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Dietary fish oil improves autistic behaviors and gut homeostasis by altering the gut microbial composition in a mouse model of fragile X syndrome

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ABSTRACT

Fragile X syndrome (FXS) is the most common inherited intellectual disability, caused by a lack of the fragile X mental retardation protein (FMRP). Individuals with neurodevelopmental disorders frequently experience gastrointestinal problems that are primarily linked to gut microbial dysbiosis, inflammation, and increased intestinal permeability. Omega-3 polyunsaturated fatty acids (omega-3 PUFAs) are non-pharmacological agents that exert potential therapeutic effects against neurological disorders. However, it is unclear whether omega-3 PUFAs improve autistic behaviors in fragile X syndrome (FXS) by altering the gut microbial composition. Here, we describe gastrointestinal problems in *Fmr1* knockout (KO) mice. FMRP deficiency causes intestinal homeostasis dysfunction in mice. Fish oil (FO) as a source of omega-3 PUFAs reduces intestinal inflammation but increases the mRNA and protein levels of *TJP3* in the colon of juvenile *Fmr1* KO mice. Fecal microbiota transplantation from FO-fed *Fmr1* KO mice increased the gut abundance of *Akkermansia* and *Gordonibacter* in recipient *Fmr1* KO mice and improved gut homeostasis and autistic behaviors. Our findings demonstrate that omega-3 PUFAs improve autistic behaviors and gut homeostasis in FMRP-deficient mice by suppressing gut microbiota dysbiosis, thereby presenting a novel therapeutic approach for juvenile FXS treatment.

1. Introduction

Autism spectrum disorder (ASD) is a group of neurodevelopmental conditions characterized by impaired social communication and interaction, as well as repetitive stereotyped behaviors. Autism affects one in every 44 children, and men have a higher prevalence rate of autism than women (Maenner et al., 2021). Genetic and environmental factors both contribute to autism (Hirota and King, 2023). Fragile X syndrome (FXS) is the most common monogenic cause of autism spectrum disorder (ASD) and the most prevalent inherited cause of intellectual disability, affecting 1 in 4000 males and 1 in 7000 females (Lozano et al., 2014). The disease stems from the expansion of a trinucleotide repeat (CGG) within the X-linked fragile X mental retardation 1 (FMR1) gene, which leads to a loss of the product of the FMR1 gene, fragile X mental retardation protein (FMRP) (Banerjee et al., 2018). The FMR1 gene is highly conserved across species, with the murine homolog Fmr1 showing 97% amino acid sequence homology (Ashley et al., 1993). Owing to the high similarity in the expression pattern of FMR1 at the mRNA and protein level in different tissues of humans and mice (Abitbol et al., 1993; Bachner et al., 1993; Hinds et al., 1993), a *Fmr1* knockout (KO) mouse model for the FXS was developed to investigate the FXS because it exhibits some characteristics comparable to those of patients with FXS, including macroorchidism, aberrant dendritic morphology, and associative cognitive deficits (Bakker et al., 1994; Bernardet and Crusio, 2006). Thus, while the mutational mechanism differs between the animal models and fragile X patients, the fragile X mouse can no longer generate normal FMRP, making the mouse an excellent animal model for investigating the physiologic function of the FMR1 gene and gaining more insight into the pathophysiological mechanisms and clinical phenotype linked to the absence of FMRP in humans (Telias, 2019).

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Despite the intense interest in FXS, particularly because of its potential as a gateway to understanding autism, the precise mechanisms underlying this rare syndrome remain unclear, and no Food and Drug Administration (FDA)- approved drug is available.

The majority of individuals with neurodevelopmental disorders have gastrointestinal (GI) problems (Holingue et al., 2018). In addition, evidence suggests a potential causal relationship between parental, particularly maternal, inflammatory bowel disease (IBD) and childhood autism (Sadik et al., 2022). There are speculations that GI symptoms in ASD are a manifestation of the underlying inflammatory process (Navarro et al., 2016). Intestinal inflammation and disruption of the intestinal barrier increase intestinal permeability, allowing the leakage of bacterial-derived neurotoxic peptides such as lipopolysaccharides (LPS) (D'Eufemia et al., 1996; De Angelis et al., 2015). Furthermore, GI symptoms in ASD patients are a result of gut microbial dysbiosis. Alterations in the gut microbiota composition and microbial metabolites have been observed in animal models of neurodevelopmental disorders and children with ASD (Buffington et al., 2016; Connolly et al., 2016). However, GI problems in FXS patients have not been investigated.

Omega-3 polyunsaturated fatty acids (PUFAs) are enriched in fish oil (FO). Supplementation with omega-3 PUFAs has been proposed as a potential treatment for depression (Jazayeri et al., 2008; Mozaffari-Khosravi et al., 2013). Emerging evidence indicates that omega-3 PUFAs partially alleviate neuroinflammation and autistic behaviors in *Fmr1* knockout (KO) mice (Pietropaolo et al., 2014). Additionally, omega-3 PUFAs were found to potentially decrease intestinal inflammation by regulating mice gut microbiota (Kaliannan et al., 2015). However, the effects of omega-3 PUFAs on gut microbiota composition and intestinal inflammation in *Fmr1* KO mice remain unclear.

The hypothesis tested here is that FO improves GI problems and autistic behaviors in *Fmr1* KO mice by altering the gut microbiota. We show that FMRP deficiency causes GI problems and intestinal homeostasis dysfunction in mice. Fish oil (FO) administered orally as a source of omega-3 PUFAs, reduces intestinal inflammation but increases mRNA and protein levels of *TJP3* in the colon of juvenile *Fmr1* KO mice. Fecal microbiota transplantation from FO-fed *Fmr1* KO mice increased the gut abundance of *Akkermansia* and *Gordonibacter* in recipient *Fmr1* KO mice, and improved gut homeostasis and autistic behaviors. As such, this work uncovered a previously unknown role for FMRP and gut homeostasis in maintaining juvenile cognitive ability induced by omega-3 PUFAs administration and present a novel therapeutic strategy for FXS treatment.

2. Materials and methods

2.1. Animals

All animal procedures adhered to protocols approved by the Nankai University Experimental Animal Ethics Committee and Tianjin University of Traditional Chinese Medicine Care and Use Committee, respectively. C57BL/6 mice were given free access to water and food and were maintained in a temperature-controlled (22 °C) room with a 12:12-h light/dark cycle (8:00 AM, lights on; 8:00 PM, lights off). FMR1 is an X-chromosome-linked gene, and male *Fmr1* KO mice have significantly reduced fertility; in this view, we used male littermate WT and *Fmr1* KO mice for the present investigation. All behavioral analyses were performed by experimenters who were blinded to sample identity and treatments.

2.2. Supplementation with Fish oil (FO)

Four-week-old male WT and *Fmr1* KO mice were randomly divided into two groups: one group received Phosphate Buffered Saline (PBS, Vehicle), while the other received FO (2.5 mL/kg, Sigma Aldrich). FO was administered by gavage at a predetermined time each day for four weeks (Liu et al., 2020).

2.3. Novel object recognition (NOR) test

The novel object recognition test is based on the natural preference of rodents to explore unfamiliar objects over familiar ones. The experiment was performed as previously described (Li et al., 2016; Li et al., 2018). On the first experimental day, mice were placed in an L-shaped maze and allowed to move freely for 10 min. On the second experimental day, two objects of the same shape and size, made of the same material (old objects), were placed symmetrically at both ends of the L maze, and mice were allowed to freely explore the objects for 10 min. On the third experimental day, one of the old objects was replaced with a new object, and mice were allowed to explore the objects freely for 10 min. The equipment and the objects were wiped with 75% alcohol before placing each mouse to prevent interference from other environmental factors. The amount of time each mouse spent exploring new and old objects was recorded. The identification index was defined as the difference in the exploration time of the novel and familiar objects. The discrimination index was calculated as follows: Discrimination Index = [(time spent in exploring the new object/total exploration time) × 100] – [(time spent in exploring the familiar object/total exploration time) × 100]. On the last experimental day, the exploration behavior of all mice was videotaped to ensure objectivity.

2.4. Three-chamber sociability test

The three-chamber sociability test was designed to evaluate social behavior and social novelty in mice. The experiment was performed as previously described (Shen et al., 2019). Briefly, a topless transparent plastic box (60 × 40 × 23 cm) was divided into three chambers (left, middle, and right). In the first phase, the mice were placed in the middle chamber of a clean, empty apparatus and allowed to explore the entire apparatus for 10 min. After habituation, an age- and sex-matched stranger mouse (mouse 1) of the same strain that had never been exposed to the test mouse was placed in a wire cage in a side chamber, and an empty wire cage was placed on the opposite side, as an inanimate object, (for sociability tests). Mice were free to explore the apparatus for 10 min. The stranger mouse was randomly placed into the left or right chambers. In the third phase, another stranger mouse (mouse 2) was introduced into the empty wire cage (for the social recognition test), and the test mouse was allowed to freely explore for 10 min. The orientation of the two wire cages containing mouse 1 or mouse 2 (or empty) was counterbalanced for each set of experiments. Movements of mice were recorded using a camera outfitted with software that provided a real-time readout of the relative locations of the mice in the three chambers. To ensure objectivity, all experiments were videotaped and scored by scientists who were blinded to the experimental conditions. The discrimination index was calculated as follows: Discrimination Index = [(time spent in exploring the unfamiliar mouse 2/total exploration time) × 100] – [(time spent in exploring the familiar mouse 1/total exploration time) × 100].

2.5. Novel location recognition (NLR) test

This test assesses spatial memory by evaluating the ability of mice to recognize the new location of a familiar object, assisted by spatial cues. The experiment was performed as previously described (Li et al., 2016; Li et al., 2018). Briefly, mice were exposed to two identical objects for 6 min during the acquisition phase three times and tested 3 min later. Object preferences were evaluated during each session. Of note, one of the objects was moved to a novel location during the trial session. The mice were allowed to explore the objects for 6 min, and the total time spent exploring each object was recorded. Exploration was defined as any investigative behavior (i.e., head orientation and sniffing within < 1.0 cm) or deliberate contact with each object. The discrimination index was calculated formulas follows: Discrimination Index = [(time spent in exploring the novel location/total exploration time) × 100] – [(time

spent in exploring the old location/total exploration time) × 100]). All experiments were videotaped, and scientists who were blinded to the experimental conditions scored the data.

2.6. Open-field activity test

All mice were tested individually in a black box (50 × 50 × 50 cm) as described previously (Li et al., 2016; Li et al., 2018). Mice were placed at the corner of the device and habituated for 3 min. The mice were allowed to move freely for 30 min; their movement was recorded using a video-computerized tracking system. The total distance covered and the mean speed served as activity indicators.

2.7. Self-grooming test

The self-grooming test was performed as previously described (Hong et al., 2014). Mice were placed individually in a clean cage lined with a thin layer of bedding (approximately 1 cm) to reduce neophobia and prevent digging, a potentially competing behavior. The mice were allowed to habituate for 10 min. Each mouse was examined for 10 min, and the cumulative time spent in grooming was calculated. Experimenters blinded to the experimental conditions used the self-grooming time to evaluate anxiety behaviors.

2.8. Tissue harvesting

Mice were sacrificed after fasting for 12 h, and colon and blood samples were collected from all mice. Blood was drawn from the eyeball directly into Microvette® tubes and kept at room temperature. The colon tissues were used for biochemical analyses. Moreover, the feces were gently flushed out and homogenized with ice-cold phosphate-buffered saline (PBS). All tissues were placed in liquid nitrogen for short-term storage and then transferred to a −80 °C refrigerator. Plasma was separated from the blood that had been kept at room temperature for 1 h by centrifugation (1000 rpm at 4 °C for 15 min).

2.9. RNA extraction and quantitative PCR (qPCR) analysis

Total RNA was extracted from the colon tissue using the TRIzol reagent (Solarbio, China). The quality and concentration of the extracted RNA were assessed using BioDrop (Thermo, USA). Next, 1 μg of RNA was reverse transcribed using the Revert Aid First Strand cDNA Synthesis Kit (Thermo, USA). Real-time PCR assays were performed using the SYBR qPCR master mix (MCE, USA). GAPDH served as an internal control. Primer sequences are depicted in Supplementary Table 1.

2.10. Gut microbiota analysis

Relative bacterial populations in the feces were assessed by qPCR. Total bacteria in feces were represented as 16S rRNA gene copies per mg of wet feces (copies/mg). Primer sequences are provided in Supplementary Table 1.

2.11. Western blot analysis

Total cellular protein was extracted from the colon by lysing frozen tissues in the RIPA lysis buffer containing protease and phosphatase inhibitors. Denatured proteins were resolved by SDS-PAGE, and transferred to PVDF membranes (Sigma, USA). The membranes were blocked in Tris-buffered saline with Tween-20 containing 5% skim milk at room temperature, probed with the indicated primary antibodies, and incubated with the appropriate HRP-conjugated secondary antibodies. The following antibodies were used: anti-TJP3 (1:2000, Immunoway) and anti-GAPDH (1:10000, Immunoway).

2.12. Elisa

Serum samples were diluted (1:10 to 1:1000) in DEPC water (Solarbio, China) and heated at 70 °C for 10–15 min. The serum levels of lipopolysaccharide (LPS) were evaluated using an ELISA kit (ZciBio, China). The obtained OD values were converted to concentration values.

2.13. Fecal DNA extraction and 16S rRNA gene sequencing

Fresh fecal samples (80 mg) were frozen at −80 °C until further processing. DNA was extracted from the fecal samples using the E.Z.N.A. ® soil DNA Kit (Omega Bio-Tek, Norcross, GA, U.S.), according to the manufacturer's protocol. The hypervariable region V3-V4 of the bacterial 16S rRNA gene were amplified with primer pairs 338F (5′-ACTCCTACGGGAGGCAGCAG-3′) and 806R(5′-GGACTACHVGGGTWTCTAAT-3′) by an ABI GeneAmp® 9700 PCR thermocycler (ABI, CA, USA). Purified amplicons were pooled in equimolar amounts and paired-end sequenced on an Illumina MiSeq PE300 platform (Illumina, San Diego, USA) according to the standard protocols by Majorbio Bio-Pharm Technology Co. Ltd. (Shanghai, China). The composition, correlation and function prediction of gut microbiota were analyzed using the Majorbio microbial diversity cloud platform (https://cloud.majorbio.com/).

2.14. Intestinal permeability analysis

Intestinal permeability was assessed using the 4 kDa fluorescent dextran (MCE, USA). Mice were administered fluorescein isothiocyanate (FITC)-dextran (50 mg/mL) after a 4-hour fast via gavage (600 mg/kg) (Chevalier et al., 2020). After 1 h, 200 μL of the blood was collected into a Microvette® tube. The tubes were centrifuged at 10,000 × g for 10 min at room temperature to collect serum. Collected sera were diluted with the same volume of PBS and FITC concentration (excitation 485 nm, emission 535 nm) was analyzed using a microplate reader (Cytation 5, USA).

2.15. Fecal microbiota transplantation (FMT)

Stool samples from *Fmr1* KO mice treated with vehicle or FO for 4 weeks were collected daily under sterile conditions. Fresh stool samples (100 mg) were resuspended in 1 mL of sterile saline, and vortexed. The suspension was then centrifuged at 1000 rpm for 3 min. The supernatant was prepared the same day and transplanted within 10 min using a gavage. Each recipient mouse (4-week-old *Fmr1* KO mouse) received 200 μL of bacterial suspension daily for 7 days. *Fmr1* KO mice that received feces from vehicle-fed *Fmr1* KO mice were denoted as the "KO-FMT" group, while *Fmr1* KO mice that received feces from FO-fed *Fmr1* KO mice were denoted as the "KOFO-FMT" group.

2.16. Statistical analysis

Statistical analysis was performed using analysis of variance (ANOVA) and Student's *t*-test, unless specified, with the GraphPad Prism software. Two-tailed and unpaired *t*-test was used to compare two conditions. The two-way analysis of variance (ANOVA) was used to compare multiple experimental conditions. Tukey's post hoc test was used for multiple comparisons. All data were presented as mean with standard error of the mean (mean ± S.E.M). Probabilities of $P < 0.05$ were considered significant.

3. Results

3.1. FMRP deficiency causes gut microbial dysbiosis in mice

The gut microbial composition of 8-week-old WT and *Fmr1* KO mice housed separately was examined using 16S rRNA gene sequencing.

Principal component analysis of Bray-Curtis distances showed different fecal microbiota clusters between WT and *Fmr1* KO mice (Fig. 1A). On the other hand, the Shannon index revealed no differences in the richness and diversity of microbial species between WT and Fmr1 KO mice (Fig. 1B). In WT mice, Firmicutes (49.51%), Bacteroidota (40.25%), Desulfobacterota (2.73%), Actinobacteria (2.87%), and Verrucomicrobiota (2.66%) were most abundant at the phylum level. In *Fmr1* KO mice, Firmicutes (49.67%), Bacteroidota (45.00%), Desulfobacterota (2.21%), and Actinobacteria (1.68%) predominated (Fig. 1C). The proportion of Verrucomicrobiota was significantly higher in WT mice than in *Fmr1* KO mice. Differences in bacterial abundance were found to be associated with inflammation and short-chain fatty acids (SCFAs) synthesis in WT and *Fmr1* KO mice, including the *Lachnospiraceae NK4A136 group*, *Faecalibaculum*, *Turicibacter*, *Akkermansia*, and *Gordonibacter* (Fig. 1D). In a nutshell, the gut microbiota differed significantly between WT and *Fmr1* KO mice, particularly those associated with inflammation and synthesis of SCFAs.

3.2. FMRP deficiency influences intestinal homeostasis in mice

Alterations in the gut microbiota may cause a range of conditions, including inflammation, increased intestinal permeability, and elevated serum LPS levels (Cani et al., 2008). To assess for potential inflammation and GI problems in *Fmr1* KO mice, we evaluated the mRNA expression of pro-inflammatory cytokines in the colon. Our analysis revealed significantly high mRNA expression levels of *TNF-α* mRNA in the colon of *Fmr1* KO mice (Fig. 2A: t = 2.303, df = 10, p = 0.0440) (Fig. 2A). Furthermore, we evaluated the related expressions of colonic tight junction proteins (TJPs) (Figure S1) and discovered that the mRNA and protein levels of TJP3 were lower in the colon of *Fmr1* KO mice than in the colon of WT mice (Fig. 2B:t = 3.358, df = 12, p = 0.0057; Fig. 2D:t = 11.29, df = 4, p = 0.0004) (Fig. 2B-D). Subsequently, we measured intestinal permeability with FITC-dextran and discovered no significant differences between WT and *Fmr1* KO mice (Fig. 2E: t = 0.1789, df = 14, p = 0.8606) (Fig. 2E). Lastly, we measured serum LPS levels and found significantly high serum LPS levels in *Fmr1* KO mice (Fig. 2F:t = 3.583, df = 14, p = 0.0030) (Fig. 2F). These data imply that FMRP deficiency

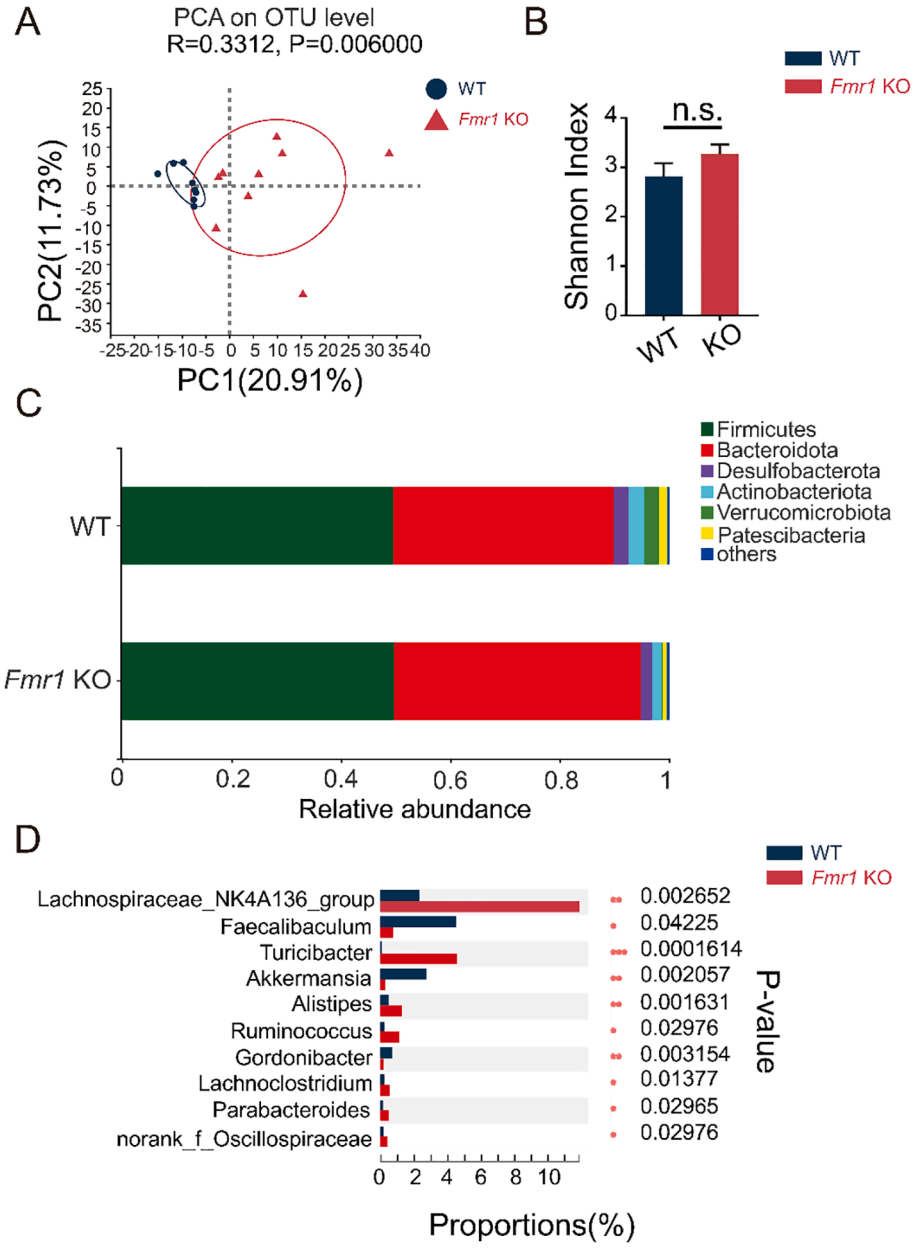

Fig. 1. FMRP deficiency triggers gut microbial dysbiosis in mice. (A) Principal component analysis of Bray-Curtis distances in the beta diversity of gut microbiome in WT and *Fmr1* KO mice (WT = 8 mice, *Fmr1* KO = 9 mice). (B) Quantitative comparison of microbial species richness and α-diversity in WT and *Fmr1* KO mice (N = 8 mice per group). (C) Analysis of the relative bacterial abundance at the phylum level in WT and *Fmr1* KO mice (N = 8 mice per group). (D) Proportional abundance analysis at the genus level in WT and *Fmr1* KO mice (N = 8 mice per group). *p < 0.05; **p < 0.01; ***p < 0.001; n.s., no significant difference.

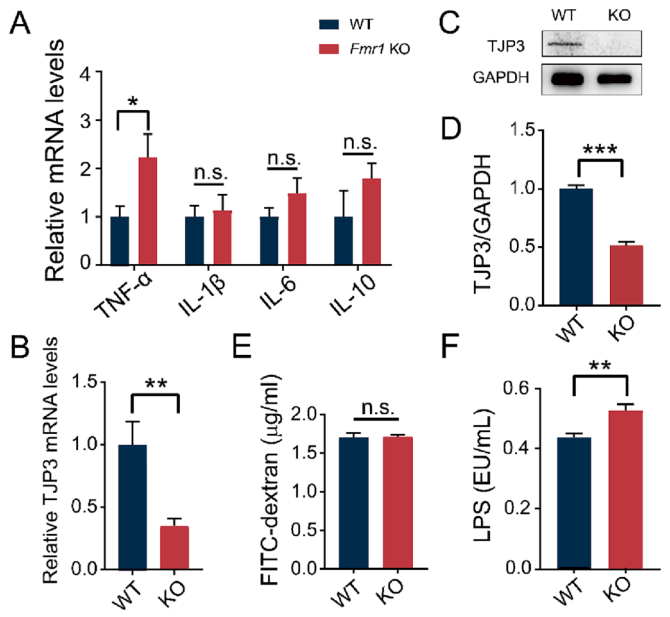

Fig. 2. *Fmr1* KO mice exhibit GI symptoms. (A) Quantitative real-time PCR analyses of mRNA expression level of pro-inflammatory factors, such as *TNF-α*, *IL-1β*,*IL-6* and *IL-10* in colon tissues from *Fmr1* KO mice and WT littermates (N = 6–8 mice per group). (B) Quantitative real-time PCR analyses of *TJP3 mRNA* expression in colon tissues from *Fmr1* KO mice and WT littermates (N = 7 mice per group). (C-D) Western blot analyses of TJP3 protein expression levels in colon tissues from *Fmr1* KO mice and WT littermates (N = 3 mice per group). (E) Quantitative comparison of the intestinal permeability between *Fmr1* KO mice and WT littermates (WT = 7 mice, *Fmr1* KO = 8 mice). (F) Quantitative comparison of the serum endotoxin levels between *Fmr1* KO mice and WT littermates (N = 8 mice per group). *p < 0.05; **p < 0.01; ***p < 0.001; n.s., no significant difference.

causes intestinal homeostasis dysfunction in mice.

3.3. FO supplementation improves autistic behaviors in Fmr1 KO mice

A PUFA-rich diet can improve cognitive and behavioral deficits in individuals with autism and has been suggested as a potential therapeutic intervention for several neurological and psychiatric disorders (Baron-Mendoza and Gonzalez-Arenas, 2022; Pietropaolo et al., 2014). In this view, we assessed whether FO supplementation could improve autistic behaviors in *Fmr1* KO mice (Fig. 3 and Figure S2). As a result, 4-week-old *Fmr1* KO mice and their WT littermates were subjected to 4-week treatment with FO, and their cognitive functions were assessed using a novel location test (NLT), object recognition test (NOR), three-chamber sociability test, and self-grooming test (Fig. 3A and Fig. S2A). FO supplementation had no significant effect on overall health (Fig. S2B: $F_{(1,16)} = 0.4924$, $P = 0.4930$) and locomotor activity in WT and *Fmr1* KO mice (Fig. S2C: $F_{(1,20)} = 0.02$, $p = 0.8889$; Fig. S2D: $F_{(1,20)} = 0.3497$, $p = 0.5609$) (Fig. S2B-D). *Fmr1* KO mice exhibited impaired spatial learning in NLT and objective learning in NOR compared with WT littermates, as evidenced by a lower discrimination index (Fig. 3B-E). FO supplementation rescued cognitive deficits of *Fmr1* KO mice in both NLT and NOR but had no significant effect on WT mice (Fig. 3C: $F_{(1,28)} = 12.66$, $p = 0.0014$; Fig. 3E: $F_{(1,28)} = 11.54$, $p = 0.0021$) (Fig. 3B-E). Meanwhile, *Fmr1* KO mice exhibited impaired social behaviors in the three-chamber sociability test and anxiety in the self-grooming test compared with the WT littermates, as evidenced by a lower discrimination index and decreased self-grooming time (Fig. 3F-I). FO supplementation improved social interaction ability and anxiety in *Fmr1* KO mice but had no discernible effect on WT mice (Fig. 3G: $F_{(1,28)} = 6.256$, $p = 0.0185$; Fig. 3I: $F_{(1,28)} = 9.062$, $p = 0.0055$) (Fig. 3F-I).

3.4. FO supplementation influences gut microbiota in Fmr1 KO mice

Diet can influence the composition of gut microbes and the production of SCFAs (Gibiino et al., 2021). Nutritional supplements have been demonstrated to improve gut microbial composition (Tomova et al., 2020). In the present work, we evaluated the effects of FO supplementation on gut microbiota through 16S rRNA gene sequencing of the feces of 8-week-old *Fmr1* KO mice treated with either vehicle or FO housed in the same environment. FO supplementation altered the gut microbial composition in *Fmr1* KO mice. The PCA plot depicted a distinction in vehicle- and FO-treated *Fmr1* KO mice (Fig. 4A and Figure S4), whereas the Shannon index revealed no significant differences in microbial diversity (Fig. 4B). Moreover, the Firmicutes/Bacteroidota ratio along with the proportion of Verrucomicrobiota and Actinobacteria increased in *Fmr1* KO mice treated with FO (Fig. 4C). In the subsequent tests, we adopted the linear discriminant analysis effect size (LefSe) to verify that there were significant differences between the aforementioned two groups. FO supplementation significantly affected some anti-inflammatory molecules- and SCFAs-producing bacteria by suppressing gut microbiota dysbiosis in *Fmr1* KO mice. The abundance of *Akkermansia* and *Gordonibacter*, originally abundant in WT mice, increased in *Fmr1* KO mice treated with FO (Fig. 4D). These results demonstrate that FO supplementation potentially influences the composition of gut microbiota in *Fmr1* KO mice.

3.5. FO improves autistic behaviors by regulating gut microbiota in Fmr1 KO mice

Compelling evidence shows that gut microbes play a significant role in regulating autistic behaviors in mice (Buffington et al., 2016; Buffington et al., 2021; Hsiao et al., 2013). In this view, we used FMT to investigate whether FO improves autistic behaviors by altering the gut microbial composition. We did not use antibiotics in our experiments because long-term antibiotics administration has negative effects on the nervous system, gut microbes, and autistic behaviors (Aguilera et al., 2013). Four-week-old *Fmr1* KO mice received feces from 8-week-old *Fmr1* KO mice treated with either vehicle or FO (Fig. 5A and Fig. S3A). FMT from either vehicle- or FO-fed *Fmr1* KO mice showed no significant effect on overall health (Fig. S3B: $t = 1.562$, $df = 14$, $p = 0.1406$) or locomotor activity in recipient *Fmr1* KO mice (Fig. S3C: $t = 2.201$, $df = 10$, $p = 0.0524$; Fig. 3D: $t = 2.201$, $df = 10$, $p = 0.0523$) (Fig. S3B-D). FMT from FO-fed *Fmr1* KO mice improved spatial learning and objective cognition in recipient *Fmr1* KO mice in both NLT and NOR (Fig. 5C: $t = 4.051$, $df = 14$, $p = 0.012$; Fig. 5E: $t = 4.675$, $df = 14$, $p = 0.0004$) (Fig. 5B-E).

FMT from FO-fed *Fmr1* KO mice improved social interaction ability (Fig. 5G: $t = 3.932$, $df = 14$, $p = 0.0015$) (Fig. 5F-G) and alleviated anxiety, as evidenced by decreased grooming time in recipient *Fmr1* KO mice (Fig. 5I: $t = 2.211$, $df = 14$, $p = 0.0442$) (Fig. 5H-I). These results demonstrate that FO improved cognitive function in a Fragile X mouse model in part due to changes in the microbiota.

3.6. FMT from FO-fed Fmr1 KO mice improves gut homeostasis and increases Akkermansia and Gordonibacter abundance in recipient Fmr1 KO mice

FO supplementation decreased *TNF-α* mRNA levels while increasing mRNA and protein levels of *TJP3* in the colon of *Fmr1* KO mice (Fig. S5A: $t = 2.731$, $df = 9$, $p = 0.0232$; Fig. S5B: $t = 3.42$, $df = 12$, $p = 0.0051$; Fig. S5D: $t = 6.485$, $df = 4$, $p = 0.0029$) (Fig. S5A-D). Furthermore, we investigated whether FO supplementation improved intestinal homeostasis by examining the mRNA expression of pro-inflammatory factors in *Fmr1* KO mice who received FMT from either vehicle- or FO-fed *Fmr1* KO mice. FMT from FO-fed *Fmr1* KO mice significantly decreased the *TNF-α* mRNA expression in the colon of recipient *Fmr1* KO mice (Fig. 6A: $t = 5.65$, $df = 13$, $p < 0.0001$) (Fig. 6A). On contrary, FMT from FO-fed *Fmr1*

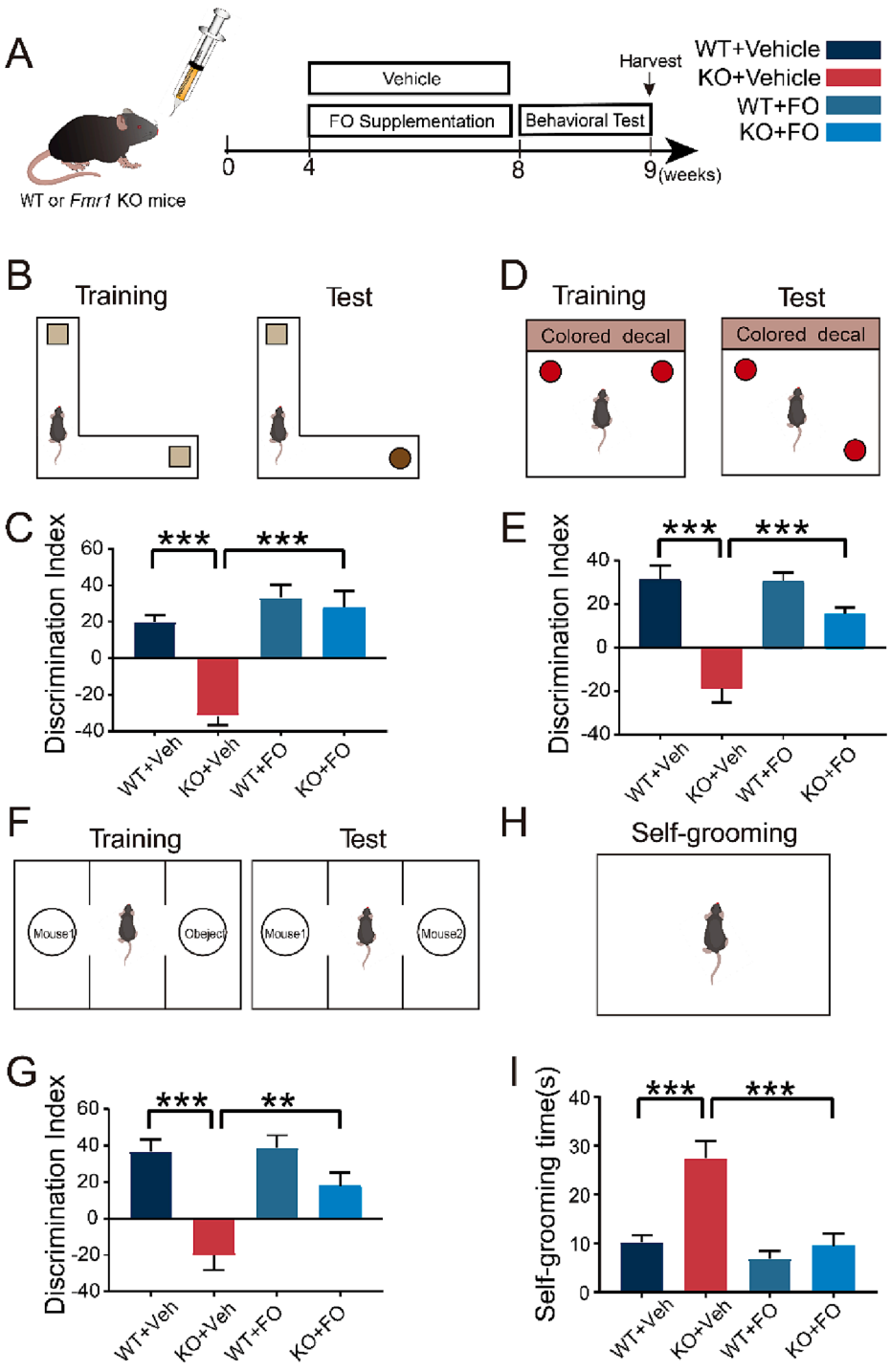

Fig. 3. Oral administration of FO improves autistic behaviors in *Fmr1* KO mice. (A) Experimental scheme designed to assess cognitive functions in WT and *Fmr1* KO mice treated with either vehicle or FO. (B-C) FO administration completely rescues novel objection recognition in *Fmr1* KO mice (N = 8 mice per group). (D-E) FO administration completely rescues spatial learning deficits in *Fmr1* KO mice (N = 8 mice per group). (F-G) FO administration completely rescues social interaction deficits in *Fmr1* KO mice (N = 8 mice per group). (H-I) FO administration completely rescues anxiety in *Fmr1* KO mice (N = 8 mice per group). **p < 0.01; ***p < 0.001; n.s., no significant difference.

KO mice significantly increased mRNA and protein levels of *TJP3* in the colon of recipient *Fmr1* KO mice (Fig. 6B: t = 3.891, df = 14, p = 0.0016; Fig. 6D: t = 5.664, df = 14, p = 0.0048) (Fig. 6B-D). In addition, FMT from FO-fed *Fmr1* KO mice was found to significantly decrease serum LPS levels in the colon of recipient *Fmr1* KO mice (Fig. 6E: t = 2.761, df = 10, p = 0.0201) (Fig. 6E). Assessment of the abundance of *Akkermansia* and *Gordonibacter* in feces showed FMT from FO-fed *Fmr1* KO mice significantly increased the abundance of these bacteria in the colon of recipient *Fmr1* KO mice (Fig. 6F: t = 2.657, df = 8, p = 0.0289; Fig. 6G: t = 4.381, df = 8, p = 0.0023) (Fig. 6F-G). These findings strongly demonstrate that FO supplementation improved intestinal homeostasis in *Fmr1* KO mice by regulating gut microbiota and increasing the abundance of *Akkermansia* and *Gordonibacter*, respectively.

4. Discussion

The exact etiology of ASD is complex and multifactorial. GI problems are a common comorbidity in patients with ASD which manifest as abdominal pain, gaseousness, diarrhea, constipation and flatulence. These GI symptoms are triggered by intestinal inflammation, increased intestinal permeability, elevated blood LPS levels, and altered gut microbiota (Al-Beltagi, 2021; Li et al., 2017; Pietropaolo et al., 2014). We demonstrate that GI diseases also exist in a mouse model of FXS. In addition, our results show that FO can resolve GI problems and rescue

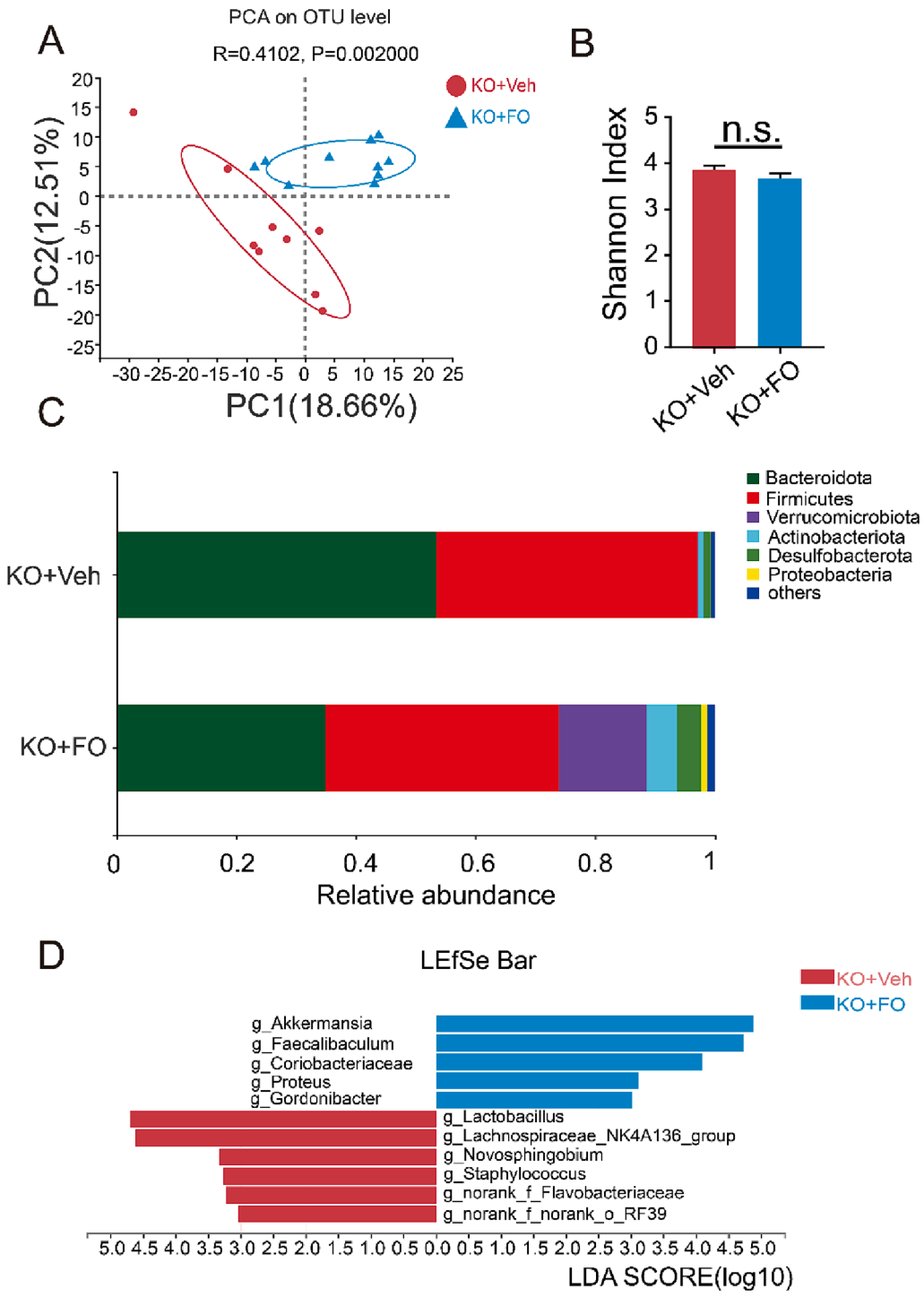

Fig. 4. Oral administration of FO alters the composition of gut microbiota in *Fmr1* KO mice. (A) Quantitative comparison of beta diversity of gut microbiome at OTU level in colon tissues from *Fmr1* KO mice treated with either vehicle or FO (KO + Veh = 9 mice, KO + FO = 10 mice). (B) Quantitative comparison of α-diversity as shown in Shannon index in colon tissues from *Fmr1* KO mice treated with either vehicle or FO (N = 8 mice per group). (C) Quantitative comparison of the abundance of bacteria at the phylum level in colon tissues from *Fmr1* KO mice treated with vehicle or FO (N = 8 mice per group). (D) Quantitative analysis of the microbial taxa represented by LEfSe scores in colon tissues from *Fmr1* KO mice treated with either vehicle or FO (N = 8 mice per group). n. s., no significant difference.

autistic behaviors in a fragile X mouse model by modulating gut microbiota. These exciting results provide a new treatment strategy for FXS, especially for juvenile individuals with FXS.

LPS is usually produced by intestinal microbes. Microbial dysbiosis can increase serum LPS levels due to increased intestinal leakage and disruption of the intestinal barrier, thereby causing systemic inflammation (Raman and Ghosh, 2019). The intestinal barrier is mainly composed of tight junction proteins, including claudins, TJ-associated marvel proteins (TAMPs), such as occludin and junctional adhesion molecules (JAMs), and cytoplasmic zonula occludens family proteins (TJP1, TJP2, and TJP3) (Kyuno et al., 2021). A decrease in TJP3 expression has been linked to intestinal barrier damage (Su et al., 2021). The present results showed that *TNF-α* mRNA level was elevated, but the mRNA and protein levels of TJP3 were downregulated in the colon of *Fmr1* KO mice, and this was accompanied by an increase in serum LPS levels. However, there were no significant alteration in the intestinal permeability in the colon of *Fmr1* KO mice. These results indicate that LPS may enter the bloodstream through other routes, such as intestinal epithelial cells or chylomicrons (Ghoshal et al., 2009). Omega-3 PUFAs deficiency has been shown to negatively affect the maternal mental health and neurodevelopment in fetus and infant (Nishi et al., 2016). However, the mechanism by which supplementation of PUFAs-rich diet improves cognition and behavioral deficits in individuals with autism is not fully understood (Nishi et al., 2016). This study shows that FO

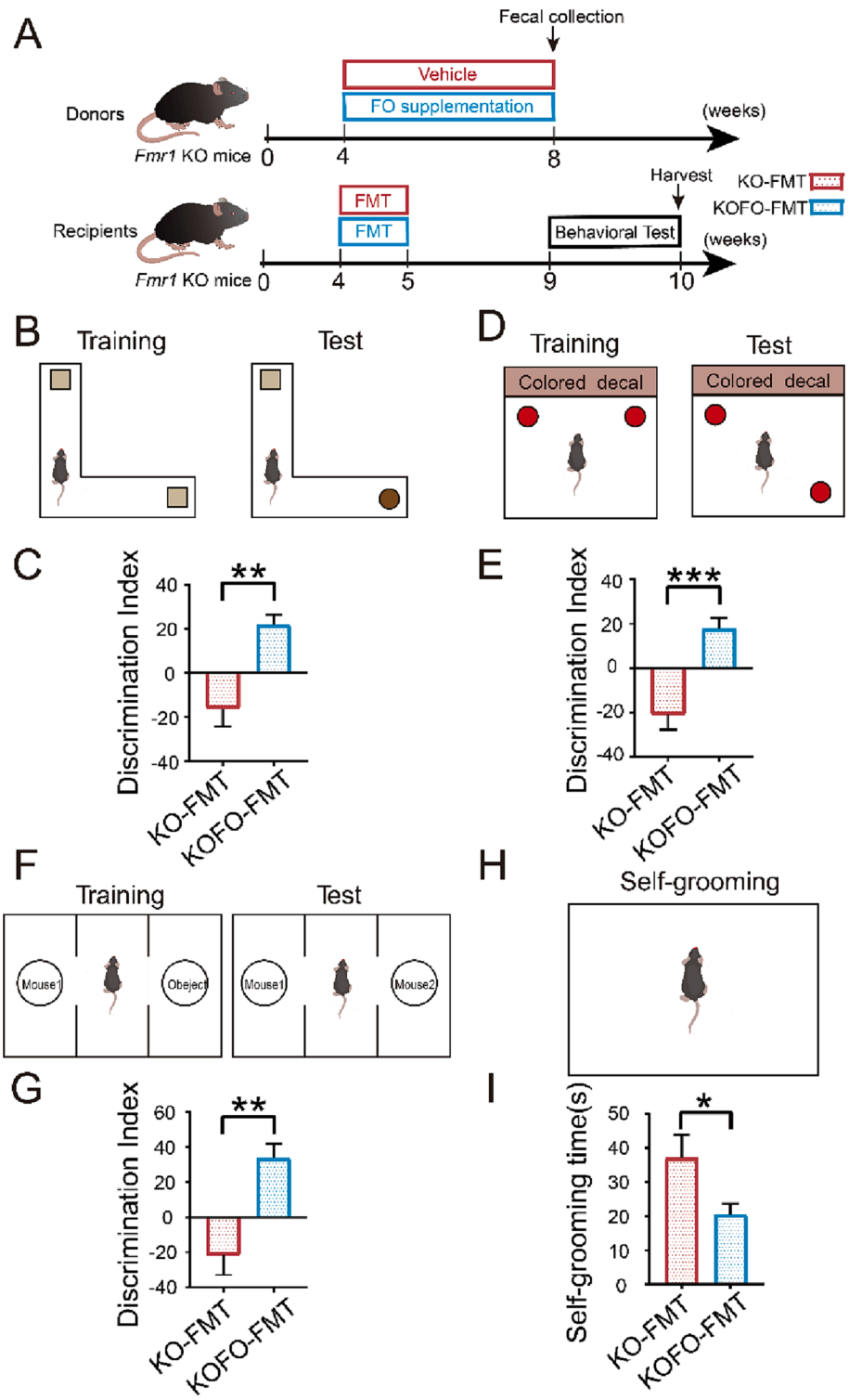

Fig. 5. Fecal microbiota transplantation (FMT) from FO-fed *Fmr1* KO mice improves autistic behaviors in recipient *Fmr1* KO mice. (A) Experimental scheme designed to assess cognitive function in *Fmr1* KO mice administered with FMT from either vehicle- or FO-fed *Fmr1* KO mice. (B-C) FMT from FO-fed *Fmr1* KO mice fully rescues novel objection recognition in recipient *Fmr1* KO mice (N = 8 mice per group). (D-E) FMT from FO supplementation in *Fmr1* KO mice completely rescues spatial learning deficits in recipient *Fmr1* KO mice (N = 8 mice per group). (F-G) FMT from FO-fed *Fmr1* KO mice completely rescues social interaction deficits in recipient *Fmr1* KO mice (N = 8 mice per group). (H-I) FMT from FO-fed *Fmr1* KO mice completely rescues anxiety in recipient *Fmr1* KO mice (N = 8 mice per group). *p < 0.05, **p < 0.01, and ***p < 0.001.

supplementation can ameliorate cognitive deficits in *Fmr1* KO mice by maintaining gut homeostasis in a mouse model of FXS. This shows the therapeutic role of nutritional intervention of omega-3 PUFA as the treatment for neurodevelopmental disorder-related signs and core symptoms.

Alterations to the composition of gut microbiome have been reported in patients with ASD (Sgritta et al., 2019). This study demonstrates a significant difference in Verrucomicrobiota between WT and *Fmr1* KO mice at the phylum level, which is consistent with findings from a previous study (Altimiras et al., 2021). At the genus level, changes in the top 10 groups were identified in WT and *Fmr1* KO mice, including the *Lachnospiraceae NK4A136 group*, *Akkermansia*, *Turicibacter*, *Prevotellaceae UCG-001*, *Alistipes, Enterorhabdus*, the *Eubacterium Xylanophilum_group*, *Gordonibacter*, *Lachnoclostridium*, and *A2*. Among them, seven bacterial genera, including the *Lachnospiraceae NK4A136 group*, *Turicibacter*, *Prevotellaceae UCG-001*, *Alistipes*, the *Eubacterium Xylanophilum group*, *Lachnoclostridium*, and *A2* were more abundant in *Fmr1* KO mice. These results further revealed a potential association of gut microbiome with FXS, providing new ideas for developing reliable treatments and non-invasive biomarkers.

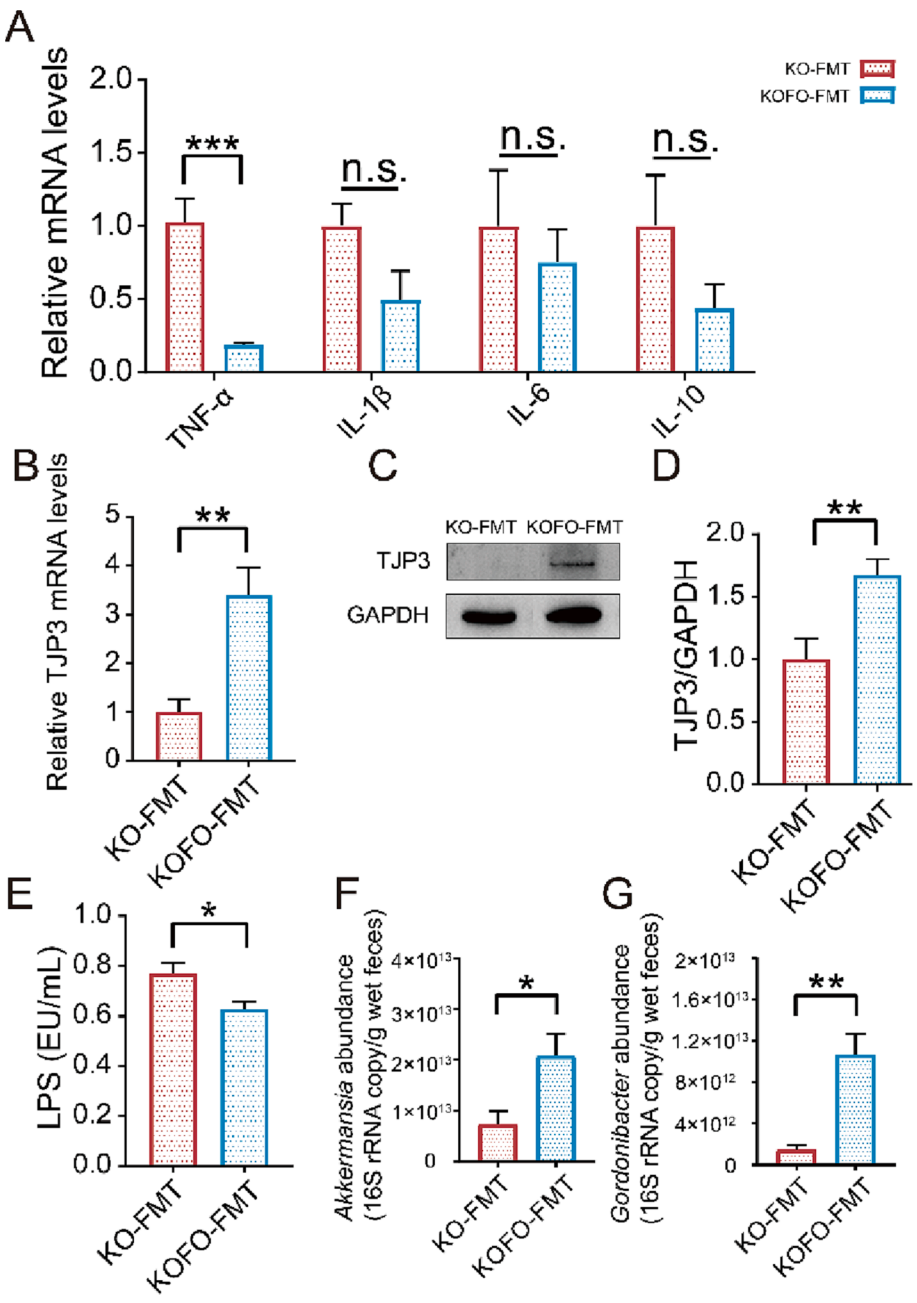

Fig. 6. Fecal microbiota transplantation (FMT) derived from FO-fed *Fmr1* KO mice improves intestinal homeostasis and increases gut abundance of *Akkermansia* and *Gordonibacter* in recipient *Fmr1* KO mice. (A) Quantitative real-time PCR analyses of *mRNA* expression levels of pro-inflammatory factors, such as *TNF-α*,*IL-1β*,*IL-6* and *IL-10* in colon tissues from *Fmr1* KO mice administered with FMT from either vehicle- or FO-fed *Fmr1* KO mice (N = 6–8 mice per group). (B) Quantitative real-time PCR analyses of *TJP3* mRNA level in colon tissues from *Fmr1* KO mice administered with FMT from either vehicle- or FO-fed *Fmr1* KO mice (N = 8 mice per group). (C-D) Western blot analyses of TJP3 protein expression in colon tissues from *Fmr1* KO mice administered with FMT from vehicle- or FO-fed *Fmr1* KO mice (N = 3 mice per group). (E) Quantitative comparison of serum endotoxin levels in colon tissues from *Fmr1* KO mice administered with FMT from vehicle- or FO-fed *Fmr1* KO mice (N = 6 mice per group). (F) Quantitative comparison of the abundance of *Akkermansia* in colon tissues from *Fmr1* KO mice administered with FMT from vehicle- or FO-fed *Fmr1* KO mice (N = 5 mice per group). (G) Quantitative comparison of the abundance of *Gordonibacter* in colon tissues from *Fmr1* KO mice administered with FMT from vehicle- or FO-fed *Fmr1* KO mice (N = 5 mice per group). *p < 0.05, **p < 0.01, and ***p < 0.001; n.s., no significant difference.

High omega-6/omega-3 ratio in the blood has been reported in patients with psychiatric disorders (Stevens et al., 2003; Stevens et al., 1995). Omega-3 PUFAs as nutritional supplements can improve inflammation and ASD symptoms (Chang and Su, 2020). In clinical trials, the commonly used dosages of PUFAs range between 1.3 and 1.5 g/day and the duration of treatment ranges from 6 to 24 weeks (Cheng et al., 2017). For instance, a 6-month, randomized, placebo-controlled trial of omega-3 fatty acid supplements (1.5 g) was conducted to determine whether nutritional supplement can effectively improve the severity of autism symptoms and externalize symptoms in young children with autism (Mankad et al., 2015). However, in another trial, a group of young children with autism were randomized to receive dietary DHA supplementation of 200 mg/day or a placebo for 6 months and their behaviors were evaluated (Voigt et al., 2014). Despite this promising evidence, randomized controlled trials examining the effect of PUFAs in reducing symptoms of ASD have yielded inconclusive results due to the inadequate sample size and small overall effects of studies. Therefore, large-scale randomized clinical trials are still needed to elucidate the effects of PUFAs on ASD (Cheng et al., 2017; Mazahery et al., 2017).

A previous study found that gut microbial dysbiosis in juvenile mouse was associated with important morphological and functional changes in the enteric nervous system (ENS), and resulted in occurrence of gastrointestinal disorders (Yang et al., 2010). Changes in intestinal microbiota in early life alter the expression of molecular factors involved in neural development in both the ENS and central nervous system (CNS). In some clinal trials, microbiota transfer therapy has shown good therapeutic effects on children with autism including improving gastrointestinal and autism symptoms (Kang et al., 2019; Kang et al., 2017). Furthermore, recent studied based on animal models have shown that therapeutic interventions during sensitive periods, such as juvenile stage, can ameliorate the development of specific pathologies associated with behavioral outcomes (Marin, 2016). Since gut microbiota potentially plays a crucial role in the immunologic response of the host during

early life (Marrs et al., 2021), PUFAs supplementation at an early age can be effective in modulating gut microbes. Thus, juvenile mice were fed on PUFAs derived from the FDA-recommended dosage for human about 1.5 g/day using the Meeh-Rubner formula for 4 weeks (Nishi et al., 2016). Since different concentrations of PUFAs may have different effects on gut microbiota and cognitive function in individuals with autism of different ages, future studies are needed to further investigate the therapeutic effects of PUFAs in FXS or ASD.

Transplantation of gut microbes from WT mice improved autistic behaviors in *Fmr1* KO mice by increasing the abundance of *Akkermansia muciniphila* in gut (Goo et al., 2020). *A. muciniphila* belonging to the phylum Verrucomicrobia is a promising candidate for use as a probiotic for inhibiting intestinal inflammation (Goo et al., 2020; Wu et al., 2021). *Fmr1* KO mice exhibited neuroinflammation as evident by increased levels of pro-inflammatory cytokines in the prefrontal cortex (PFC) and hippocampus (Pietropaolo et al., 2014). Moreover, compared with WT microglia, FMRP-deficient microglia produce significantly higher levels of pro-inflammatory cytokines following LPS stimulation (Altimiras et al., 2021). Similarly, acute elevation in serum levels of IL-1β and IL-6 has been reported in *Fmr1* KO mice at 4 h post-LPS exposure (Hodges et al., 2020). Gut microbial dysbiosis causes elevation in LPS levels which may trigger inflammation in *Fmr1* KO mice, whereas increased abundance of *Akkermansia* might inhibit LPS-induced inflammation in these mice. Furthermore, the gut abundance of *Akkermansia* has been shown to suppress inflammation and increase levels of SCFA (Lee et al., 2022). Disruption of GI function can cause systemic inflammation and trigger cognitive impairment (Gampierakis et al., 2021; Yang et al., 2019). Although this study demonstrates that FMT from FO-fed *Fmr1* KO mice rescues cognitive deficits in recipient *Fmr1* KO mice by improving gut homeostasis, direct evidences to support the role of gut dysbiosis in triggering inflammation within the brain, and its influence on autistic behaviors in FXS or ASD is lacking, and need to be further investigated.

It has been shown that PUFAs-rich diet improves cognition and behavioral deficits in individuals with autism, characterized by enhanced atypical brain plasticity, including synaptic transmission and neurotransmitters release (Baron-Mendoza and Gonzalez-Arenas, 2022). Exposure to PUFA can increase adult hippocampal neurogenesis which alters cognitive outcomes and synaptic plasticity by improving long-term potentiation (LTP) and regulating expression of synaptic proteins to stimulate the dendritic arborization and formation of new spines (Crupi et al., 2013). Gut microbiome has been implicated in hippocampal neurogenesis, which contributes to the pathogenesis of neurological disorders (Dohm-Hansen et al., 2022). Our previous studies revealed that aberrant hippocampal neurogenesis can lead to FXS cognitive impairment (Li et al., 2016; Li et al., 2018; Liu et al., 2018). In the present study, oral FO supplementation improved autistic behaviors in *Fmr1* KO mice. Furthermore, FO positively modulated gut microbiota in *Fmr1* KO mice by reducing intestinal inflammation. FMT from FO-fed *Fmr1* KO mice also induced similar effects on recipient *Fmr1* KO mice. Considering the lack of evidence to support the direct links among PUFA administration, gut microbiota-regulated hippocampal neurogenesis and etiology or pathophysiology of FXS, future studies should explore how current discoveries comprise circumstantial links for clinically relevant research of neurodevelopmental disorders and FXS in humans.

In summary, this study reveals that gastrointestinal disturbance in a Fragile X mouse model results in gut microbiota dysbiosis and intestinal inflammation which affects the initial gut microbiota composition and gut homeostasis. Supplementation of omega-3 PUFAs during early life restores the balance in gut microbiota and ameliorates autistic behaviors, providing a promising therapeutic strategy for FXS (Fig. 7).

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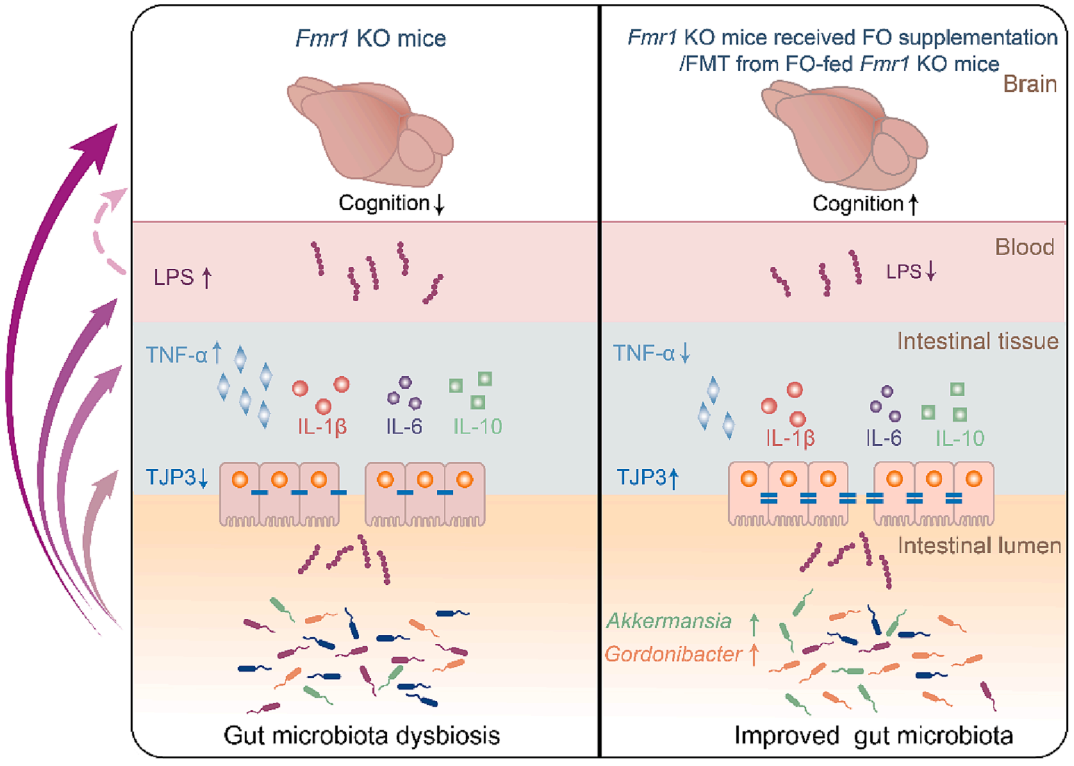

Fig. 7. The proposed mechanism underlying the regulatory effects of FO and/or FMT on the composition of gut microbiota in relation to cognition in the FXS mouse model.

Declaration of Competing Interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

Data availability

Data will be made available on request.

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Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.bbi.2023.02.019.

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