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Fructooligosaccharide modulates the intestinal exosomal miRNAs to reshape the gut microbiota

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ABSTRACT: Fructooligosaccharide (FOS), a well-recognized prebiotic, is generally considered non-absorbable in the upper gastrointestinal tract, allowing it to reach the colon and exert its prebiotic effects. In this study, we uncovered a novel function of FOS: it directly modulates the expression profile of miRNAs in exosomes derived from intestinal epithelial cells (IECs) and in the feces of antibiotic-induced pseudo-germ-free (PGF) mice, indicating that FOS-mediated regulation of intestinal exosomal miRNAs is independent of its prebiotic role. Furthermore, these miRNAs modified by FOS in turn reconstruct the gut microbial structure, notably elevating the relative abundance of *Eubacterium*, a component of the core human gut microbiome that is beneficial to gut health. Specifically, supplementation with FOS at 0.6 and 1.2 g/kg/d significantly reduced fecal miR-690 expression in PGF mice by 4.3-fold (from 768.3 ± 92.0 to 177.0 ± 87.5) and 7.8-fold (to 98.7 ± 12.4), respectively ($P < 0.05$). Subsequent *in vitro* co-culture experiments demonstrated that synthetic miR-690 mimics (2.5 nM) selectively inhibited the growth of *Eubacterium limosum*. Our findings provide new insights into a novel molecular mechanism by which FOS reshapes the gut microbiome through the regulation of exosomal miRNA expression in IECs.

Key words: Fructooligosaccharide, intestinal exosomal miRNA, miR-690, gut microbiota, *Eubacterium*

1. Introduction

The gut microbiota, the most complex micro-ecosystem in the human body, maintains dynamic equilibrium in healthy individuals[1,2]. Recent genomic and metabolomic studies have revealed that it plays a pivotal role in host health by regulating nutrient metabolism and immune responses[3,4]. Imbalances in intestinal microecology may lead to IBD, neurological, and autoimmune diseases[5-8]. Numerous studies have established that dietary habits are a key determinant of gut microbiota composition and structure, which in turn regulates critical bodily functions and overall health[9-12].

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Functional oligosaccharides (FOs), composed of 2-10 monosaccharides linked by glycosidic bonds, are resistant to human digestive enzymes. They pass through the upper gastrointestinal tract intact, reaching the colon where they selectively promote the growth of probiotics like *Lactobacillus* and *Bifidobacterium*[13,15]. As prebiotics, FOs serve to shape the gut microbiota and contribute to intestinal health by promoting a balanced microbial community[13-15]. Fructooligosaccharide (FOS), a naturally occurring member of the FO family, is not digested in the small intestine but induces a dose-dependent increase in *Bifidobacterium* levels[15,16]. FOS also restores gut microbiota homeostasis and reduces inflammation in patients with ulcerative colitis (UC) by enhancing short-chain fatty acid (SCFA) production[17,18]. Owing to these benefits, FOS has become a common additive in various food products and infant formulas[19].

Exosomes, 30-150 nm extracellular nanovesicles secreted by various live cells, carry bioactive components like proteins, nucleic acids, and lipids, particularly microRNAs (miRNAs)[20-24]. The discovery of miRNAs was recognized with the 2024 Nobel Prize in Physiology. miRNAs, a type of non-coding small-molecule RNAs with a length of 18-25 nucleotides, regulate gene expression by inhibiting its transcription or translation after binding to the 3'-untranslated region (3'-UTR) of target mRNAs[25,26]. Liu et al. found that intestinal epithelial cells (IECs) are a main source of exosomal miRNAs in feces, which can enter gut bacteria, specifically regulating their gene expression and growth[27]. Some specific miRNAs have been shown to alleviate colitis, and oral transfer of fecal miRNAs from colitis-recovered mice prevented DSS-induced colitis in a microbe-dependent manner[28-30]. Together, these findings demonstrate that IEC-derived miRNAs functionally shape the composition of the gut microbiota.

Is there a regulatory axis between functional oligosaccharides, small intestinal epithelial cell miRNAs, and the gut microbiota? Intriguingly, our recent work showed that stachyose, a tetrasaccharide naturally abundant in vegetables, beans and other plants, alters the profile of IECs-derived exosomal miRNAs independently of its prebiotic role, and these miRNAs were then released into the colon lumen to reshape the gut microbiota[31]. However, it remains unknown whether this mechanism extends to other FOs. To address this issue, this study focused on FOS, a typical functional oligosaccharide, and investigated the interactions between FOS, intestinal exosomal miRNAs and gut microbiota. The impacts of FOS on the intestinal exosomal miRNAs were assessed in pseudo-germ-free mice (PGF mice). The regulatory effects of FOS-modified intestinal exosomal miRNAs on the gut microbiota were assessed via fecal miRNA transplantation. This study provides additional scientific evidence for expanding the nutritional theory of FOs.

2. Materials and methods

2.1 Cell culture and MTT assay

Mouse small intestinal epithelial MODE-K cells were cultured in Dulbecco's modified Eagle's medium (DMEM) containing 10% fetal bovine serum (FBS) and 1% penicillin/streptomycin (Pen/Strep), and then maintained at 37°C with 5% CO_2. The cells were sub-cultured every three days with trypsin-EDTA. The FOS (Cas No. 308066-66-2) used in this study was obtained from Shanghai yuanye Bio-Technology Co.,

Ltd. (Shanghai, China). The FOS composition consisted of 1-kestose (58.1%), nystose (35.3%), and fructosylnystose (3.0%), with an average molecular weight of 560 Da.

Cells in the logarithmic growth phase were digested, resuspended, and seeded into 96-well plates at a density of 1.2 × 10^5 cells/mL (200 μL per well). The experimental design comprised a negative control group, a blank control group (without cells), and seven FOS-treated groups (0.05, 0.1, 0.2, 0.4, 0.6, 0.8, and 1 mg/mL). Each group contained three replicate wells, and two identical plates were prepared. The culture medium was replaced with FOS-containing medium 24 h and 48 h prior to the end of the incubation period, and the plates were returned to the CO_2 incubator. After treatment, the supernatant was aspirated, and 90 μL of fresh complete medium along with 10 μL of MTT solution (5 mg/mL) were added to each well. Following 4 h of incubation, the liquid was removed, and 110 μL of formazan dissolution solution was added. The plates were gently shaken for 10 min to completely dissolve the formazan crystals. Absorbance was measured at 490 nm using a microplate reader.

2.2 Preparation and characterization of cellular exosomes

Cells were cultured in complete medium supplemented with 10% exosome-removed FBS for 24 h. The cell supernatant was collected and centrifugated at 2000 × g for 30 min, 10000 × g for 45 min at 4℃ to remove cell fragments and metabolites. Subsequently, the obtained liquid was filtered through 0.22 μm membrane. The cellular exosomes were then separated by ultracentrifugation twice at 120000 × g for 70 min at 4℃, resuspended in cold phosphate buffer saline (PBS), and stored at -80°C for later use. The morphologic characterization of exosomes was analyzed by Transmission Electron Microscopy (TEM, Hitachi HT-7700, Japan) and the size distribution was analyzed by Nano-315 flowcytometry N30E (NanoFCM INC., China).

2.3 Animal experimental design

The C57BL/6 mice of SPF grade (4 weeks old) from Laboratory Animal Center of Shaanxi Normal University were raised in standard cages under a 12h/12h light/dark cycle with controlled conditions (temperature: 22 ± 2°C; humidity: 45 ± 5%). All experimental procedures comply with the ARRIVE guidelines and were approved by the Regulations of Experimental Animal Administration of Shaanxi Normal University Committee on Animal Care and Use (approval number: 32272309).

1. FOS intervention experiment in PGF mice. A mixture of broad-spectrum antibiotics (100 mg/mL neomycin, 50 mg/mL Streptomycin, 100 mg/mL metronidazole, 1 mg/mL Bacitracin, 125 mg/mL ciprofloxacin, 100 mg/mL ceftazidime, 170 mg/mL gentamicin) were used to remove the gut microbiota during the whole experimental period[27]. After given antibiotics for two weeks, all mice were randomly assigned into three groups (n = 10/group): (1) antibiotics plus normal chow (Ab-NC); (2) antibiotics plus low dose of FOS (0.6 g/kg·bw, Ab-LF); (3) antibiotics plus high dose of FOS (1.2 g/kg·bw, Ab-HF). The recommended daily intake of fructooligosaccharides for a healthy adult weighing 70 kg is 10 g/d. After conversion, the intragastric dose of FOS for the recipient mice was 1.2 g/kg body weight. The removal efficiency of gut microbes was analyzed by qPCR analysis on the 16S constant region in mice feces. “The

removal efficiency of gut microbes was analyzed by qPCR analysis on the 16S constant region in mice feces. The 16S universal primer sequences are:

27F: 5'AGAGTTTGATCCTGGCTCAG3'

1492R: 5'TACGGTTACCTTGTTACGACTT3'.

2. Fecal miRNA transplantation experiment. All mice were given a mixture of antibiotics for two weeks. Then, the mice were allowed to spontaneously restore the gut microbiota and randomly assigned into three groups (n = 10/group): (1) transplantation of fecal miRNAs isolated from mice in Ab-NC group (miR-Ab-NC); (2) transplantation of fecal miRNAs isolated from mice in Ab-LF group (miR-Ab-LF); (3) transplantation of fecal miRNAs isolated from mice in Ab-HF group (miR-Ab-HF). The feces were freshly collected every day.

Throughout the experimental period, the body weight of the mice was recorded weekly. At the end of experiment, the cecum contents were collected and soaked in RNA storage solution for 16S rRNA gene sequencing analysis. The mouse colons were collected for sectioning and H&E staining.

2.4 Preparation of fecal miRNA

After FOS intervention for one week, mice feces were freshly collected every day. Fecal samples of equal weight (100 mg) were collected from each group to ensure consistency in miRNA extraction. Each sample was suspended in 1 mL of sterile water, thoroughly homogenized, and centrifuged at 2000 rpm for 3 min. The collected supernatant was heated at 80°C for 60 min to kill bacteria, and then centrifuged at 8000 rpm for 3 min. The supernatant with fecal miRNAs was then transferred to new tubes for later use[31].

2.5 qPCR analysis for 16S rRNA gene

Total RNA of the exosomes, cells and feces was extracted using TRIzol (CWBIO, China) as instructed, and the quality of the extracted RNA was assessed as A260/A280 ratio using NanoDrop One (Thermo Fisher Scientific, USA). The qualified RNA was reverse transcribed into complementary DNA using MightyScript First Strand cDNA Synthesis Master Mix (Sangon Biotech, Shanghai) and qPCRs were performed with the Cobas z480 analyzer (Roche, Switzerland) using 2×SG Fast qPCR Master Mix (Sangon Biotech, Shanghai). All samples were run in 96-well reaction plates, and data were analyzed according to the $2^{-\Delta\Delta Ct}$ method.

2.6 Small RNA sequencing

The cecal contents of mice or the exosomes of MODE-K cells were collected for Small RNA Sequencing. The Raw Data of the results were filtered to obtain high-quality Clean Data, and then the sequences with base length of 18-26 nt were screened for downstream analysis. The remaining sequences within a specific range were further filtered against various RNA databases (RFam database, Repbase database, etc.) to remove common RNA families and repetitive sequences to obtain valid data. The ACGT101-miR (LC Sciences, Houston, Texas, USA) was used to identify the miRNAs in valid data. The identified miRNAs were mapped to the miRbase database and its own reference genome, and then

quantified and normalized. Subsequent data processing and analysis were performed using standardized data.

2.7 miRNA quantification by qPCR

MODE-K cells-secreted exosomal miRNAs and fecal miRNAs were quantified using the All-in-one^{TM} miRNA qRT-PCR reagent kits (Gene Copoeia, USA) according to the instructions. The relative expression of miRNA was normalized to U6 levels and calculated by $2^{-\Delta\Delta Ct}$. The forward sequences of miRNA primers were designed by Sangon Biotech (Shanghai China), and the primer sequences are shown below (U6: 5'CAGCACAAAAGGAAACTCACC3', miR-690: 5'GCGCTAAAGGCTAGGCTCACAACC3', miR-182-5p: 5'GCTTTGGCAATGGTAGAACTCACACCG3', miR-125b-5p: 5'GCGCTCCCTGAGACCCTAACTTGTGA3', let-7a-5p: 5'CGGCCGGCTGAGGTAGTAGGTTGTA3')

2.8 Gut microbiota analysis by 16S rRNA gene sequencing

To decipher the composition of the gut microbiome, the cecal contents of mice were collected to sequence the V3-V4 hypervariable region of 16S rDNA. Total DNA in feces was extracted using the CTAB method according to the manufacturer's instructions. After confirming that the DNA samples were qualified, 16S rRNA gene sequencing was performed by Lianchuan Bio (Hangzhou, China). The Raw Data were spliced using overlap and were filtered to obtain Clean Data. Divisive Amplicon Denoising Algorithm 2 (DADA2) was used for length filtering and denoising to obtain the ASV feature table and feature sequence. Alpha diversity is mainly reflected by observed_species, shannon, chao1, pielou_e, simpson, goods_coverage and other indices to show species richness and evenness. Beta diversity was mainly analyzed by principal component analysis (PCA), Principal coordinate analysis (PCoA), Clustering analysis (Clustering analysis, $P < 0.05$). UPGMA were used to identify the differences between samples.

2.9 Prediction of miRNA target genes

Using the Omicstudio online analysis platform, two target prediction algorithms (TargetScan v5.0 and miRanda, 3.3a, miranda Energy ≤ -10, Targetscan Score ≥ 50) were used to identify the binding sites of bacterial mRNA towards miRNAs. The results based on the two algorithms were integrated and the intersection was taken. GO Terms and KEGG pathways of these miRNA-targeted sequences were also annotated.

2.10 Synthesis of miRNA mimics

Synthetic miRNA mimics of NC (UUGUACUACACAAAAGUACUG), miR-690 (UAAAGGCUAGGCUCACAACC) and miR-690 scramble (UAAACAACCUCAGGUAAACC) were provided by Sangon Biotech (Shanghai, China).

2.11 Bacterial strains and growth conditions

Eubacterium limosum (*E. limosum*, ATCC 8486) was grown in modified chopped meat medium (ATCC 1490, MingZhouBio, China) at 37°C in an anaerobic incubator (Don Whitley Scientific (DWS), 433 UK). The anaerobic environment consisted of a mixture of gases (80% N_2, 10% CO_2 and 10% H_2).

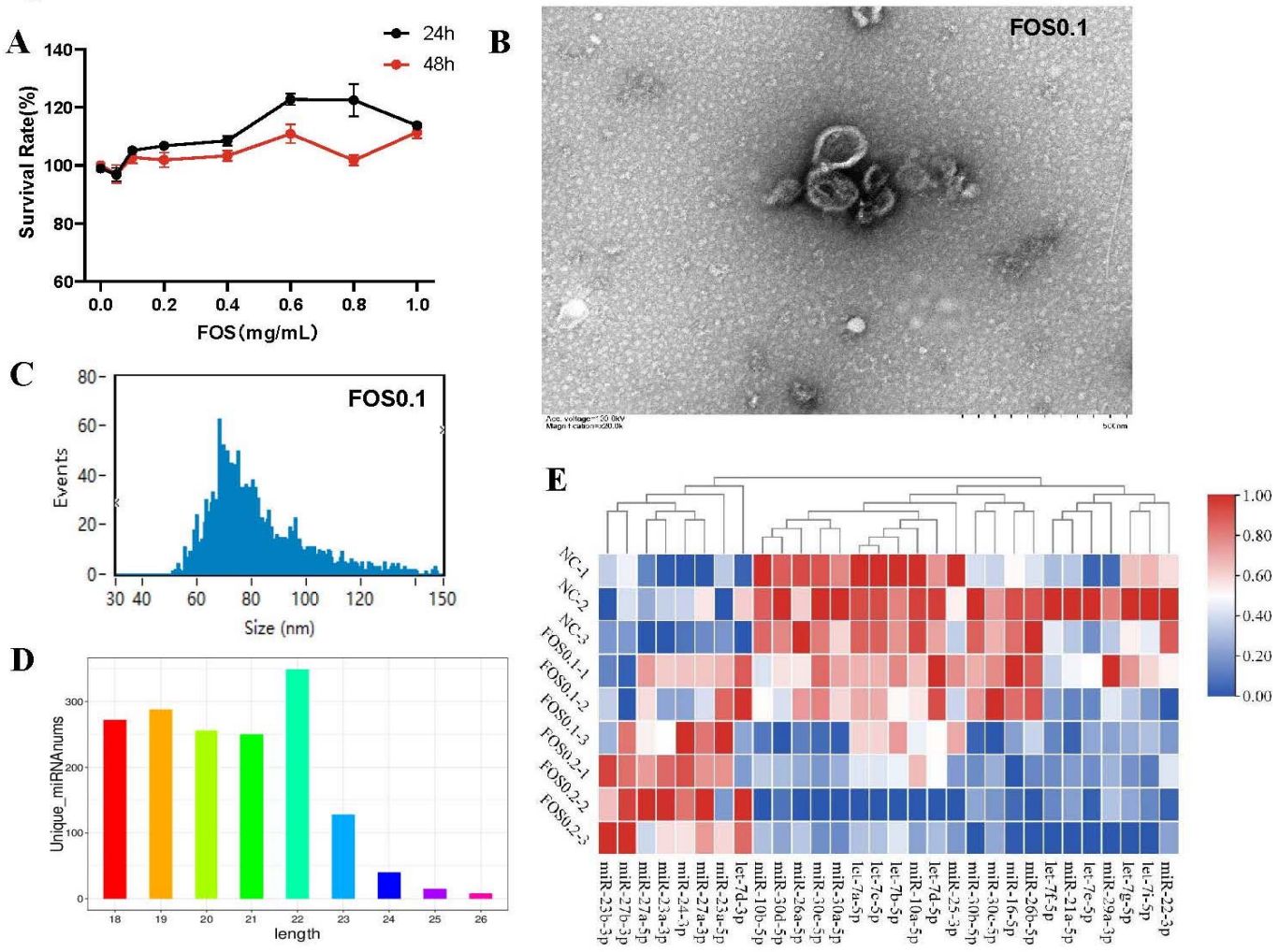
2.12 Statistical analysis

All data were presented as mean ± SEM for three or more biological replicates. 16S rRNA gene sequencing and Small RNA sequencing results were analyzed using the OmicStudio tools at https://www.omicstudio.cn/tool. After conducting the consistency of variance test, Duncan's multiple range test was used to compare the differences among various groups. Results were considered statistically significant at $P < 0.05$. Figures were made by GraphPad Prism 8.0 or TBtools.

3. Results

3.1 FOS alters the miRNA expression profile of MODE-K cells-released exosomes

As shown in MTT assay, FOS at the concentrations of 0.1 and 0.2 mg/mL were selected because they had no effect on the viability of MODE-K cells after 24 and 48 hrs culturing (**Figure 1A**). After intervening with 0.1 and 0.2 mg/mL of FOS for 24 hrs, we extracted the nanoparticles from MODE-K cells supernatant to perform TEM and nanoparticle tracking analysis (NTA). In TEM images, cup-shaped, exosome-liked particles with a bilayered membrane structure were observed (**Figure 1B and Figure S1A**). NTA anlysis confirmed that the size of these particles ranged from 40 to 150 nm (**Figure 1C and Figure S1A**). The above results indicated the successful isolation of exosomes.



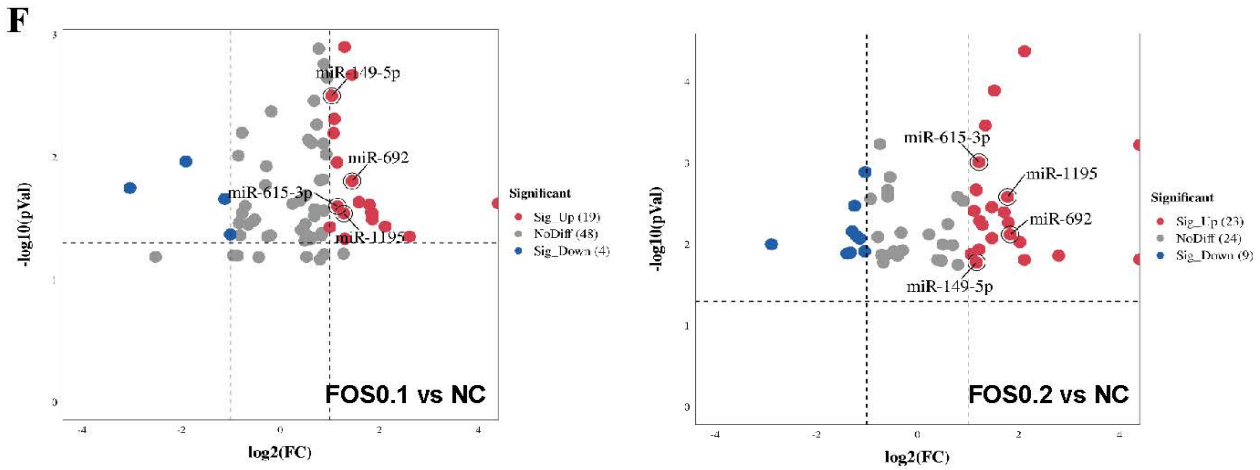


Figure 1. FOS alters the miRNA profile of exosomes released by MODE-K cells. (A) The survival rate of MODE-K cells in response to FOS intervention by MTT assay. Identification of exosomal vesicles secreted by MODE-K cells treated with 0.1 mg/mL FOS, shown as TEM (B) and NTA (C) results. (D) Length of miRNAs identified in the exosomes of MODE-K cells. (E) Profiling of exosomal miRNAs released by MODE-K cells after treated with 0, 0.1 and 0.2 mg/mL FOS based on the Small RNA sequencing results (n = 3). (F) Volcano plot of exosomal miRNA levels.

To investigate whether FOS alters the expression profile of exosomal miRNA in MODE-K cells, we performed Small RNA sequencing. Consistent with the canonical miRNA, the length of identified miRNAs is approximately 18 to 25 nucleotides (**Figure 1D**). A heatmap of highly expressed murine miRNAs (the relative expression > mean relative expression of all miRNAs) was generated to visualize miRNA expression profile. As shown in **Figure S1B and C**, both 0.1 and 0.2 mg/mL of FOS altered the exosomal miRNAs expression profile, with the higher dose of FOS inducing a more pronounced change (**Figure 1E**). By further restricting the selection criteria on Fold Change (FC) and P values ($|\text{Log}_2\text{FC}| > 1$, $P < 0.05$), the expression of 23 miRNAs (19 miRNAs were upregulated and 4 miRNAs were downregulated) responded differentially to 0.1 mg/mL FOS intervention, and 32 miRNAs (23 were upregulated and 9 were downregulated) responded differentially to 0.2 mg/mL FOS (**Figure 1F**). Especially, miR-692, miR-149-5p, miR-615-3p, and miR-1195 were all upregulated, showing a consistent variation trend under the intervention of 0.1 and 0.2 mg/mL of FOS. Accordingly, FOS intervention markedly alters the exosomal miRNA expression profile of MODE-K cells *in vitro*.

3.2 FOS modifies the host-originating fecal miRNA signature in microbiota-depleted mice

IECs are the main source of host-derived miRNAs in feces, and these miRNAs encapsulated in exosomes can be stably present in the intestinal contents[27]. Based on the pronounced expression variation of exosomal miRNAs we found *in vitro*, we further deciphered the fecal miRNA profile *in vivo*. Firstly, to exclude the prebiotic effects of FOS, we pretreated mice with antibiotics mixture to remove the intestinal bacterial[31]. Then the mice were gavaged with FOS at doses of 0.6 g/kg·bw (Ab-LF) and 1.2 g/kg·bw (Ab-HF) for 6 weeks (**Figure 2A**). During the experimental period, the body weights of all mice increased gradually (**Figure S2A-B**). The antibiotics treatment significantly reduced the fecal level of 16S rRNA (close to 0) in mice, suggesting that the PGF mouse model was successfully constructed (**Figure 2A**). And treatment of low and high doses of FOS result in more tightly arranged intestinal crypts, indicating the positive role of

FOS on the intestinal health (**Figure S2C**). Then we collected cecum contents to perform Small RNA sequencing. The sequencing results indicated that, independent of intestinal microbes, FOS altered the expression profile of mouse-derived miRNAs in feces (**Figure 2B and C**). When limited to the selection criteria of $|\mathrm{Log_2FC}| > 1$ and $P < 0.05$, we found that the miR-125b-5p was up-regulated, and the miR-690, let-7a-5p and miR-182-5p were down-regulated dose-dependently in response to FOS intervention (**Figure 2D**). Specifically, the relative expression level of miR-690 was remarkably downregulated from 768.3 ± 92.0 in Ab-NC group to 177.0 ± 87.5 in Ab-LF group and 98.7 ± 12.4 in Ab-HF group.

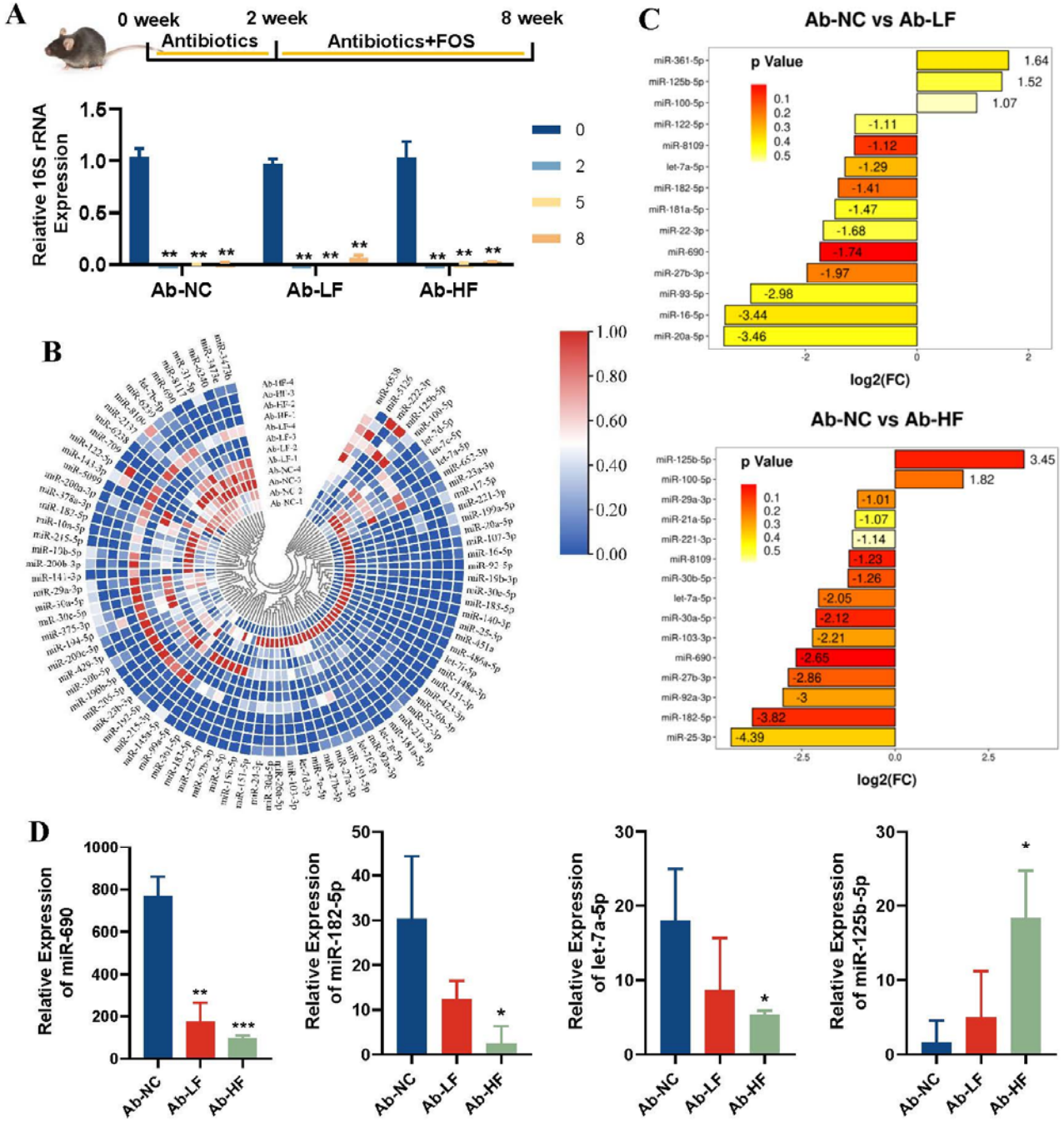
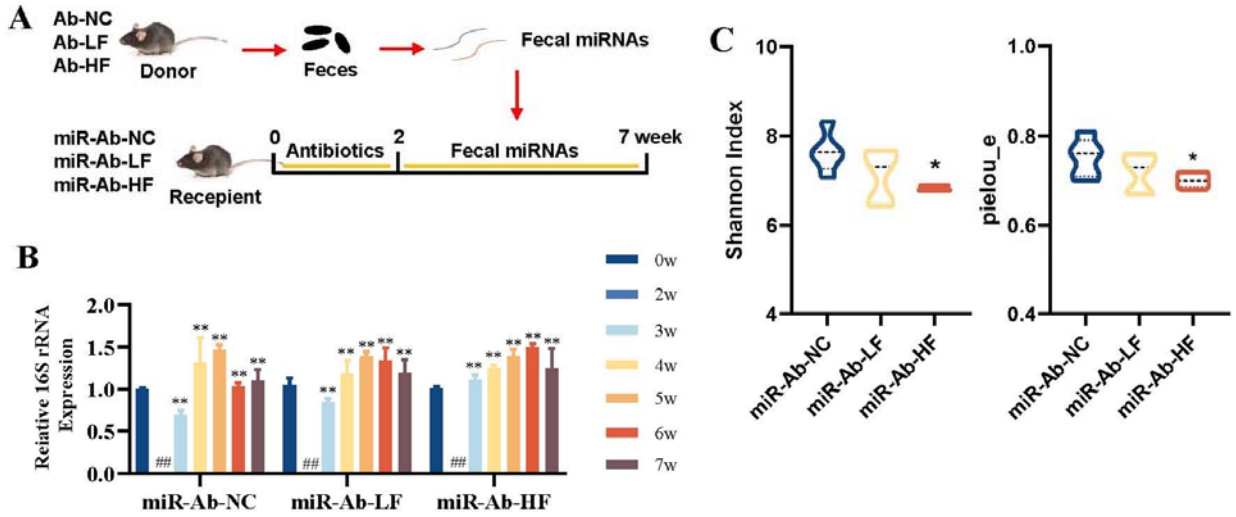


Figure 2. FOS reconstitutes the fecal miRNA profiles of PGF mice. (A) Relative expression of 16S rRNA gene in fecal samples from normal and antibiotics-treated PGF mice. The relative 16S rRNA level was calculated based on Ct values tested by qPCR analysis (n = 3) (**$P < 0.01$). (B) Fecal miRNA profile of PGF mice receiving 0 (Ab-NC), 0.6 (Ab-LF), and 1.2 (Ab-LF) g/kg·bw of FOS tested by Small RNA sequencing analysis (n = 4). (C) The fecal miRNAs that were significantly altered after FOS intervention, as compared to Ab-NC. (D) The significantly differentially expressed miRNAs with the same variation trend after both low and high doses of FOS intervention.

3.3 FOS-altered fecal miRNAs play a critical role in remodeling the gut microbiome of mice

Inspired by the fact that the host can shape the gut microbiota via the exosomal miRNAs in feces[27], we next evaluated the role of FOS-altered fecal miRNAs in the regulatory effects of FOS on the gut microbiome. To standardize gut microbiota composition across experimental subjects, we implemented a two-phase protocol (**Figure 3A**): First, mice were subjected to antibiotic pretreatment to eliminate inter-individual variations in gut microbial communities. Subsequently, during the phase of microbial recolonization, these pseudo-germ-free mice were transplanted with fecal miRNA extracts derived from FOS-treated donors through standardized microbiota transplantation procedures[27,30]. During the whole experimental period, the body weight of all mice gained steadily (**Figure S2A-B**). After treated with fecal miRNAs, the gut microbiota was successfully restored (**Figure 3B**). H&E staining revealed that, analogous to FOS, the fecal miRNAs derived from FOS-treated PGF mice elicited a tighter arrangement of colonic crypts, indicative of their beneficial impact on gut health (**Figure S2C**). To mechanistically interrogate the microbiome responsiveness to FOS-modulated fecal miRNAs, cecal luminal samples were aseptically harvested from recipient mice at the terminal experimental phase for 16S rRNA gene sequencing. More than 80,000 tags were detected for all samples. Analyses of α-diversity showed that, unlike the canonical prebiotic effect of FOS, a decrease of the richness and diversity of intestinal microbial communities in mice transplanted with FOS-altered fecal miRNAs were observed (**Figure 3C**). Principal component analysis (PCA) and Unweighted Pair Group Method with Arithmetic Mean (UPGMA)-based β diversity analyses revealed that the phylogenetic structure of mice (miR-Ab-LF, miR-Ab-HF) receiving fecal miRNAs from FOS-treated mice was different from that of mice (miR-Ab-NC) receiving fecal miRNAs from the control mice (**Figure 3D, E**).



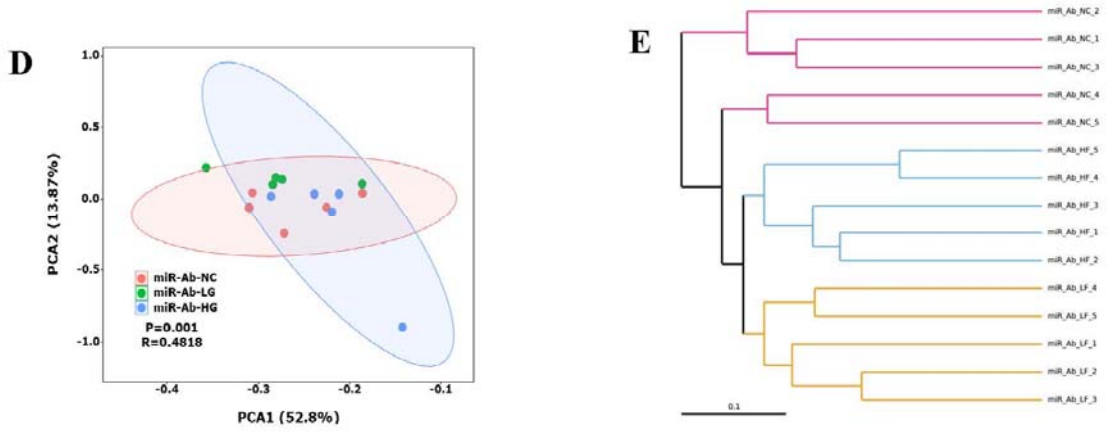


Figure 3. Structural changes in the gut microbiota of recipient mice after transplanted with FOS-altered fecal miRNAs. (A) After 2-week of pretreatment with antibiotics, fecal miRNAs collected from donor mice (Ab-NC/Ab-LF/Ab-HF) were transplanted into recipient mice (miR-Ab-NC/ miR-Ab-LF/ miR-Ab-HF). (B) Relative expression of 16S rRNA gene in fecal samples from recipient mice. The relative 16S rRNA level was calculated based on Ct values tested by qPCR analysis (n = 3). (##$P < 0.01$ compared with 0 w, **$P < 0.01$ compared with 0 w). After 5-week of FOS intervention, the feces of recipient mice were collected for 16S rRNA gene sequencing analysis. (C) Shannon index and pielou_e showed the α-diversity of gut microbiota. (D) Unweighted principal component analysis (PCA) and (E) Unweighted pair group method with arithmetic mean (UPGMA) analysis revealed the β-diversity of gut microbiota, and the scale means the self-expansion value.

To dig into the effects of FOS-altered miRNAs on the gut microbiota, we conducted a detailed analysis of microbial composition from the phylum to the genus level. At phylum level, 5 phyla with mean relative abundance > 0.5% were identified, in which Firmicutes was one of the dominant contributors **(Figure 4A)**. Analysis of bacterial composition at the genus level offered a deeper understanding of the effects of FOS-altered fecal miRNAs on the intestinal microbiota in mice (**Figure 4B**). Of the 429 genera detected in all mice, 14 genera with a statistical significance of $P < 0.05$ and a mean relative abundance > 0.1 were selected for further analysis (**Figure 4C**). Notably, 10 of these genera belonged to the phylum Firmicutes. Specifically, the relative abundance of beneficial genera such as *Eubacterium*, *Bacteroides* and *Rikenellaceae_RC9_gut_group* increased, while the detrimental genera, such as *Odoribacter* and *Oscillibacter*, decreased in response to FOS-altered fecal miRNAs (**Figure 4D**). Within these selected genera, *Eubacterium* displayed a consistent trend under the intervention of both doses of FOS and exhibited a particularly high relative abundance, which was elevated from 1.1 for the miR-Ab-NC group to 4.9 for the miR-Ab-LF group. *Eubacterium*, one of the core genera in the intestinal microbiota, plays a crucial role in maintaining intestinal balance and stability, preventing intestinal diseases, and participating in human metabolic processes[32-34]. Our data establish a pivotal role for FOS-modified fecal miRNAs in driving gut microbiota remodeling, evidenced by a marked increase in *Eubacterium* genera upon their administration.

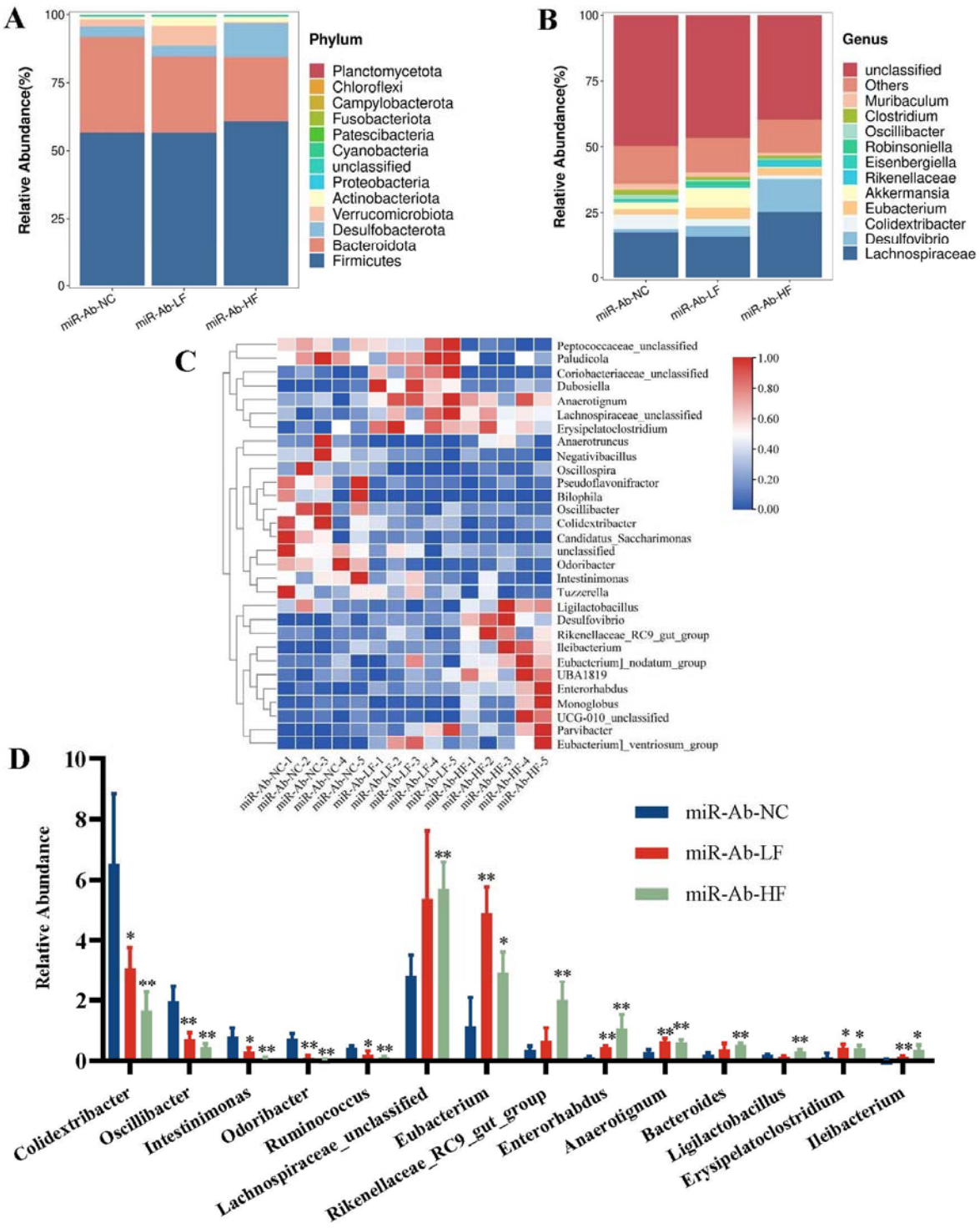


Figure 4. FOS-altered fecal miRNAs remodel the gut microbial composition of mice. Stacked bar graphs showing the relative abundance of different bacteria at the phylum (A) and genus (B) level (n = 5). (C) Heatmap of the top 30 genera that are shared by all groups at the genus level. (D) The relative abundance of 14 genera with significant changes in response to FOS-altered fecal miRNAs.

3.4 FOS-downregulated miR-690 inhibits the proliferation of E. limosum in vitro

It has been reported that miRNA could enter *Escherichia coli* thereby regulating the transcription of key genes and modulating the growth of bacteria[27,35]. Based on the small RNA sequencing results showing significant changes in miR-690, miR-182-5p, let-7a-5p and miR-125-5p, along with 16S rDNA sequencing identifying *Eubacterium* as one of the most responsive genera to FOS treatment, we next investigated which

specific miRNA might mediate the growth of *E. limosum* (a species of the genus *Eubacterium* that is well-known for its capacity to ameliorate colonic inflammation and bolster mucosal integrity)[36]. To address this issue, we firstly validated the impacts of FOS on the levels of miR-690, miR-182-5p, let-7a-5p and miR-125-5p (the 4 significantly altered miRNAs by FOS in PGF mice identified by small RNA sequencing) in the exosomes of MODE-K cells by qPCR assay. The results showed that the expression of miR-690, miR-182-5p and let-7a-5p were dose-dependently down-regulated by 0.1 and 0.2 mg/mL of FOS (**Figure 5A**). Then GO and KEGG enrichment analysis were performed based on miR-690, miR-182-5p and let-7a-5p. As shown in **Figure 5B**, these three miRNAs targeted in many signaling pathway such as Ras, mTOR, Neurotrophins and MAPK signaling pathway. KEGG pathway analysis further revealed potential functions related to membrane and protein binding, which are vital for bacterial survival (**Figure 5B**).

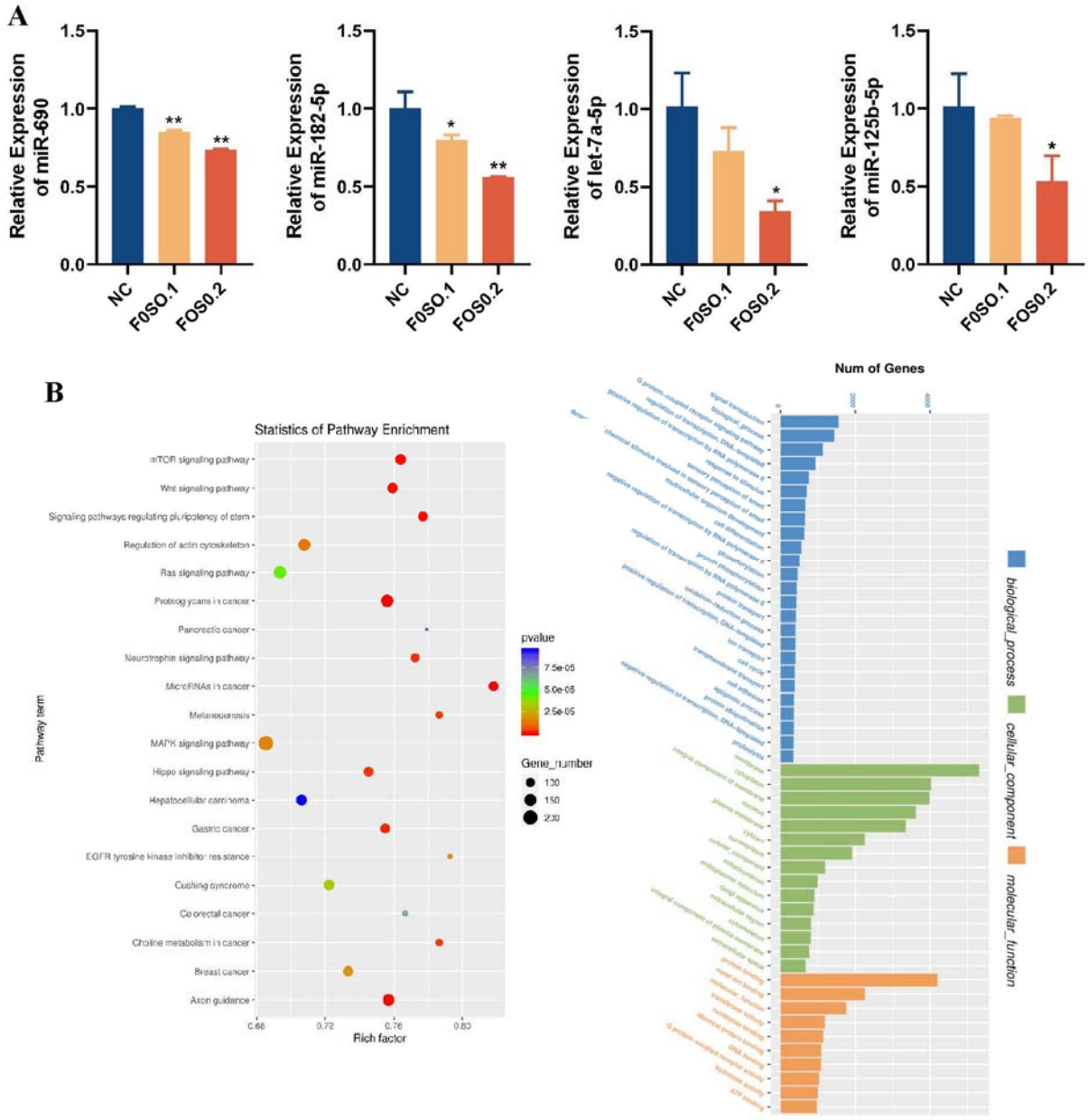
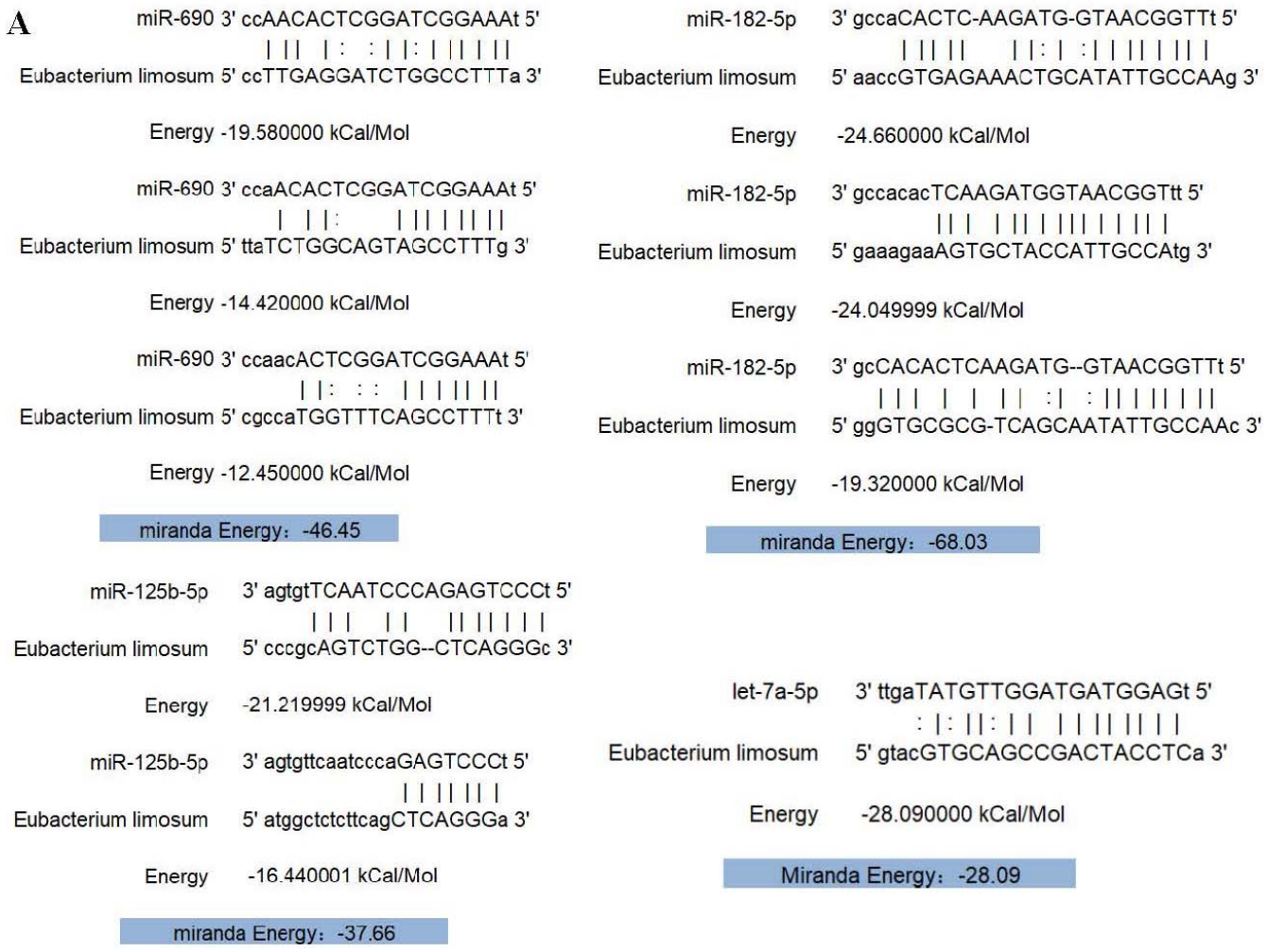


Figure 5. The potential correlation between FOS-altered fecal miRNAs and *E. limosum*. (A) The relative expression of miR-690, miR-182-5p, let-7a-5p, and miR-125-5p in MODE-K cells after 24 h of FOS treatment (0, 0.1,0.2 mg/mL). Targeting analysis of miR-690, miR-182-5p, let-7a-5p and miR-125b-5p toward *E. limosum* based on (B) KEGG pathway enrichment analysis and GO enrichment analysis.

Furthermore, as predicted by targetScan and miRanda database-based sequence alignment, miR-690 and miR-182-5p showed a high targeting potential towards the 16S mRNA of *E. limosum* (**Figure 6A**). Since the relative abundance of miR-690 in mouse feces was remarkably higher than that of miR-182-5p, we then focused on the potential relationship between miR-690 and *E. limosum*. Thus, we next synthesized miR-690 mimics in vitro and co-growth it with *E. limosum*. To establish the growth profile of *E. limosum*, we prepared series dilutions (10^1, 10^2, 10^3, 10^4, 10^5 and 10^6) and measured the growth curves of the strains under anaerobic conditions at 37°C (**Figure 6B**). As depicted in **Figure 6B**, the 10^1 dilution was identified as optimal, ensuring bacterial growth while clearly delineating the growth stages of the strains. Under the inoculation of 10^1, we found that 2.5 nM miR-690 mimics significantly inhibited the proliferation of *E. limosum.* Conversely, as a control, the scrambled miR-690 showed no discernible effect on the growth of *E. limosum*, indicating that the inhibitory effect of miR-690 on the proliferation of *E. limosum* is both targeted and sequence-specific (**Figure 6C, D**). Altogether, these findings suggest that FOS downregulate the expression of exosomal miR-690 in IECs to promote the growth of *E. limosum*, ultimately influencing intestinal health.



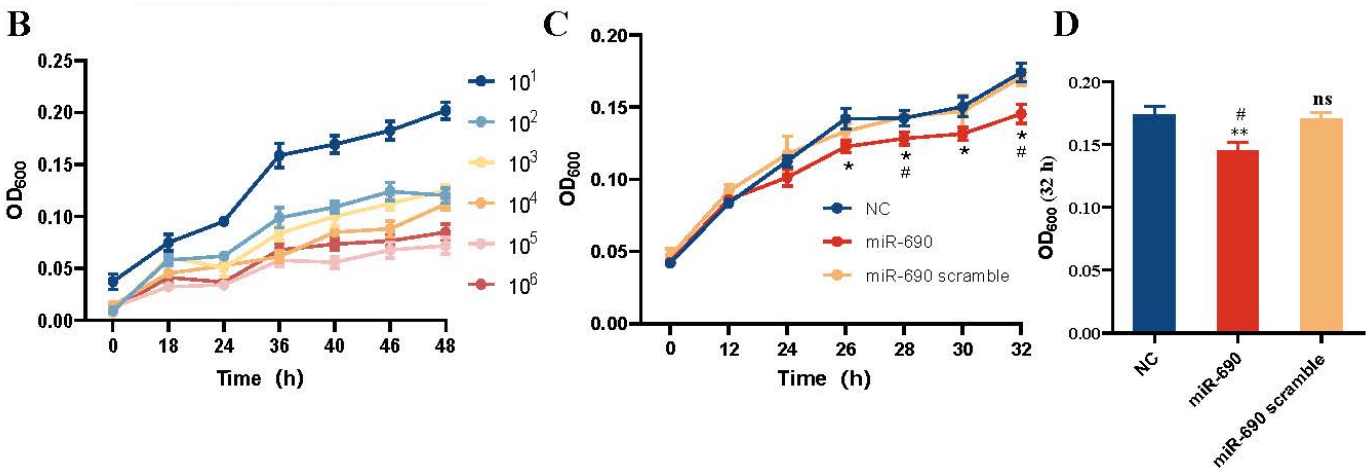


Figure 6. The targeting effects of miR-690 toward *E. limosum*. (A) The target sites of miR-690, miR-182-5p, let-7a-5p and miR-125b-5p on the 16S rRNA gene sequence of *E. limosum*. The interaction energy was measured as Targetscan score and Miranda energy. (B) Growth curves of *E. limosum* at different inoculum levels. (C) The growth curves of *E. limosum* under the dilution of 10^1, in the presence of 2.5 μM miRNA mimics or their scrambled control. Bacterial growth was monitored as absorbance at 600 nm (OD_{600}). (D) The growth status of *E. limosum* at 32 h. The values in the graph are expressed as the mean ± SEM of three samples from each group, and statistically analyzed using t-tests. *P < 0.05 compared with NC, #P < 0.05 compared with miR-690 scramble.

4. Discussion

The intestinal tract serves as a crucial organ for digestion and absorption within the human body, where the majority of nutrients undergo absorption and utilization. The gut microbiota, which establishes its presence even before birth, exhibits substantial individual variability and is highly susceptible to factors such as diet and lifestyle[37]. FOs, characterized by their low degree of polymerization, exert profoundly beneficial effects on human health. Common types of FOs encompass FOS, GOS, stachyose, raffinose, and others. Traditionally, FOs are believed to be indigestible and non-absorbable by the body, primarily serving as prebiotics to regulate the growth of beneficial bacteria. In this study, we suggest that FOS directly interacts with IECs, modifying the expression profiles of intestinal exosomal miRNAs in PGF mice, and these altered miRNAs in turn restructure the intestinal microbiome.

As a traditional functional oligosaccharide, FOS is extensively utilized in various food additives. Previous studies have demonstrated its efficacy in enhancing intestinal barrier function, mitigating intestinal inflammation, and regulating gut microbiota disturbances[15-18,38]. The mechanism underlying FOS's action involves its direct entry into the distal colon, where it serves as a substrate for specific intestinal microorganisms to produce beneficial fermentation products, thereby optimizing the intestinal environment. Intriguingly, we recently uncovered a new function of the oligosaccharide stachyose, which binds the hydrophobic residues of membranous heat shock protein (HSP) 90β on small IECs, thus reprograming the exosomal miRNA profile[31]. In recent years, accumulating studies have shown that exosomal miRNAs are able to influence the intestinal microbiota and regulate intestinal homeostasis[27,39,40]. André A. et al. found that host miR-21, which was overexpressed in the liver of mice undergoing bile duct ligation surgery, can inhibit the growth of *Lactobacillus reuteri* to promote liver dysfunction[41]. These findings inspired us to

explore the potential relationship between FOS, intestinal exosomal miRNAs and gut microbiota from *in vivo* and *in vitro* dimensions.

By conducting ultracentrifugation and Small RNA sequencing, we found that the intervention of FOS was capable of altering the expression profile of exosomal miRNAs in small intestinal cells. Subsequently, we established a group of PGF mouse models with intestinal bacteria depleted. Following a six-week period of gavage with FOS, the expression profile of miRNAs in the cecum contents of these mice changed significantly. These results demonstrated that, in the absence of intestinal bacteria, FOS intervention could directly affect the expression of IECs-released exosomal miRNAs in mice. Based on the FC value, P value, and the expression level, we then screened 4 miRNAs that exhibited significant differential expression in response to FOS intervention, namely miR-690, miR-182-5p, miR-125b-5p and let-7a-5p.

Exosomes, an emerging messenger of intercellular communication, have been shown to be able to transmit cargos to target cells[25]. It has been demonstrated that orally administered ginger-derived exosomal miRNAs can specifically target *Lactobacillus rhamnosus*[42]. To explore whether FOS-induced alterations in endogenous intestinal miRNAs influence the composition and structure of intestinal bacteria, we conducted an additional experiment using PGF mice to mimic the fecal microbiota transplantation (FMT) process. Specifically, we extracted fecal miRNAs from mice subjected to FOS interventions and administered them to recipient mice via gavage. Following five weeks of continuous gavage with these fecal miRNAs, we conducted 16S rDNA sequencing on the cecum contents of the recipient mice. Our findings revealed that FOS-altered fecal miRNAs had the capacity to reshape the gut microbial composition in mice, notably enhancing the proliferation of *Eubacterium*, a well-recognized next-generation probiotic that plays a pivotal role in protecting the intestinal mucosal barrier, regulating intestinal pH, and suppressing intestinal inflammatory response[34,43]. Indeed, *E. limosum* has been shown to alleviate experimental colitis in mice[36]. To further explore the specific miRNA who may specifically target *E. limosum*, we predicted the targeting relationship of the four screened differentially-expressed miRNAs towards *E. limosum*, and found that miR-690 and miR-182-5p showed a strong correlation with *E. limosum*. By co-culturing miRNA mimics and scrambles with *E. limosum* in vitro, we demonstrated that miR-690 significantly and sequence-specifically inhibited the proliferation of *E. limosum*. Thus, our findings suggest that FOS regulates the proliferation of *E. limosum* through the modulation of miR-690 expression in IEC exosomes.

In conclusion, our research provides a foundation for revealing the novel probiotic mechanisms of indigestible FOS. Specifically, FOS can directly communicate with IECs to alter the expression profile of their exosomal miRNAs, which are subsequently released into the intestinal lumen, thereby shaping the gut microbiota and promoting intestinal health. Notably, FOS-downregulated miR-690 significantly inhibits the proliferation of *E. limosum*. This newly discovered function contests the prevailing notion that oligosaccharides, as passersby of small intestine, traverse the upper gastrointestinal tract directly to the colon. Moreover, this innovative mechanism of FOS identified in our study underscores a fresh, active role for

exosomal miRNAs and also provides new perspectives and insights into exploring the "latent" nutritional functions driven by FOS in the upper gastrointestinal tract.

Supplementary material

The supporting information are available in “Supplementary materials”. The identification of exosome vesicles secreted by MODE-K cells and the differentially expressed exosomal miRNAs (Figure S1); and the basic physiological indicators during the animal experiment (Figure S2).

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.

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