p<0.05$, Fig. 1F). Taking WT as the central axis, it can be seen that the influence of left *Shank3*$^{-/-}$ and load swimming can be carried to the right along with FMT. Although some results do not show statistical significance, a trend of "symmetry" can still be seen.

The Effect of Load Swimming and FMT on Anxiety Behavior in Shank3$^{-/-}$ Rats

Rats were also tested in an open field to analyze their total activity. FMT-KO rats exhibited increased anxiety behavior, as indicated by the increased amount of total distance in the open field compared with WT rats ($p<0.01$). The KO group also showed a similar trend, and KLS significantly decreased ($p<0.05$) (Fig. 1H). Additionally, the motion trajectory showed fewer KO and FMT-KO groups entering the central region (Fig. 1J). The marble burying test is also mainly used to test the anxiety level of experimental rats (Fig. 1G). The data results show that the number of buried beads in the KO and FMT-KO groups tends to be greater than that in the WT group, although it is not significant. However, there is a significant difference between KLS and FMT-KO ($p<0.01$). The number of buried

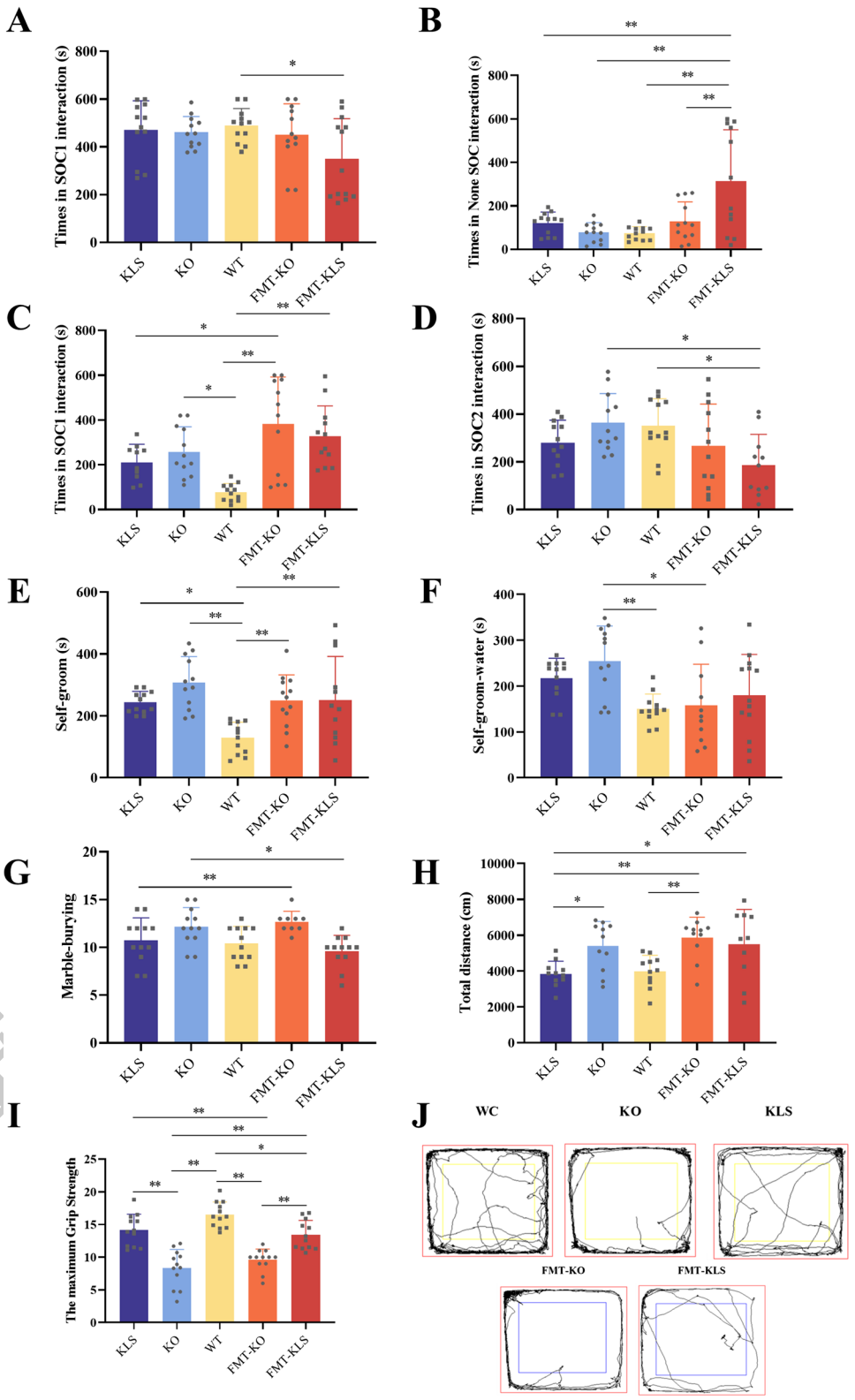


Fig. 1 The effects of load swimming and FMT on behavior in *Shank3*$^{-/-}$ rats. **A** The first stage project: times in SOC1 interaction(s). **B** The first stage project: Times in None SOC1 interaction(s). **C** The second stage project: times in SOC1 interaction(s). **D** The second stage project: times in SOC2 interaction(s). **E** Self-groom(s). **F** Self-groom-water(s). **G** Marble-burying. **H** Total distance of open field experiment. **I** The maxium grip strength. **J** Walking trajectory of open field experiment
*$p < 0.05$, **$p < 0.01$ WT rats, n=12; KO rats, n=12; KLS rats, n=12

beads in the FMT-KLS group was significantly reduced compared to the KO group (p<0.05). This provides a reference for load swimming and FMT to improve the anxiety behavior of *Shank3*$^{-/-}$ rats. We used grip strength to assess whether there was a change in muscular strength of *Shank3*$^{-/-}$ rats, and the results showed that the maximum muscle strength in the KO and FMT-KO groups decreased significantly compared to that in the WT group (p<0.01, p<0.01, Fig. 1I). Compared with the

KO group, the maximum muscle strength of the KLS and FMT-KLS groups was significantly increased ($p<0.01$, $p<0.01$), indicating that the muscle strength of the gene knockout rat was poor and significantly increased after weight-bearing swimming intervention (Fig. 1I). Although there is still a significant difference between the FMT-KLS group and the WT group ($p<0.05$), there is a significant increase compared to the KO group ($p<0.01$). Similarly, KLS also significantly increased compared to FMT-KO ($p<0.05$).

The Effect of Load Swimming and FMT on Small Intestinal HE Staining in Shank3$^{-/-}$ Rats

HE staining showed clear tissue structure of the small intestine in the WT group, while inflammatory cells infiltrated under the intestinal mucosa in the KO group. The small intestine structure and inflammatory cells in the KLS group improved more than those in the KO group (Fig. 2A). Quantitative results showed that compared to the WT group (Fig. 2B), the length of small intestinal villi in the KO group decreased significantly ($p<0.05$), and KLS could reverse the decrease in villi length caused by *Shank3* knockout ($p<0.01$). The results of villus width showed that compared with WT group, the width of small intestinal villus in KO group tended to narrow, but it was less significant than that in FMT-KO group ($p<0.01$, Fig. 2C). And compared to the KO group, the FMT-KO group had less villous width ($p<0.01$). FMT-KLS can reverse the reduction of FMT-KO ($p<0.01$) and form a significant difference with WT and KLS ($p<0.01$, $p<0.01$). The crypt depth in the KLS group was greater than that in the FMT-KLS group ($p<0.01$, Fig. 2D). The myometrial thickness was also associated with the loss of *Shank3* (Fig. 2E). Compared with the KO group, the muscle layer thickness of KLS and FMT-KLS showed a significant increase ($p<0.01$, $p<0.01$). The ratio of villus length to recess depth can reflect the intestinal absorption function (Fig. 2F). Although there was no significant difference between the groups, KO and FMT-KO still showed a decreasing trend in absorption rate, while KLS and FMT-KLS also showed an improvement trend. In addition, we also counted the number of air bubbles in the villi to reflect the degree of inflammation laterally. The results showed that (Fig. 2G), the number of air bubbles in the KO and FMT-KO group increased significantly (($p<0.01$, $p<0.01$), while the number of air bubbles in the villi in the FMT-KLS group was lower than that in the FMT-KO group ($p<0.05$).

Effects of Load Swimming on Colonic Content Microbial Diversity

ASV/OUT results showed that the abundance value of the KO group increased compared to that of the WT group and KLS group, indicating that the microbial community in the KO group was more abundant (Fig. 3A). The results of alpha diversity analysis of microbiota in small intestine contents showed that the PD indices of Chao1, observed specifications, Shannon, Simpson, Pielou's evenness, and Faith's in KO group were higher than those in WT group and KLS group; and the Good's coverage index in WT group was higher than those in KO group and KLS group (Fig. 3B, C, D). This indicates that KLS can reduce the abnormal increase in intestinal microbial abundance and diversity caused by *Shank3* knockout. The distribution of PCoA is shown in Fig. 3E. It is generally believed that when the value is less than 0.2, the results of NMDS analysis are more reliable. As shown in the Fig. 3F, a stress of 0.104 indicates that our results are reliable.

Differences in Gut Microbiota of Shank3$^{-/-}$ Rat with Loading Swimming and None

To test whether the effects of *Shank3* knockout and load swimming are related to changes in the intestinal microbiota, we analyzed the composition of the intestinal microbiota among the three groups. We used histograms and heatmaps at the phylum and genus levels to determine the abundance information of the top 10 communities to estimate the potential bacterial populations that cause intestinal disorders (Fig. 4A, C). At the phylum level, the abundance of *Firmicutes* and *Bacteroidetes* was higher in the three groups, with the abundance of *Firmicutes* and *Bacteroidetes* showing a downward and upward trend compared to WT. KLS reversed this trend (Fig. 4B). At the genus level, compared to the WT group, the abundance of *Lactobacillus* in the KO group significantly decreased ($p<0.05$), while the abundance of *Lachnospiracea* and *Alloprevotella* significantly increased ($p<0.05$, $p<0.01$) (Fig. 4D). The KLS group reversed this change, and the abundance of *Alloprevotella* was significantly higher than that in the KO group ($p<0.05$). In summary, these results provide evidence that the fecal microbiota *Shank* 3$^{-/-}$ mice is different from that of WT rat, that this difference may affect the intestinal environment, and that KLS is involved in regulating the intestinal microbial environment.

Effects of FMT on Colonic Content Microbial Diversity

The ASV/OUT results after fecal microbiota transplantation also showed that the abundance value of the FMT-KO group was higher than that of the FMT-KLS group (Fig. 5A), indicating that the effects of fecal microbiota transplantation and *Shank* 3$^{-/-}$ gene knockout on the abundance of intestinal flora were consistent. The diversity analysis results showed that the indices of the Chao1, observed specification, and Faith's PD groups in the FMT-KLS group were higher than those in the FMT-KO

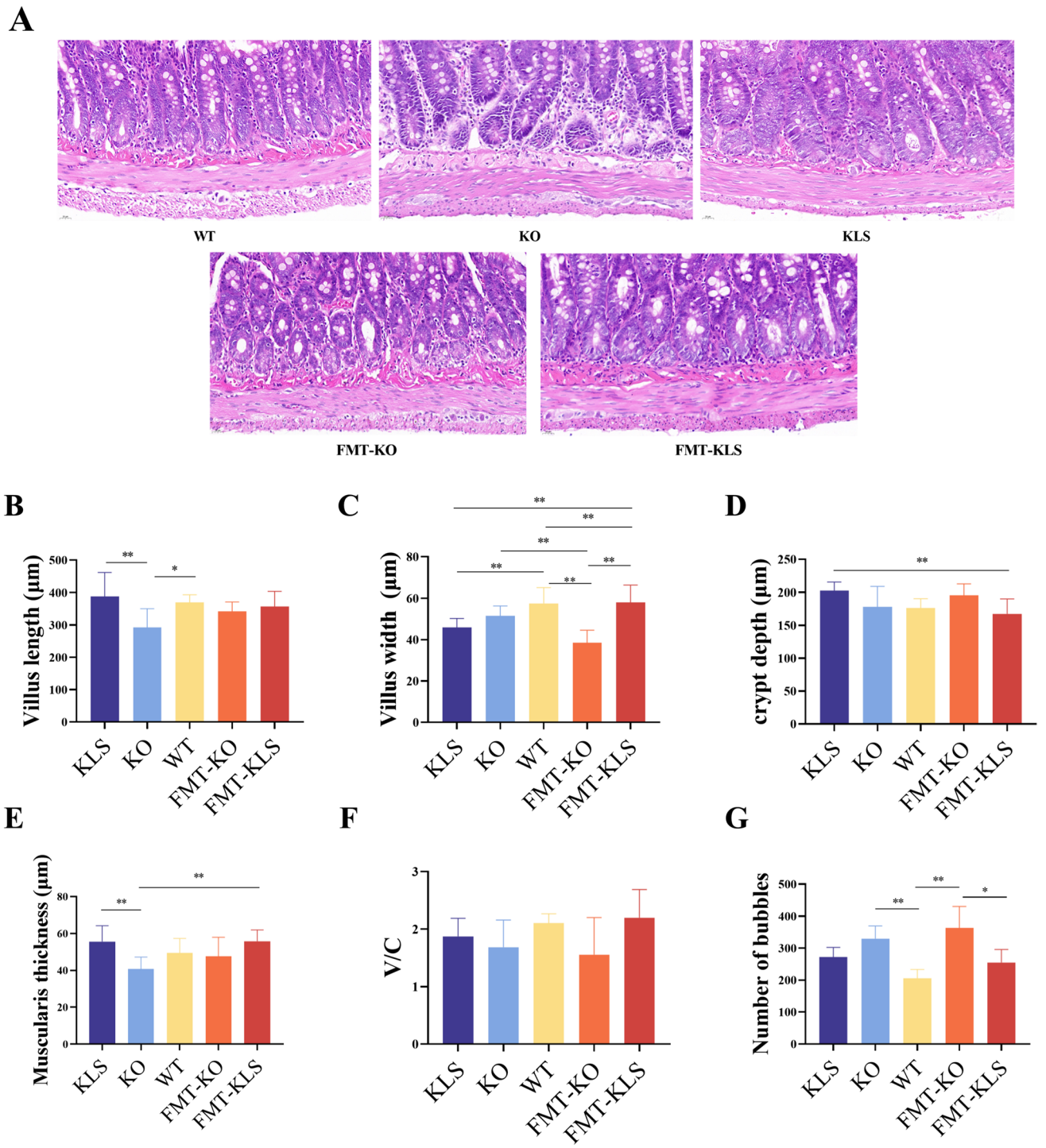
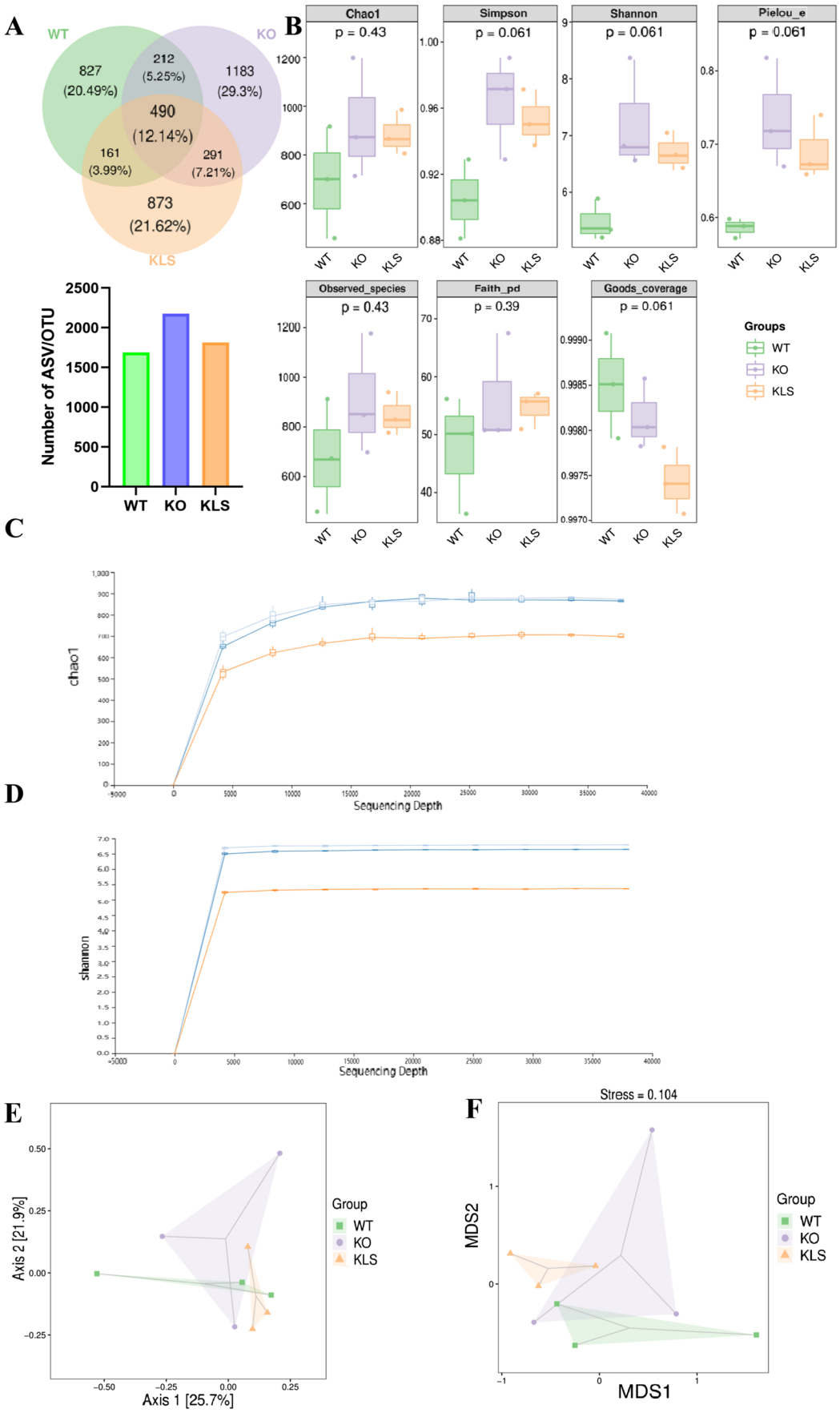


Fig. 2 HE staining of small intestinal cross sections of rats (200×). **A** Hematoxylin-eosin staining. **B** Villus length. **C** Villus width. **D** Crypt depth. **E** Musclaris thickness. **F** V/C. **G** Number of bubbles. *$p < 0.05$, **$p < 0.01$

group (Fig. 5B, C, and D), indicating that FMT can increase the diversity of intestinal microbiota in rats. The distribution of PCoA is shown in Fig. 5E. It is generally believed that when the value is less than 0.2, the results of NMDS analysis are more reliable. As shown in Fig. 5F, a stress of 0.0948 indicates that our results are reliable.


A
WT
KO
KLS
827 (20.49%)
212 (5.25%)
1183 (29.3%)
490 (12.14%)
161 (3.99%)
291 (7.21%)
873 (21.62%)
B
Chao1
p = 0.43
Simpson
p = 0.061
Shannon
p = 0.061
Pielou_e
p = 0.061
Observed_species
p = 0.43
Faith_pd
p = 0.39
Goods_coverage
p = 0.061
Groups
WT
KO
KLS
Number of ASV/OTU
C
chao1
Sequencing Depth
D
shannon
Sequencing Depth
E
Axis 2 [21.9%]
Axis 1 [25.7%]
Group
WT
KO
KLS
F
Stress = 0.104
MDS2
MDS1
Group
WT
KO
KLS

◂**Fig. 3** ASV/OYU clustering and alpha diversity of intestinal flora. **A** Number of ASV/OYU. **B** Alpha diversity of intestinal flora **C** Chao1. **D** Shannon. **E** PCoA analysis **F** NMDS analysis

Differences in Gut Microbiota of Shank3$^{-/-}$ Rat with FMT

The horizontal abundance of the transplanted phyla and genera is shown in Fig. 6A, C. At the phylum level (Fig. 6B), the top two abundances were *Firmicutes* and *Bacteroides*. The level of *Verrucomicrobia* in the FMT-KLS group was significantly higher than that in the FMT-KO group ($p<0.01$). At the genus level, compared with the FMT-KLS group (Fig. 6D), the abundance of Prevotella-9 in the FMT-KO group was significantly reduced. The abundance of *Bacteroides* first increased ($p<0.05$). In summary, these results provide evidence that fecal microbiota transplantation can affect the abundance of intestinal content, and the fecal microbiota after load swimming is different from *Shank3*$^{-/-}$, which may be the reason for behavioral changes.

Discussion

Changes in intestinal microbiota can lead to the occurrence of neurological diseases, mainly manifested in abnormal behavior and changes in tryptophan metabolism in SD rats receiving fecal microbiota from patients with depression [44]. The fecal microbiota of patients with Alzheimer's disease reduces metabolites related to the central nervous system in the feces of mice and leads to cognitive dysfunction. Therefore, changes in intestinal microflora can lead to changes in the metabolic products of the central nervous system, thereby affecting behavior. The neurodevelopmental dysfunction process of ASD has been proven to be related to the imbalance of the intestinal microbiota and its metabolites [45]. Exogenous supplementation of the intestinal flora can improve the behavior of autistic patients and animals [46–48]. Therefore, regulation of the intestinal microbiota is used as a management strategy for ASD [49]. In this study, a new gene knockout model was used to investigate the potential relationship between the intestinal microbiota and ASD after *Shank3* knockout. Load swimming was used to explore methods to improve the disease model.

Our results show that, rats with *Shank3* gene knockout exhibit abnormal intestinal microorganisms and exhibit stereotypical and anxiety like behavior. We found differences in the abundance and diversity of the intestinal microbiota in WT, KO, and KLS rats. In existing reports, children with autism have a higher risk of gastrointestinal diseases such as constipation and diarrhea, and their risk of abdominal pain is three times higher than that of normal children [50]. This supports the hypothesis that changes in the intestinal microbiota affect the development of neuropathology in ASD. Children with autism are more prone to food intolerance [51]. ASD patients with gastrointestinal complications exhibit more sleep difficulties, abnormal emotions, antagonism, provocative or destructive behavior, anxiety, sensory reactions, stiffness, obsessive compulsive behavior, self injury, aggression, and social disorders [52]. This result may explain why anxiety is the behavior that can be changed after fecal bacteria transplantation is anxiety. It has also been reported that compared to men, women with autism have more intrinsic symptoms, such as anxiety and depression, which may depend on genetics, sex hormones, or other reasons [53–55]. Although no gender comparison was conducted in this experiment, the anxiety behavior of female mice can be accompanied by fecal bacteria transplantation. The close relationship between exercise and changes in intestinal flora has been confirmed [56]. Exercise can reduce the abnormal increase in intestinal microbial abundance caused by *Shank3* knockout, which can be transferred to the recipient's intestinal content as fecal bacteria are transplanted, and can affect their anxiety symptoms. This supports the possibility of exercise affecting the treatment of neuropathology in ASD with intestinal microbiota.

The sequencing results of intestinal contents showed that two strains with higher relative abundance in the WT, KO, and KLS groups were *Firmicutes* and *Bacteroidetes*. The normal human intestinal microbiota consists of two main phyla, namely *Bacteroides* and *Thickwalled Bacteria* [57]. Reportedly, the proportion of *Firmicus/Bacteroides* was increased in ASD patients, and the proportion of *Lachnospiraceae* and *Ruminococcus* increased significantly [58]. This is consistent with our results. Traditionally, the *Firmicutes*:*Bacteroidetes* ratio has been implicated in predisposition to disease states [59]. Another recent assessment showed that with the increase in the proportion of *Bacteroides* to *Thickwalled Bacteria*, microbial diversity decreased by 25%, and the core group decreased from 29 to 12 [60]. Therefore, ensuring the relative stability of *Chlamydomonas* and *Bacteroides* has reference significance for human health. *Patescibacteria*, *Actinobacteria*, and *Protebacteria* showed increased relative abundance in the KO group. *Patescibacteria* has been reported to be associated with depression-related indicators [61]. The elevation of *Patescibacteria* is involved in the process of nerve damage [62]. In another study, the representative gate of increase in the underweight group was *Patsciberia*, which may affect muscle strength [63]. *Actinomyces* are one of the four main phyla of intestinal microbiota, and although they account for only a small proportion, they are crucial in maintaining intestinal homeostasis [64]. Current research indicates that abnormal *actinomycete* abundance is not related to the pathogenesis of inflammatory bowel disease, but changes in the intestinal environment and immune factors caused by *actinomycetes* may exacerbate inflammation-induced damage

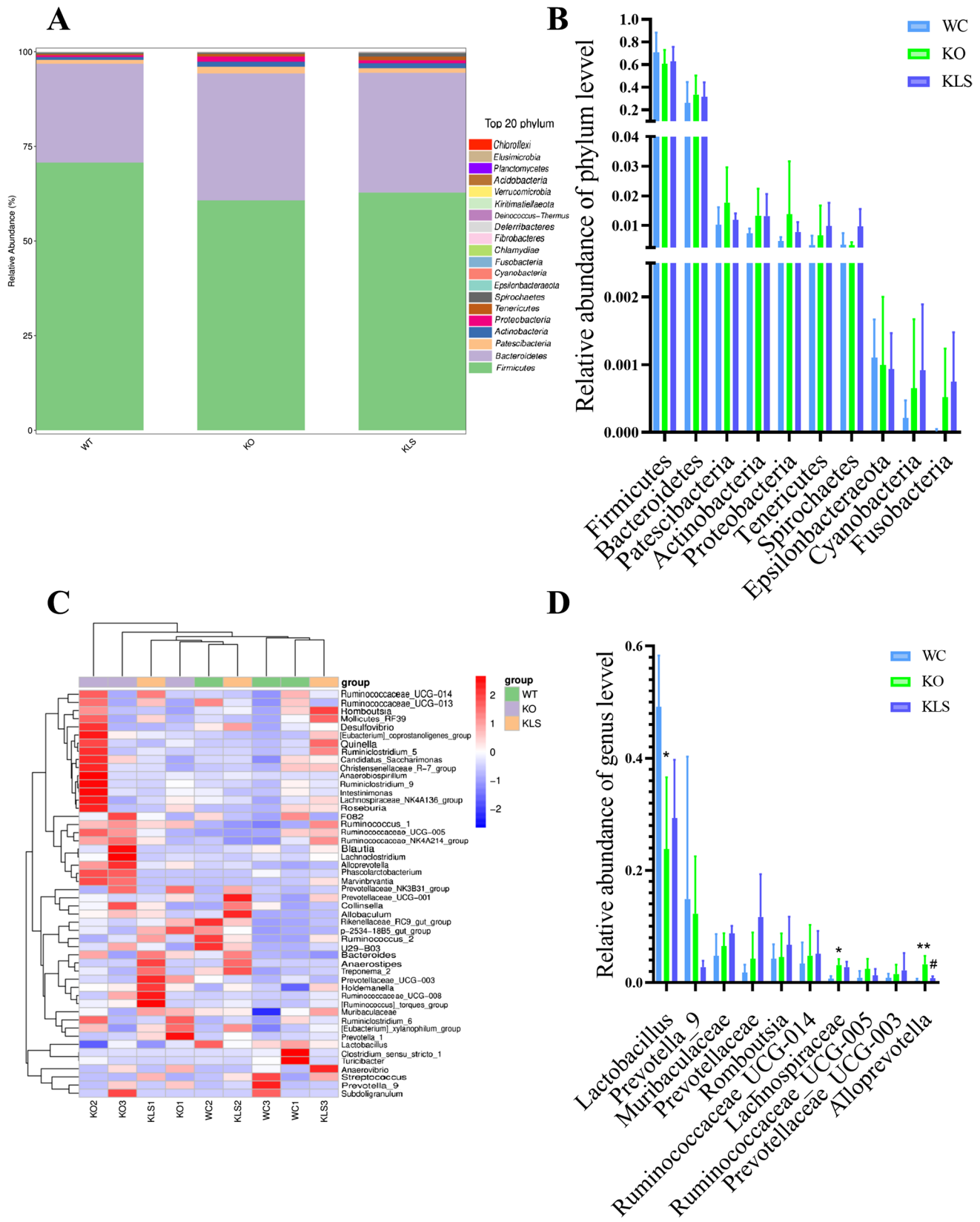


Fig. 4 Effects of WT, KO, and KLS on species at the phylum and genus levels of rats colonic intestinal flora. **A** Relative abundance of top 20 at phylum level. **B** Relative abundance of top 10 at phylum level. **C** Heat map showed the changes of intestinal microorganisms in each group of rats at genus level. **D** Relative abundance of top 10 at genus level. *$p < 0.05$ WT vs. KO, **$p < 0.01$ WT vs. KO, $^{\#}p < 0.05$ KO vs. KLS, $^{\#\#}p < 0.05$ KO vs. KLS

[65]. An environmental or drug-related colitis survey of the microbiome of horse feces showed a significant increase in the abundance of *Protebacteria* [66].

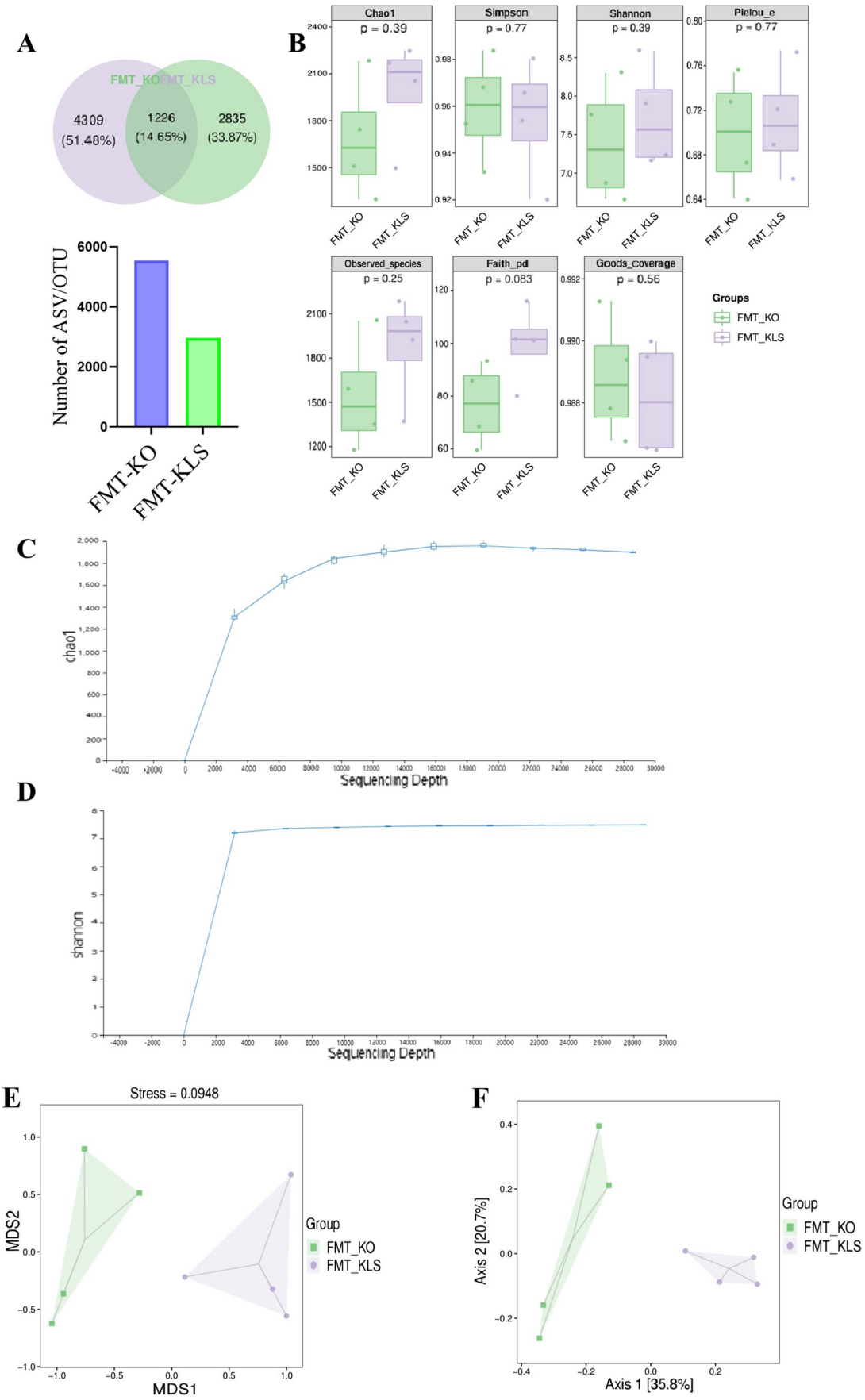
Through fecal microbial abundance detection, we found that *Shank3* lacked the abnormality of *Lactobacillus* in the feces of female rats compared with normal female rats. *Lactobacillus i*mproved the imbalance of intestinal microbiota, promoted the diversity of intestinal microbiota, reduced proinflammatory bacteria, and increased anti-inflammatory bacteria [67]. We found that the relative abundance of *Lactobacillus* in *Shank* 3 $^{-/-}$ rats increased after exercise, which may be beneficial for inflammatory resistance. In addition, the relative abundance of *Lachnospiraceae* also increased significantly after *Shank3* deletion. *Lachnospiraceae* is a large family of bacteria, and its members may be related to health and disease [68] .Compared with the healthy control group, the number of *Lachnospiraceae* bacteria in children with ASD increased, and microbiota changes in patients with autumn spectrum disorders [69]. And *Lachnospiraceae* integration into the gut microbiome at key time points in early life is linked to infant neurodevelopment [70]. The controversial role of human gut *Lachnospiraceae*: their disorder is related to many other chronic diseases, such as inflammatory bowel disease, kidney disease, liver disease, and neurobehavioral disease [68]. High moderate-to-vigorous physical activity (MVPA) levels, *Paraprevotellaceae*, *Lachnospiraceae*, and *Lachnospira* were enriched, among students [71]. Finally, we found that the relative abundance of *Alloprevotella* significantly increased in the KO group, and load swimming reversed this trend. *Alloprevotella* has been reported to be enriched in cancer tissue [72]. Moreover, an increase in the abundance of *Alloprevotella* was also found in mice with ulcerative colitis [73]. It has been shown that the abundance of *Alloprevotella* is related to intestinal inflammation and carcinogenesis. From the morphological quantitative perspective, the number of vacuoles in the KLS group was also lower than that in the KO group, indicating that the inflammatory situation had improved.

Epsilonbacteraeota was significantly higher in the FMT-KLS group than in the FMT-KO group in intestinal content sequencing after fecal microbiota transplantation. *Epsilonbacteraeota* has also been reported to be associated with the occurrence and development of ulcerative colitis, intestinal tumors, inflammatory bowel diseases, and other diseases, but further research and confirmation are needed. In addition, we also found a decrease in *Prevotella* in the KLS and FMT-KLS groups. Metagenomic analysis of 30 cases of C-ASD and its age-matched TD showed a decrease in *Prevotella* and *Bacteroides* species and associated metabolic activity dysregulation, which may be related to the pathogenesis of C-ASD [74]. Another cohort study also confirmed that a decrease in gut microbial diversity in children with autism was associated with a decrease in the relative abundance of the phylum most closely related to *Prevotella* [69].

Similarities between the microbiome of highly frail nursing home residents (≥65 years) and the malnourished population both showed an increase in *Ruminococcus* gnavus, a species recently associated with inflammatory joint disease [75].On the other hand, the statistically significant presence of *Ruminococcus* (*Ruminococcaceae*) in ASD patients without GI symptoms may corroborate the idea that a starch-degrading bacterium may, in any case, improve GI wellness, prevent from symptoms, and respond to nutritional triggers rather than to host innate immunity [76]. Similar to our results, the relative abundance of *Romboutsia i*n children with neurodevelopmental disorders increased significantly [77]. In addition, the relative abundance of *Romboutsia* and circulating inflammation (IL-1 β) were correlated with behavioral output [78]. This change may be related to abnormal inflammatory factors in autistic patients. The high abundance of M was also confirmed in KO. *Bifidobacterium* and *Muribaculaceae* have beneficial effects on intestinal dysbiosis through immunoregulation and modulation of gut homeostasis [79]. *Blautia* is one of the major intestinal microbes often found in human fecal samples [80]. There is a strong relationship between decreased levels of the genus *Blautia* and diseases. Increasing the ratio of *Blautia* in the intestine might be beneficial for health [81]. Similarly, in Chen Y's study, it was found that intestinal barrier damage and intestinal microbiota imbalance are characterized by depletion of the prevalent family [82]. In the KLS group after fecal microbiota transplantation, we found a significant increase in the abundance of the dominant beneficial bacteria *Bacteroides*, which are reported to metabolize polysaccharides and oligosaccharides, providing nutrients and vitamins to the host and other intestinal microorganisms [83].

The effect of flora transplantation also plays a role in different organ changes, such as muscle. Of the eight stool microbiota transplants, 75% showed that the recipient mice successfully copied the muscle phenotype of the donor [84]. This can explain the replication of the muscle phenotype by load swimming after our transplantation.

The load swimming effect after transplantation increased the intestinal content abundance of KO rats. In rodent studies where exercise and nutrition are more controlled, regular physical activity is associated with an elevated bacterial diversity and richness in the gut/fecal microbiota [85]. The nature and/or intensity of exercises may differentially alter the microbiota composition (voluntary exercise vs. forced exercised [86]. In rodents with voluntary access to the wheel exercise, the bacterial diversity and main phyla abundance were not strongly altered, but forced exercise (treadmill) led


A
FMT_KO
FMT_KLS
4309
(51.48%)
1226
(14.65%)
2835
(33.87%)
Number of ASV/OTU
6000
4000
2000
0
FMT-KO
FMT-KLS
B
Chao1
p = 0.39
Simpson
p = 0.77
Shannon
p = 0.39
Pielou_e
p = 0.77
Observed_species
p = 0.25
Faith_pd
p = 0.083
Goods_coverage
p = 0.56
Groups
FMT_KO
FMT_KLS
C
chao1
Sequencing Depth
D
shannon
Sequencing Depth
E
Stress = 0.0948
MDS2
MDS1
Group
FMT_KO
FMT_KLS
F
Axis 2 [20.7%]
Axis 1 [35.8%]
Group
FMT_KO
FMT_KLS

◂**Fig. 5** ASV/OYU clustering and alpha diversity of intestinal flora of fecal microbiota transplant rats. **A** Number of ASV/OYU. **B** Alpha diversity of intestinal flora. **C** Chao1. **D** Shannon. **E** PCoA analysis. **F** NMDS analysis

to an increased proportion of harmful and pro-inflammatory bacteria vs. free-exercised animals and sedentary controls [87]. A study demonstrated that forced treadmill exercise exacerbates inflammation and causes mortality while voluntary wheel training is protective in a mouse model of colitis [88]. FMT from mice with free access to running wheels for 6 weeks in germ-free mice subjected to inflamed colon (colitis), 49 allowed higher microbiota diversity, limited colitis, and improved body mass compared with the same animals transplanted with microbiota from sedentary mice [89]. Therefore, the effect of reasonable exercise on intestinal flora

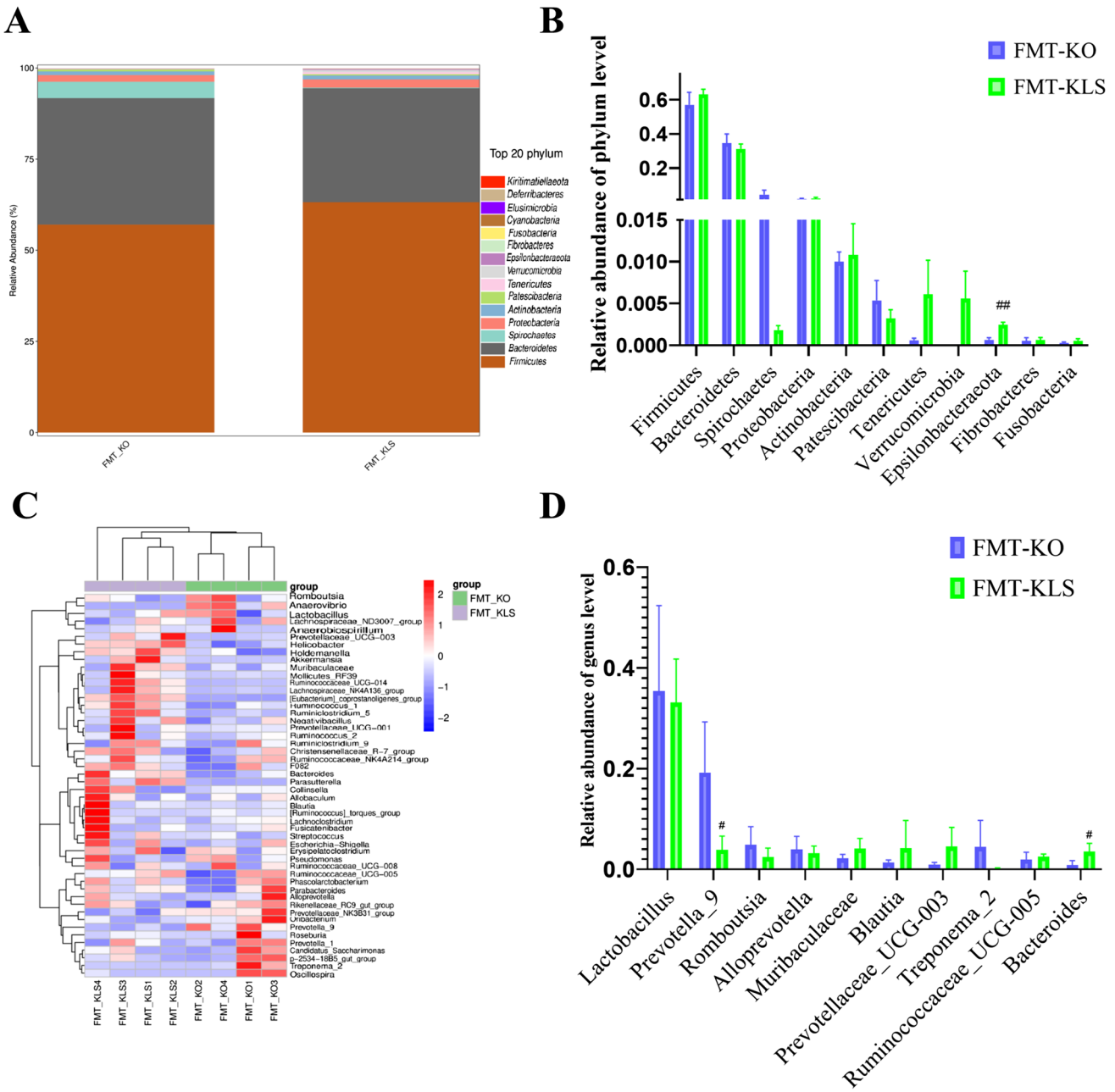


Fig. 6 Effects of FMT-KLS and FMT-KO on species at the phylum and genus levels of rats colonic intestinal flora. **A** Relative abundance of top 20 at phylum level. **B** Relative abundance of top 10 at phylum level. **C** Heat map showed the changes of intestinal microorganisms in each group of rats at genus level. **D** Relative abundance of top 10 at genus level. $^{\#}p < 0.05$ FMT-KO vs. FMT-KLS, $^{\#\#}p < 0.05$ FMT-KO vs. FMT-KLS

is beneficial, and this effect can be better reflected through flora transplantation.

Author Contributions All authors contributed to the study conception and design. Material preparation, data collection, and analysis were performed by Shasha An, Zhiping Zhen, Shijiao Wang, Mingze Sang, and Shuai Zhang. The first draft of the manuscript was written by Shasha An, and all authors commented on previous versions of the manuscript. All authors read and approved the final manuscript.

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Data Availability The data that support the findings of this study are available from the corresponding author upon reasonable request.

Declarations

Ethics Approval All animal experiments were conducted following the Peking University Animal Care and Use Committee (ethics approval ID, LA2015204).

Consent for Publication Not applicable.

Consent to Participate Not applicable.

Competing Interests The authors declare no competing interests.

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