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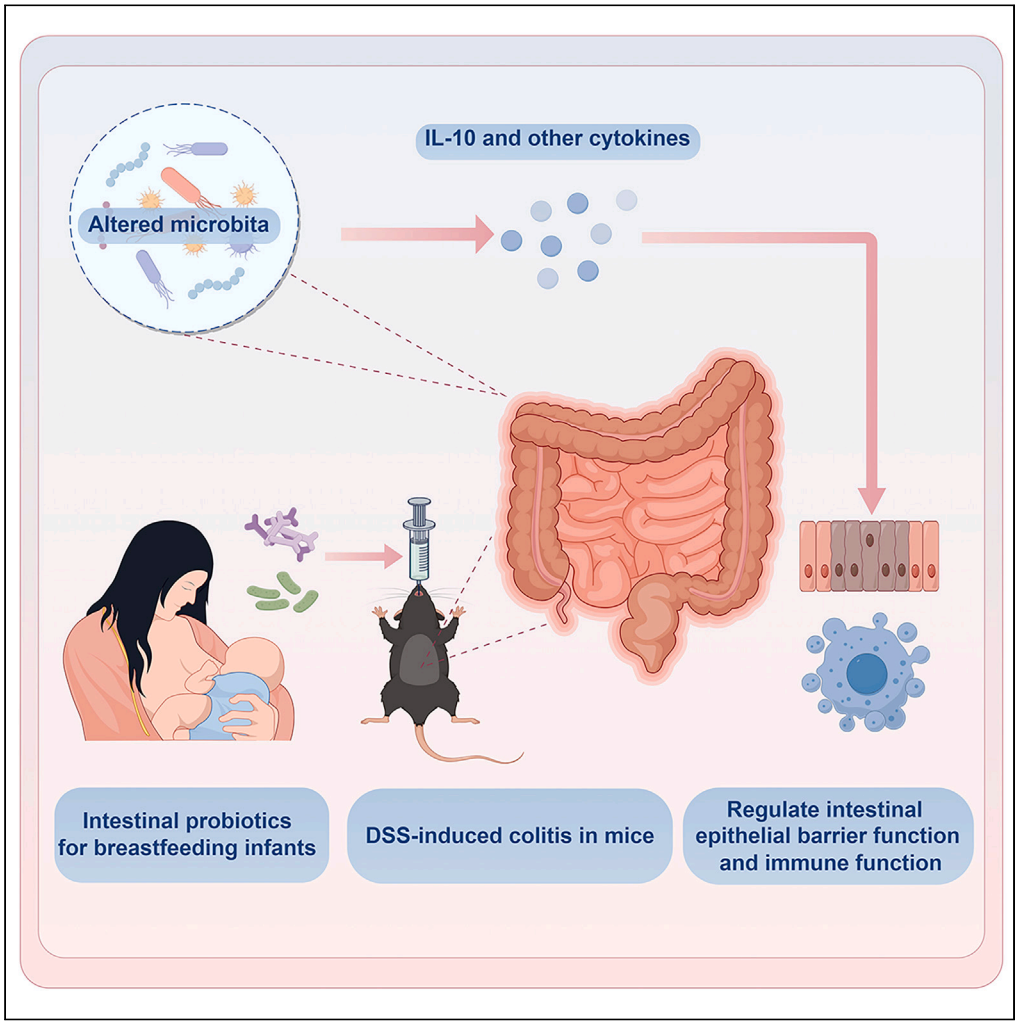


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## Highlights

New probiotic strains were isolated from feces of exclusively breastfed infants

Limosilactobacillus *reuteri* FPHC 2951 improved DSS-induced IBD symptoms

It can affect intestinal immunity and barrier function by regulating gut flora

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# Effects of breast-fed infants-derived *Limosilactobacillus reuteri* and *Bifidobacterium breve* ameliorate DSS-induced colitis in mice

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## SUMMARY

**Studies have shown that breastfeeding can reduce the risk and severity of inflammatory bowel disease (IBD) in children and adults. Probiotics in breast milk have also been isolated and their effects on IBD have been studied. However, based on current evidence, the exact efficacy and mechanisms of probiotics in the treatment of IBD cannot be determined. In this study, *Bifidobacterium breve* FPHC4024 (*BB* FPHC4024) and *Limosilactobacillus reuteri* FPHC2951 (*LR* FPHC2951) were isolated from feces of exclusively breastfed healthy infants and administered by gavage to dextran sulfate sodium (DSS)-induced IBD mice. The results showed that *LR* FPHC2951 improved the symptoms of DSS-induced IBD, increased the expression of interleukin (IL)-10 mRNA and upregulated the abundance of *Verrucomicrobiaceae Akkermansia*. Combined with Kyoto Encyclopedia of Genes and Genomes (KEGG)-based Phylogenetic Investigation of Communities by Reconstruction of Unobserved States (PICRUSt) function prediction results, we hypothesized that *LR* FPHC2951 improved DSS-induced colitis symptoms in mice by increasing of IL-10 mRNA, altering the structure of intestinal flora, and reducing proinflammatory pathways and enhancing pathways associated with anti-inflammatory and intestinal protection.**

## INTRODUCTION

Breastfeeding has always been considered as the most beneficial feeding method for the health and development of the offspring. In recent years, studies have confirmed that breastfeeding can improve the survival rate and health level of newborns, reduce the risk of infection and various developmental disorders. At the same time, some evidence supports the association between breastfeeding and intelligence, obesity, diabetes in adulthood.[1] Breast milk is the most important source of intestinal flora in infants, and the changes of intestinal flora in infants are related to the proportion of breastfeeding in a dose-dependent manner.[2] Studies have shown that infants who received their mothers' own breast milk had higher gut microbial diversity than those who received formula and donated milk,[3] and compared with preterm infants fed different proportions of infant formula, exclusively breastfed preterm infants had higher gut flora richness and greater differences in microbial composition, with formula-fed infants having higher levels of *Escherichia coli* and *Clostridium*.[4] Therefore, the composition of fecal microflora of breastfed infants is similar to that of breast milk.[5] The abundant microflora in breast milk can be vertically transmitted to the infant through the way of lactation, among which *Bifidobacterium* and *Lactobacillus* act as main probiotics, and together with human milk oligosaccharides (HMOs), promote the growth and colonization of gastrointestinal microbiota.[6–9] These probiotics provide many benefits for infants, including growth, development of lung, immune and nervous system, and a reduction in the risk of allergic diseases, above all especially the building of the intestinal barrier and regulation of the intestinal immune responses.[10]

Inflammatory bowel disease (IBD) is a chronic immune-mediated disease that affects the gastrointestinal tract. IBD consists of 2 subtypes: ulcerative colitis and Crohn's disease.[11] IBD is thought to result from the interplay of environmental, microbial, and immune-mediated factors in a genetically susceptible host.[12,13] Because of the important role of the microbiome in the pathogenesis of IBD,[14] approaches have been

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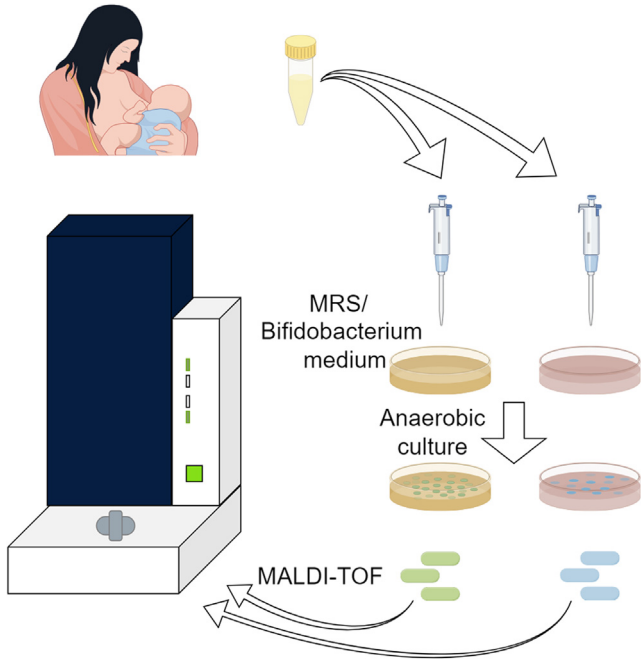


**Figure 1. Exclusive breastfeeding maternal and infant cohort bacteria identification process (by Figdraw)**

taken to modulate the gut microbiota to provide therapeutic benefits to IBD patients. A meta-analysis concluded that breastfeeding protects against the development of IBD,[15] and the application of probiotics was also confirmed to be beneficial to the maintenance of remission in patients with mild-to-moderate UC.[16]

Probiotic therapy is a natural, safe and useful intervention for the treatment of gastrointestinal disorders.[17,18] Among many probiotics, *Lactobacillus* and *Bifidobacterium* have particularly prominent probiotic functions. Previous studies have confirmed that *Bifidobacterium breve* has the functions of anti-infection, anti-depression, regulating host immune system, and promoting host nutrient absorption.[19] It has also been approved that *Limosilactobacillus reuteri* is beneficial for health, reducing infection, feeding tolerance, and could regulate immune responses of host, improve intestinal mucosal integrity, and reduce bacterial translocation.[20] Though breast milk has been proven to have good preventive and therapeutic effects on IBD,[21] there is still a lack of evidence to prove that breast milk-derived *Bifidobacterium breve* and *Limosilactobacillus reuteri* can have a beneficial effect on inflammatory bowel disease in adults. Previous studies have showed that mature human milk is a major driver of infant gut microbial development,[22–24] and considering the ability of strains to colonize the gut, we chose to isolate effective strains from the feces of exclusively breastfed infants to represent the strains from breast milk sources. Therefore, in this study, *Limosilactobacillus reuteri* and *Bifidobacterium breve* were isolated from feces of exclusively breastfed healthy infants to investigate the effects of these two probiotics on dextran sulfate sodium (DSS)-induced IBD and explore the possible mechanisms.

## RESULTS

### Bacterial isolation results of exclusive breastfeeding maternal and infant cohort

A total of 1,071 strains of bacteria were isolated from breast milk and meconium from a cohort of exclusively breastfed mothers and infants, and 780 strains belonging to the food additive genus, including *Limosilactobacillus reuteri* (173), *Lactobacillus crispatus* (86), *Lactobacillus gasseri* (192), *Lactobacillus salivarius* (51), *Lactobacillus rhamnosus* (73), *Lactobacillus paracasei* (14), *Bifidobacterium longum* (29), *Bifidobacterium bifidum* (4), *Bifidobacterium adolescentis* (3), *Bifidobacterium breve* (52), and *Bifidobacterium animalis* (103) (Figure 1; Table S4). To begin, acid tolerance, tolerance to bile salt and intestinal fluid, surface hydrophobic properties, and bacterial autoaggregation were assessed, and strains with the best overall performance were chosen for the strain adhesion test. Finally, the two strains with the best performance were chosen after a thorough evaluation of the aforementioned five indexes, namely *Bifidobacterium breve* FPHC4024 (*BB* FPHC4024) and *Limosilactobacillus reuteri* FPHC2951 (*LR* FPHC2951). (Figure S1; Table S5).

### *LR* FPHC2951 effectively alleviated the symptoms of DSS-induced colitis in mice

Male C57BL/6J mice at 15 weeks old were used for establish the DSS-induced colitis model, through being given 2% DSS in sterile water for 6 days and followed by recovery with regular drinking water for an additional 8 days. During the 14 days, mice in experimental group were fed with *BB* FPHC4024 or *LR* FPHC2951 via intragastric administration, and mice of control group were fed with skimmed milk. Body weight, stool consistency, and blood in feces were recorded daily throughout the experiment. On day 14, feces were collected for gut microbiota analysis

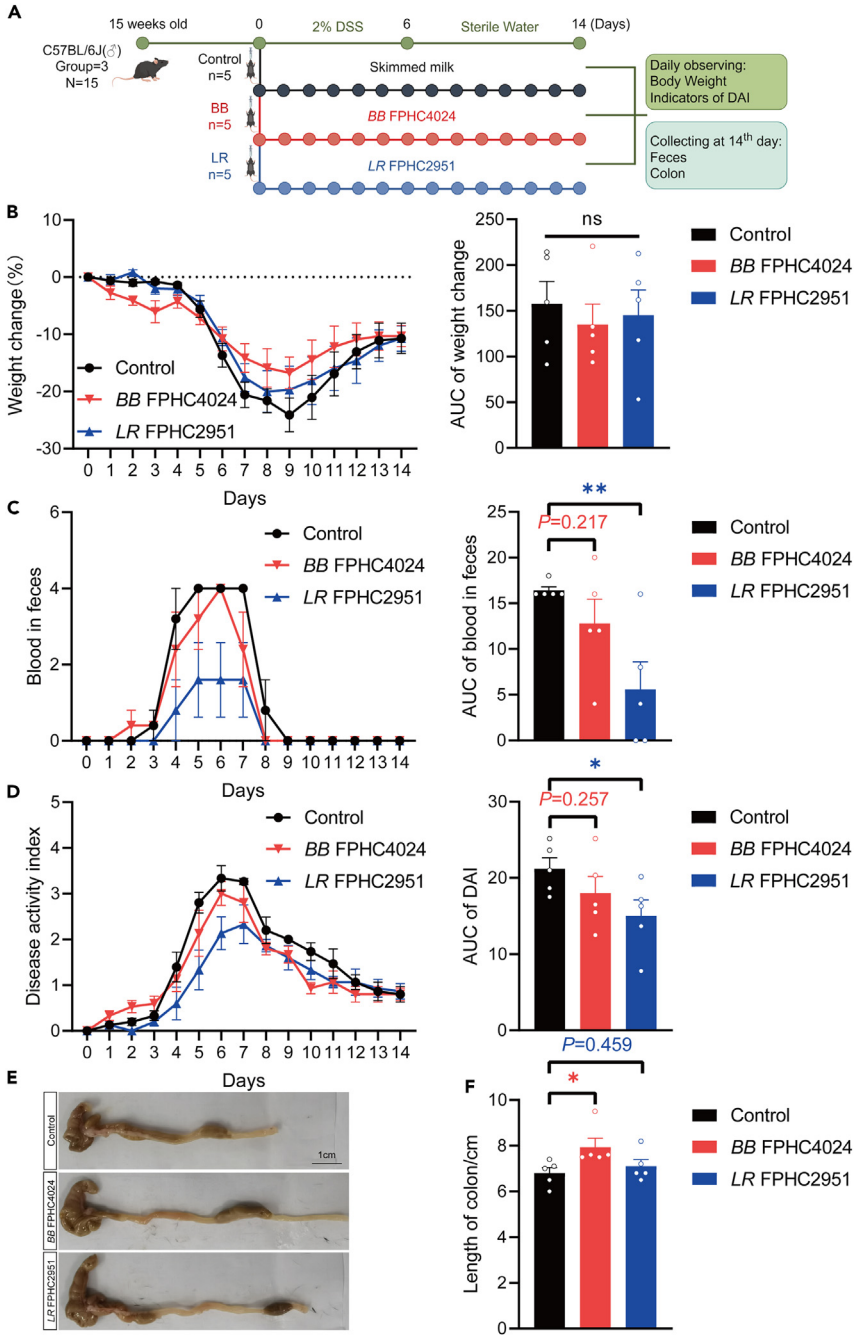


**Figure 2. *BB* FPHC4024 and *LR* FPHC2951 effectively alleviated the symptoms of DSS-induced colitis in mice**

(A) IBD model made by 2% DSS in mice and probiotic treatment process. By Figdraw.

(B–D) Daily weight change, blood in feces, disease activity index score, and area under the curve.

(E) Representative images of colons for each group.

(F) The length of colon from each group. Statistics were calculated with Student's t test. *, $p < 0.05$; **, $p < 0.01$. Data are presented as the mean ± SEM.

by 16S rRNA gene sequencing, and colon tissues were collected after mice were sacrificed (Figure 2A). Compared with the control group, there was no significant difference in weight change between the two probiotic groups (Figure 2B). The performance of blood in feces of *LR* FPHC2951 group was significantly better than that of the control group ($p = 0.007$) (Figure 2C). The DAI scores of of *LR* FPHC2951 group were also significantly lower than that of the control group ($p = 0.041$) (Figure 2D). Compared with the control group, the blood in feces ($p = 0.217$) and DAI score ($p = 0.257$) of the *BB* FPHC4024 group did not decrease significantly. *BB* FPHC4024 reduced the colon shortening

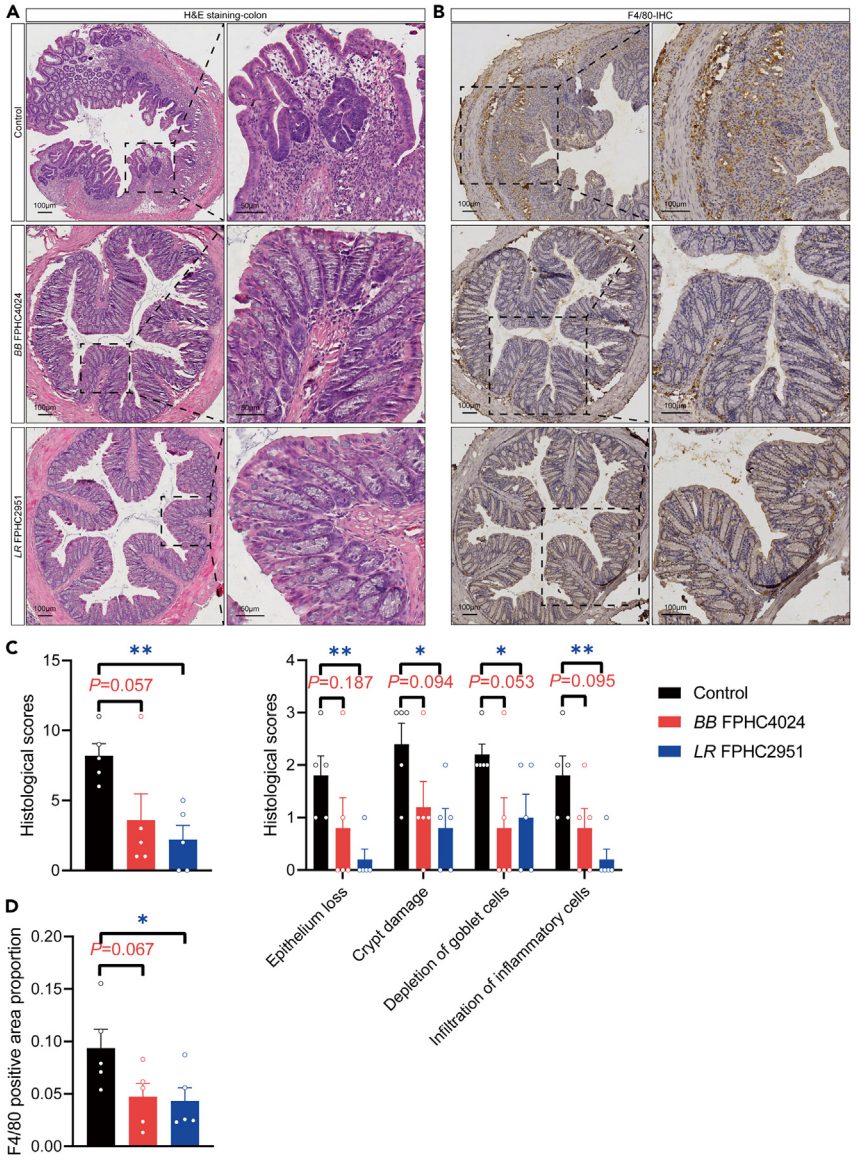


**Figure 3. *LR* FPHC2951 protected colon structure and reduced macrophage infiltration**

(A and C) Representative images of H&E staining and histology scores of colons.

(B and D) Representative images of F4/80 immunohistochemistry (IHC) and F4/80 positive area proportion of colon. Statistics were calculated with Student's t test. *, $p < 0.05$; **, $p < 0.01$. Data are presented as the mean ± SEM.

induced by DSS than the control group ($p = 0.039$), but there was no significant difference between *LR* FPHC2951 group and control group ($p = 0.459$) (Figures 2E and 2F).

## *LR* FPHC2951 reduced the histological damage to the colonic mucosa and macrophage infiltration

HE and IHC-F4/80 staining of colon tissues were adopted to evaluate the histological damage to the colonic mucosa. In the control group, the colon tissue sections showed obvious pathological damage, such as discontinuity of mucosal layer, loss of crypts, and infiltration of inflammatory cells (Figure 3A). *LR* FPHC2951 significantly reduced the histological score ($p = 0.008$) and macrophage infiltration than the control group (Figure 3C). *BB* FPHC4024 also reduced them though there was no significant difference ($p = 0.057$) (Figure 3C). F4/80-IHC staining showed that the infiltrated area of macrophages decreased after probiotic intervention (Figure 3B), and there was a significant difference between *LR* FPHC2951 group and control group ($p = 0.049$) (Figure 3D). However, *BB* FPHC4024 also reduced them but there was no significant difference ($p = 0.067$) (Figure 3D).

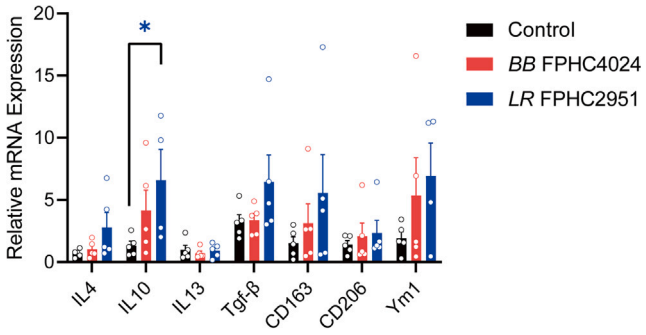


**Figure 4. *LR* FPHC2951 increased the expression of IL-10 mRNA**

Statistics were calculated with Student's t test. *, $p < 0.05$; **, $p < 0.01$. Data are presented as the mean ± SEM.

### *LR* FPHC2951 increased the expression of IL-10 mRNA

Expression of anti-inflammatory cytokines genes and intestinal barrier related genes were examined by qPCR as described previously. Compared with the control group, the expression of interleukin (IL)-10 in *LR* FPHC2951 group increased significantly ($p = 0.032$) (Figure 4). The expression of IL-4, transforming growth factor β (TGF-β), CD163, CD206, and Ym1 also increased in *LR* FPHC2951 group, though did not reach statistical significance (Figure 4). The expression of IL-10, CD163, CD206, and Ym1 showed a tendency of increasing in *BB* FPHC4024 group (Figure 4). In addition to the tendency for Cav1 to increase, there was no significant difference in the expression of other intestinal barrier related genes (Figure S2).

### *BB* FPHC4024 and *LR* FPHC2951 changed the structure of microbiota of DSS-induced colitis mice

We collected feces on day 14 for 16S rRNA sequencing and analyzed at the genus level. The top 10 bacterial genera with relative abundance are presented by a bar graph (Figure 5A). The results showed no significant difference in α-diversity (Shannon index) between the control and two probiotic intervention groups (Figure 5B). Furthermore, β-diversity principal coordinate analysis (PCoA) using the Bray-Curtis distance metric showed that the gut microbiota of the control group was clearly separated into distinct clusters from the *BB* FPHC4024 group and *LR* FPHC2951 group of bacterial communities (Figures 5C and 5D).

### Identification of signature gut microbiota in colitis mouse model by random forest

To explore the role of microbiota changes in the alleviation of enteritis symptoms, we used random forest analysis and 10 trials of 5-fold cross-validation (RFCV) to screen a number of potentially important key species (Figure 6A). The *Verrucomicrobiaceae Akkermansia* (V1) in *BB* FPHC4024 and *LR* FPHC2951 groups was significantly higher than that in the control group. The abundance levels of *Bacteroidia Bacteroidales* (V26), *Rikenellaceae Rikenella* (V42), and *Aerococcaceae Aerococcus* (V87) increased only in the *LR* FPHC2951 group (Figure 6B). Combining the microbiota structural changes and phenotypic data between groups, we plotted the co-occurrence network to explore the association between them (Figure 6C). Meanwhile, the microbiota associated with the phenotypic data were demonstrated by heat maps (Figure 6D). We found that *Verrucomicrobiaceae Akkermansia* was significantly negatively correlated with F4/80, DAI score, histological scores and stool consistency. In addition, *Rikenellaceae Rikenella* was positively correlated with the expression of TGF-β. Multiple genera such as *Enterobacteriales Enterobacteriaceae* (V5) and *Streptococcaceae Streptococcus* (V15) were significantly positively correlated with DAI score, histological scores, stool consistency and blood in feces.

### Changes in microbiota structure might ameliorate symptoms of DSS-induced colitis by regulating L-fucose degradation and sucrose degradation IV

Sankey map was used to analyze the multiple correlation between flora abundance, KEGG-pathway and phenotypic data. There were 10 bacteria genera that could be associated with the KEGG-pathway and phenotypic data, and only *Verrucomicrobiaceae Akkermansia* (V1) belonged to the core microorganism. L-fucose degradation (FUCCAT-PWY) and sucrose degradation IV (PWY-5384) have been associated with reduced IBD symptoms in previous studies.[25–27] We found that *Verrucomicrobiaceae Akkermansia* could alleviate F4/80, DAI score, histological scores and stool consistency by upregulating the FUCCAT-PWY and PWY-5384 pathways (Figure 7A). In order to explore the differences between the *BB* FPHC4024 and *LR* FPHC2951 groups, we performed multiple correlation analyses between the two probiotic groups and the control group, respectively. *Verrucomicrobiaceae Akkermansia* (V1) and *Bacteroidia Bacteroidales* (V26) in the *LR* FPHC2951 group belong to the core microorganisms and are positively correlated with FUCCAT-PWY. The pathway was negatively correlated with DAI score and blood in feces (Figure 7B). In the *BB* FPHC4024 group, only *Verrucomicrobiaceae Akkermansia* (V1) belonged to the core microorganism and was positively correlated with FUCCAT-PWY, which was negatively correlated with histological scores and stool consistency (Figure 7C).

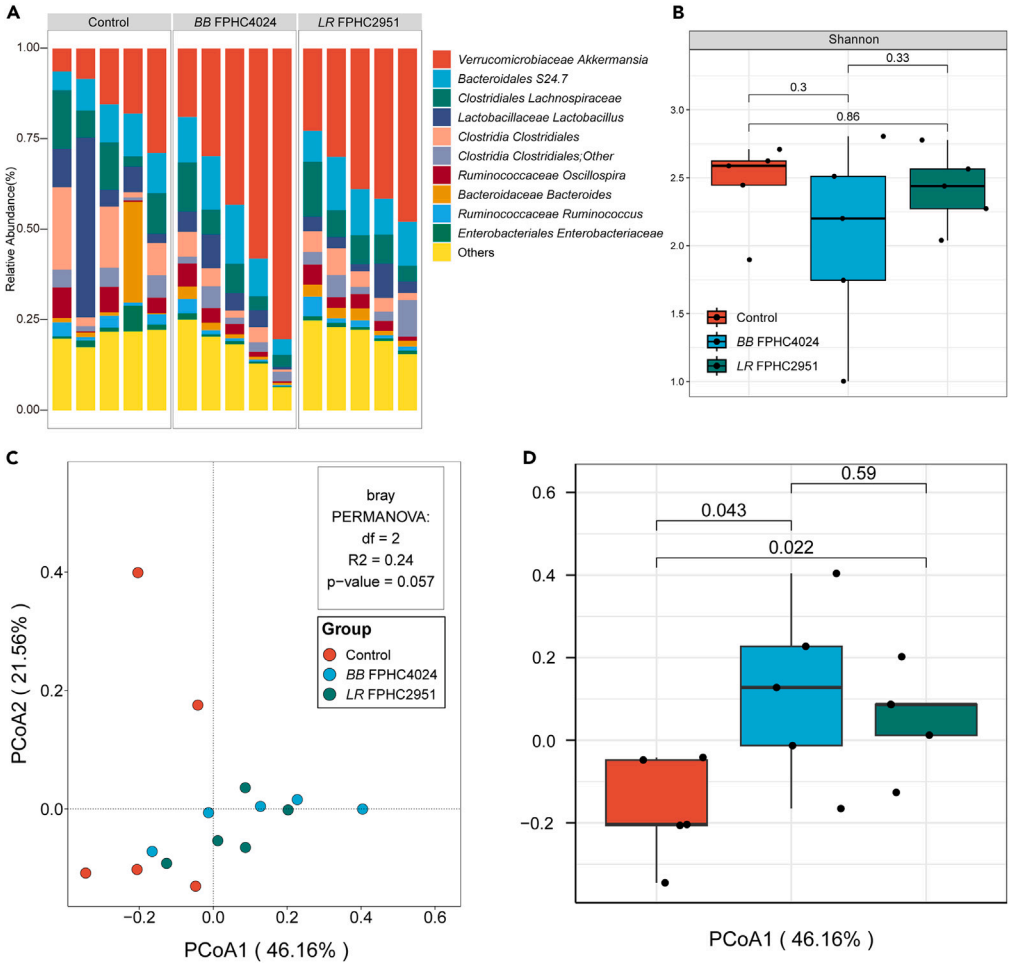


**Figure 5. *BB* FPHC4024 and *LR* FPHC2951 changed the structure of microbiota of DSS-induced colitis mice**

(A) Structure plot of the top 10 abundances at the genus level in three groups.

(B) α-diversity is represented by the boxplot of the Shannon index. Statistics were calculated with U test.

(C and D) The PCoA of β-diversity based on genus-level microbiota as assessed by a Bray-Curtis matrix between three groups. Data are presented as the mean ± SEM.

## *LR* FPHC2951 affected inflammatory pathways such as arginine succinyltransferase

The PICRUSt function predicts that differential analysis based on the KEGG KO module can help us more precisely target the genes that actually mediate colitis remission. *LR* FPHC2951 group might reduce the inflammatory response by reducing the enrichment of arginine succinyltransferase pathway and phosphatidylethanolamine (PE) biosynthesis pathway, and enhancing the enrichment of chondroitin sulfate degradation, cysteine biosynthesis, NADH: quinone oxidoreductase pathway to protect the intestinal barrier function (Figures 8 and S3A). Chondroitin sulfate degradation and cysteine biosynthesis were also enriched in *BB* FPHC4024 group (Figure S3B).

## DISCUSSION

In this study, we isolated and screened two strains, named *BB* FPHC4024 and *LR* FPHC2951, from feces of exclusively breastfed healthy infants and investigated the effects of these two probiotic strains on a DSS-induced IBD mouse model by gavage. We observed that *LR* FPHC2951 improved the symptoms of DSS-induced colitis in mice, including weight loss, blood stool, DAI score, histological damage, and macrophage infiltration, while *BB* FPHC4024 only improved colon shortening. The results confirmed the safety of two probiotic strains isolated from infant feces and the effectiveness of *LR* FPHC2951 against adult IBD, which will help to further understand the microorganisms in infants gut inherited from breast milk and identify strains with important probiotic effects for human health.

*Bifidobacterium breve* and *Limosilactobacillus reuteri* are common probiotics, and numerous studies have investigated their relationship with colitis as well. Clinical studies have shown that *Bifidobacterium breve* that produce conjugated linoleic acid can reduce intestinal mucosal damage and prevent further deterioration of DSS induced colitis.[28] Previous studies showed that deletion of CARD9 makes mice more

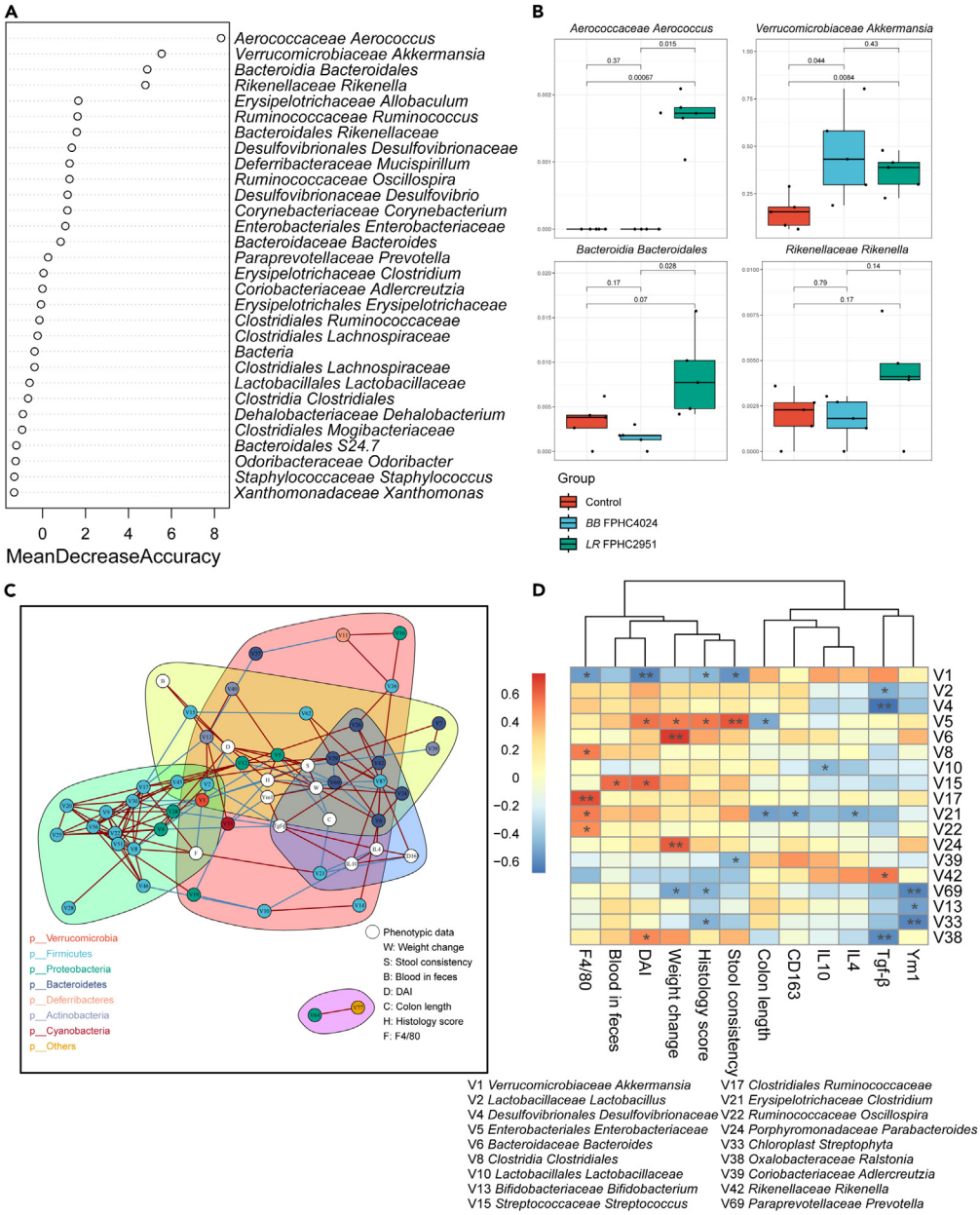


**Figure 6. Identification of signature gut microbiota in colitis mouse model by random forest**

(A) Mean decrease accuracy (MDA) was used to measure the relative abundance of each bacterium at the genus level in the predictive model.

(B) Relative abundance of four taxa in the two groups. Statistical analysis was calculated with a two-tailed Student's t test. *, $p<0.05$; **, $p<0.01$, ***, $p<0.001$.

(C) Cooccurrence network map captured the complexity of network interactions between gut microbiota and phenotypic data. Nodes were colored according to the phylum they belong to. Edges were estimated by Spearman's rank correlation coefficient, a red line between nodes represented a positive correlation, and a blue line represented a negative correlation ($p<0.05$).

(D) Heat maps showed correlations between microbial and phenotypic data. Edges were estimated by Spearman's rank correlation coefficient. *, $p < 0.05$; **, $p < 0.01$; ***, $p < 0.001$. Data are presented as the mean ± SEM.

susceptible to induced colitis due to altered microbiome resulting in impaired tryptophan metabolism.[29] However, CARD9$^{-/-}$ mice gavaged with *Limosilactobacillus reuteri* cultured from the intestinal microbiota of CARD9$^{+/+}$ wild-type mice showed increased AhR function and reduced colitis. Researchers found that *Limosilactobacillus reuteri* could influence microbiome diversity by limiting the colonization of pathogenic microorganisms, mainly through driving the production of the anti-inflammatory cytokines, including IL-22, IL-10, and prostaglandin E2 (PGE2) by innate lymphocytes type 3 (ILC3s).[30–33] Both our study and these studies demonstrated the benefit of *Bifidobacterium breve* and

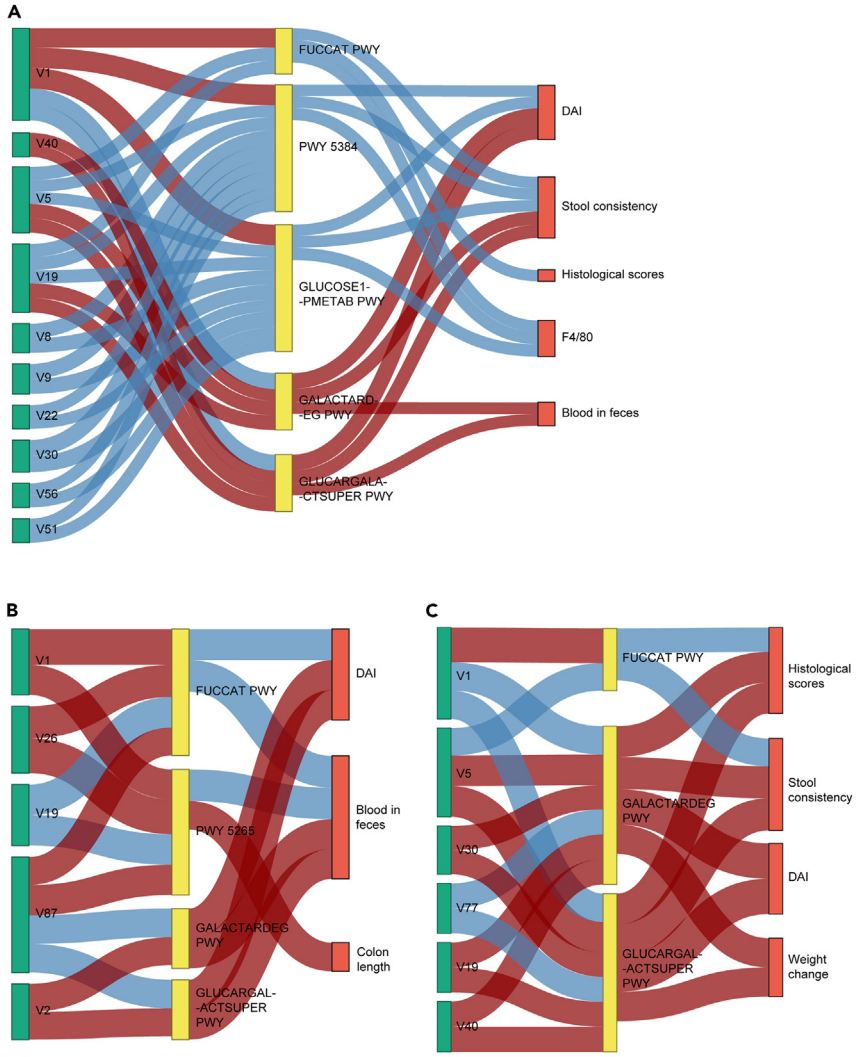


**Figure 7. Sankey map was used to analyze the multiple correlation between flora abundance, KEGG pathway and phenotypic data**

(A) Three group together.

(B) *LR* FPHC2951 group and control group.

(C) *BB* FPHC4024 group and control group.

*Limosilactobacillus reuteri* in alleviating IBD. However, in our study, *BB* FPHC4024 and *LR* FPHC2951 were derived from feces of exclusively breastfed healthy infants and our study showed that *LR* FPHC2951 was superior to *BB* FPHC4024 in ameliorating symptoms of DSS-induced IBD.

To explore possible mechanisms for the remission of IBD by infant feces-derived *BB* FPHC4024 and *LR* FPHC2951, we measured the expression levels of anti-inflammatory cytokines mRNA of colonic mucosa and performed 16S rRNA sequencing of fecal samples in experimental mice. We found that *LR* FPHC2951 significantly increased IL-10 mRNA expression in DSS-induced colitis mice. As also mentioned previously, studies on *Limosilactobacillus reuteri* and IBD have confirmed that *Limosilactobacillus reuteri* can increase the expression of IL-10 mRNA, which is consistent with our research results. IL-10 is a cytokine that plays an important role in the pathogenesis of IBD, and spontaneous IBD occurs in both mice and humans when IL-10 or its receptors are genetically disrupted.[34–38] The role of IL-10 is to maintain the $CD206^{+}$ regulatory phenotype of lamina propria macrophages, which is essential for maintaining intestinal immune tolerance.[39,40] Therefore, *Limosilactobacillus reuteri* may have a positive regulatory effect on intestinal immunity by increasing the expression of IL-10.

IBD is thought to be the result of host-microbial interactions, including gut microbiome factors, abnormal immune responses, and compromised intestinal mucosal barriers. Imbalance of microbial homeostasis leads to colonization and invasion of opportunistic pathogens in the gut, increasing the risk of host immune response and promoting the occurrence of IBD.[41–44] There has been conclusive evidence that the intestinal flora of IBD patients is significantly different from that of healthy people.[14] Therefore, we performed 16S rRNA sequencing on the stool of experimental mice, and found that the flora structure of *LR* FPHC2951 group and *BB* FPHC4024 group was significantly different from

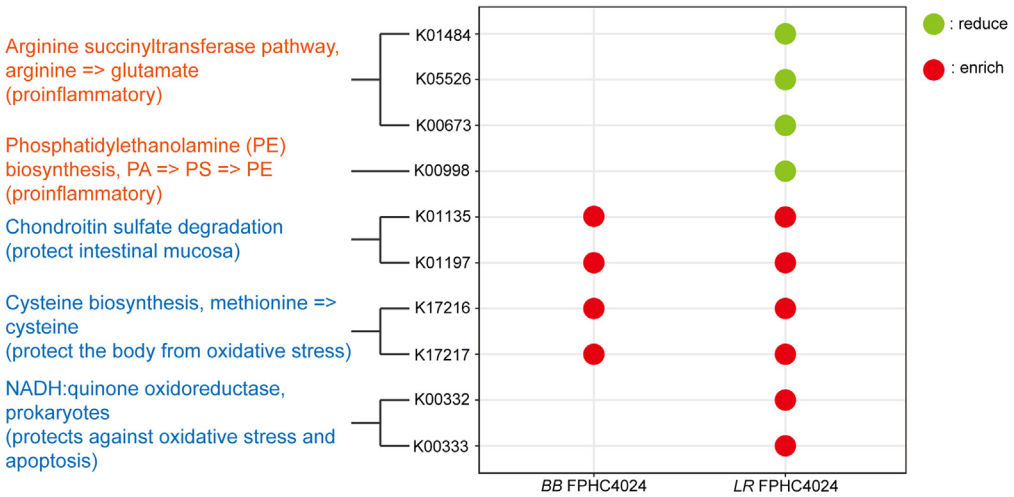


**Figure 8. *LR* FPHC2951 group and *BB* FPHC4024 group were enriched in multiple predictions of anti-inflammatory and protective intestinal barrier function**

Summarizing KO-module with significant differences associated with colitis in the PICRUSt metagenomic functional prediction map.

that of the control group. *LR* FPHC2951 group and *BB* FPHC4024 group both showed increased abundance of *Verrucomicrobiaceae Akkermansia*, which could alleviate F4/80, DAI score, histological scores and stool consistency by upregulating the FUCCAT-PWY and PWY-5384 pathways. *Verrucomicrobiaceae Akkermansia* is widely considered a promising candidate for the next generation of probiotics (NGP) because of its inverse association with a number of metabolic diseases, such as overweight, obesity, and type 2 diabetes.[45,46] In recent years, *Verrucomicrobiaceae Akkermansia* has been shown to be associated with colitis in humans and mice, and a review of differences in the gut microbiota between IBD patients and healthy people mentioned that *Verrucomicrobiaceae Akkermansia* was reduced in IBD patients.[47] Previous studies have shown that the ketogenic diet changed the gut microbiota of DSS-induced IBD mice, mainly through greatly increasing the abundance of *Verrucomicrobiaceae Akkermansia*, which significantly reduced the inflammatory response and protected the intestinal barrier function.[48] Studies have found a reduction in *Verrucomicrobiaceae Akkermansia* in the intestinal microbiota of IL-10$^{-/-}$ mice, which is associated with damage to the intestinal barrier.[49] In addition, some studies have also found that *Verrucomicrobiaceae Akkermansia* and its secretions can increase the level of serum IL-10 and reduce the pathological manifestations of colitis in mice.[50] In addition, the *LR* FPHC2951 group also showed increased abundance of *Bacteroidia Bacteroidales* and *Rikenellaceae Rikenella*. *Bacteroidia Bacteroidales* and *Rikenellaceae Rikenella* have also been found in previous studies to be increased in abundance in DSS-induced IBD models and associated with downregulation of proinflammatory factors and improvement in IBD.[51,52] These evidences helped us to conclude that infant feces-derived *LR* FPHC2951 and *BB* FPHC4024 altered the structure of the intestinal microbiota in mice, specifically increasing the abundance of *Verrucomicrobiaceae Akkermansia* to regulate immune response and protected intestinal barrier function, thereby reducing the symptoms of DSS-induced IBD, and IL-10 may played a crucial role in this process. And *LR* FPHC2951 increased the abundance of a variety of probiotics that are beneficial for IBD may contribute to the result that *LR* FPHC2951 showed a more significant IBD improvement effect than *BB* FPHC4024.

Prediction of PICRUSt function based on KEGG suggested that infant feces-derived *LR* FPHC2951 and *BB* FPHC4024 increased chondroitin sulfate degradation and cysteine biosynthesis. Chondroitin sulfate (CS) is a glycosaminoglycan, widely present in the extracellular matrix and cell surface of animal cartilage tissue, and can be used as an anti-inflammatory drug and an anti-osteoarthritis food supplement.[53] Since CS has been shown not to metabolize in the stomach or small intestine, its degradation is mainly performed by gut microbes.[54] The increased degradation of chondroitin sulfate may indicated its increased availability and function *in vivo*, including the protective effect on intestinal mucosa.[55,56] Cysteine plays a crucial role in REDOX homeostasis, it is a component of the main antioxidant glutathione, and is also a potent antioxidant in its own right.[57,58] CS and cysteine have also been shown to increase IL-10 mRNA expression levels to achieve anti-inflammatory effects,[59,60] which is consistent with the mechanism described previously. *LR* FPHC2951 reduced the enrichment of arginine succinyltransferase pathway and PE biosynthesis pathway. The *Escherichia coli*-driven arginine succinyltransferase pathway was significantly increased in rheumatoid arthritis and positively correlated with the elevation of rheumatoid factor. In IEC cell lines exposed to the pathogenic colitis-related organism enteropathegenic *Escherichia coli*, ethanolamine expression is increased in apoptotic cells and contributes to bacterial adhesion.[61] Lipidomic analysis of UC patients revealed a significant increase in PE in the mucosa.[62] Therefore, educing the enrichment of arginine succinyltransferase and PE may be related to reduce the inflammatory response. In addition, we found that *LR* FPHC2951 enhanced the enrichment of NADH: quinone oxidoreductase pathway. Many studies have linked $NAD^+$ metabolism to inflammatory diseases, gut homeostasis, and IBD.[63] Niacin and nicotinamide metabolism are the main metabolic features of inflammatory tissues in UC.[64] Gut homeostasis in IBD requires a balance between $NAD^+$ production and depletion, and the increased activity of $NAD^+$-consuming enzymes in IBD is associated with the occurrence of intestinal inflammation.[65] Therefore, NADH: quinone oxidoreductase may exert a protective effect on the intestinal barrier by increasing $NAD^+$ levels, possibly preventing the progression of IBD.

Taken together, infant fecal-derived *LR* FPHC2951 may regulate intestinal immune response, maintain intestinal mucosa and protect against oxidative stress by reducing proinflammatory pathways and enhancing pathways associated with anti-inflammatory and intestinal protection. At the same time, *LR* FPHC2951 increasing the expression of IL-10 mRNA through several possible pathways can also reduce the inflammatory response of IBD.

### Conclusion

We isolated two probiotics, *BB* FPHC4024 and *LR* FPHC2951, from feces of exclusively breastfed healthy infants and found that *LR* FPHC2951improved DSS-induced IBD symptoms and histological damage. This may be related to increasing of IL-10 mRNA in DSS-induced IBD mice, changing the structure of intestinal flora, and reducing proinflammatory pathways and enhancing pathways associated with anti-inflammatory and intestinal protection. The study identified a new strain of infants vertically transmitted from breast milk that can alleviate colitis, which will provide new strategies for clinical treatment of IBD.

### Limitations of the study

There are some limitations in this study and also some aspects worth further research. First of all, previous studies have shown that mixed probiotics often have better therapeutic effects than single bacteria, but this study only compared the improvement effect of two strains on DSS induced IBD, and further research on the mixing of two strains is needed. Secondly, the metabolites of probiotics during the improvement of IBD may serve as more convenient and direct exogenous supplements.

## RESOURCE AVAILABILITY

### Lead contact

Further information and requests for resources and reagents should be directed to and will be fulfilled by the lead contact, Dr. Liwei Xie (xielw@gdim.cn).

### Materials availability

This study did not generate new unique reagents.

### Data and code availability

- Data and images that support the findings of this study are available on request from the lead contact.
- This paper does not report original code.
- Any additional information required to reanalyze the data in this paper is available from the lead contact upon request.

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## AUTHOR CONTRIBUTIONS

L. Xie: conceptualization, methodology, supervision, funding acquisition and writing – reviewing and editing. Z.H.: investigation, data curation, formal analysis and writing – original draft. B.L.: data curation, formal analysis and visualization. L. Xiao: investigation. M.L.: investigation. Liujing Huang: software. X.Z.: investigation. K.M.: resources. R.W.: resources. F.J.: resources. W.L.: resources. Liping Huang: project administration and resources.

## DECLARATION OF INTERESTS

The authors declare no conflict of interest.

## STAR★METHODS

Detailed methods are provided in the online version of this paper and include the following:

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## SUPPLEMENTAL INFORMATION

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## STAR★METHODS

### KEY RESOURCES TABLE

| REAGENT or RESOURCE | SOURCE | IDENTIFIER |
|---|---|---|
| Antibodies | | |
| Occludin Rabbit mAb | ABclonal | Cat#A22898; RRID: N/A |
| F4/80 Rabbit pAb | Servicebio | Cat#GB11027; RRID:AB_2814687 |
| HRP, Anti-Rabbit IgG | ABclonal | Cat#AS014; RRID:AB_2769854 |
| Bacterial and virus strains | | |
| *Limosilactobacillus reuteri* FPHC2951 | Human | GDMCC: No.62946 |
| *Bifidobacterium breve* FPHC4024 | Human | GDMCC: No.62945 |
| Biological samples | | |
| Feces of exclusively breastfed healthy infants | Zhujiang Hospital of Southern Medical University | Ethics number NCT05462366 |
| Chemicals, peptides, and recombinant proteins | | |
| Phosphate buffered saline (PBS) | Solarbio | N/A |
| MRS Broth | Hopebio | HB0384-1 |
| Bifidobacterium medium | Hopebio | HB8527 |
| Dextran sulfate sodium (DSS) | MP Biomedicals | lot No. S2839 |
| TRIzol reagent | Ambion | lot 410404 |
| Critical commercial assays | | |
| MoBio PowerSoil DNA extraction kit | Qiagen | N/A |
| Software and algorithms | | |
| Fiji | NIH | https://imagej.net/software/fiji/ |
| Endnote | Clarivate | https://endnote.com/ |
| Graphpad Prism v9 | GraphPad Software, Inc. | https://www.graphpad.com/ |
| R 4.3.2 | R Core Team | https://www.R-project.org/ |

### EXPERIMENTAL MODEL AND STUDY PARTICIPANT DETAILS

#### Feces of exclusively breastfed healthy infants

The study was screened from a cohort of healthy mothers and infants admitted to the Department of Obstetrics at Zhujiang Hospital of Southern Medical University from June to October 2021. The inclusion criteria were as follows: (1) healthy pregnant women aged 20–40 years (2) vaginal delivery (3) BMI<23.5 before pregnancy (4) exclusive breastfeeding of postpartum infants (5) birth weight of infants between 2500 and 4000 g. The exclusion criteria were as follows: (1) gestational diabetes, gestational depression, gestational hypertension, or other disease during pregnancy; (2) probiotic or antibiotic treatment from 3 months before pregnancy to 3 months postpartum; (3) history of smoking or alcohol consumption; and (4) history of diarrhea from 3 months before pregnancy to 3 months postpartum. A total of 34 groups of exclusively breastfed infants were collected. Our study obtained ethical approval, registered with ClinicalTrials.gov, and passed the review (ethics number NCT05462366).[66]

#### Mice

Male C57BL/6J mice at 15 weeks old were purchased from the Hangzhou Ziyuan Laboratory Animal Technology Co.,Ltd and raised in a specific-pathogen-free (SPF) animal facility at the Institute of Microbiology of the Guangdong Academy of Sciences. The animal facility was maintained with 12-h-light/12-h-dark cycles and was temperature and humidity controlled, and all experimental animals were provided *ad libitum* access to food and water. All animal operations and procedures were approved by the Animal Protection and Utilization Committee of the Institute of Microbiology, Guangdong Academy of Sciences (permission no. SCXK [Guangdong] 2018-0034).

### METHOD DETAILS

#### Bacterial culture

*Bifidobacterium breve* FPHC4024 was cultured in bifidobacterium medium (HB8527; Hopebio, Qingdao, China), and *Limosilactobacillus reuteri* FPHC2951 was cultured in MRS broth (HB0384-1; Hopebio, Qingdao, China), both at 37°C for 24–48 h, as described previously.[66]

### Probiotic screening process

Gastrointestinal tolerance and adhesion were important indicators of probiotics. Relevant screening methods have been confirmed in previous literature,[67,68] and the detailed methods are as follows. Lactobacillus and Bifidobacterium were ranked in descending order according to the following five experimental results, and the five indicators were comprehensively scored with the same weight.

### Evaluation of strain acid tolerance

Single colonies were selected and inoculated in 5 mL of MRS broth liquid medium or bifidobacterium liquid medium for expanded culture. Eighteen hours later, the bacterial solution was transferred to a 15 mL centrifuge tube, centrifuged at 4500 rpm for 5 min, and the supernatant was discarded to collect the bacterial pellet. The bacterial pellet was washed once with 0.85% NaCl and then re-suspended with 5 mL of gastric juice to make a bacterial suspension (bacterial concentration reached 10^8 CFU). Pepsin (0.0006 g) and gastric mucin (0.002 g) were added to each 1 mL of artificial gastric juice. A 500 μL bacterial suspension was mixed with 4.5 mL of artificial gastric juice (pH 2.5), incubated at 37°C, and plated using the gradient dilution method after 0 h and 3 h, respectively. The survival rate (%) was counted after 48 h. Survival rate (%) = (number of viable bacteria at 3 h/number of viable bacteria at 0 h) × 100 %.

### Evaluation of strain tolerance to bile salt and intestinal fluid

Single colonies were selected and inoculated in 5 mL of MRS broth liquid medium or bifidobacterium liquid medium for expanded culture for 18 h. Add 0.006 g of pancreatin and 0.008 g of bile salt (pH 7.0) to each 1 mL of artificial intestinal fluid. Centrifuge at 4500 rpm for 5 min and discard the supernatant to collect the bacterial pellet. Wash the bacterial pellet with 0.85% NaCl once, centrifuge again, and discard the supernatant. Use the prepared artificial intestinal fluid to make a bacterial suspension (bacterial concentration up to 10^8 CFU). Incubate at 37°C, then plate using the gradient dilution method after 0 h and 3 h, respectively. The survival rate (%) was counted after 48 h. Survival rate (%) = (number of viable bacteria at 3 h/number of viable bacteria at 0 h) × 100 %.

### Evaluation of surface hydrophobic characteristics of strains

The hydrophobicity of the strain was determined by xylene extraction. The absorbance of the tested bacterial suspension was adjusted with 0.85% NaCl to OD600 = 0.6 ± 0.02. Take 2 mL of the suspended bacteria in a test tube, add 1 mL of xylene, swirl for 3 min, and then let it stand at room temperature for 1 h (at this time, a two-phase system is formed). The water phase is absorbed, and the absorbance at 600 nm is determined (A). A0 is the absorbance of the bacterial suspension to be measured before extraction. Hydrophobicity rate (%) = [(A0 − A)/A0] × 100%.

### Evaluation of bacterial autoaggregation

The absorbance of the tested bacterial suspension was adjusted with 0.85% NaCl to OD600 = 0.6 ± 0.02. Take 2 mL of the bacterial solution and swirl for 10 s, then measure its absorbance (A0). Let it stand at 37°C for 2 h, carefully absorb the supernatant, and measure its absorbance (At) at 600 nm. Autoaggregation (%) = [(A0 − At)/A0] × 100%.

### Evaluation of strain adhesion (Caco-2 cell culture plate colony counting method)

Caco-2 cells were inoculated into a 12-well plate and cultured to a polarized state. The single-layer Caco-2 cells in the 12-well plate were washed twice with sterile PBS buffer, and 1 mL of bacteria with a concentration of 10^8 CFU/mL (V0) was added to each well. The initial bacterial count was determined, and then the 12-well plates were cultured at 37°C for 2 h. The single cell layer of each well in the 12-well plate was washed with PBS solution more than 3 times to elute the non-adherent bacteria and metabolic secretions. Then, 500 μL of 0.25% trypsin-EDTA was added to each well for digestion for 10 min. Next, 500 μL of DMEM medium was added to terminate the digestion. After a 10-fold gradient dilution, plate colony counts were performed to obtain the number of adherent bacteria (V1). The adhesion ratio (%) can be calculated as follows: Adhesion ratio (%) = (V1/V0) × 100%.

### Dextran sulfate sodium (DSS) mouse colitis model and probiotic intervention

Fifteen mice were randomly divided into three groups ($n$ = 5) and named the control group, *Bifidobacterium breve* FPHC4024 (*BB* FPHC4024) group, and *Limosilactobacillus reuteri* FPHC2951 (*LR* FPHC2951) group. All the mice were given 2% dextran sulfate sodium (DSS) (lot no. S2839; molecular weight, 36,000 to 50,000 Da; MP Biomedicals, LLC, Solon, OH, USA) in sterile water for 6 days to establish a DSS-induced colitis mouse model, followed by recovery with regular drinking water for an additional 8 days. Mice of *BB* FPHC4024 group and *LR* FPHC2951 group were given intragastric administration of 300 μL (2 × 10^10 CFU/300 μL) probiotic strains each time for the 14 days, once a day. Mice of the control group were fed with 300 μL skimmed milk for 14 days, once a day. The mice were sacrificed after the fecal samples, colon tissue, and intestinal epithelial scrapings were collected.

### Weight, length of colon, and disease activity index (DAI)

The body weights of mice were measured everyday, and disease activity index was also calculated everyday, including loss of weight (%), stool consistency, and blood in feces. The weight change is the weight of day 14 minus the weight of day 0, and the specific calculation method of DAI is shown in Table S1. The colon was taken in the end of the experiment and the length was measured from cecum to distal colon.

### HE and IHC-F4/80 staining

The colon was dissected along the longitudinal mesentery, and then the distal colon was fixed in 4% paraformaldehyde at room temperature for 48 h, dehydrated, embedded in paraffin, and sectioned into 4-μm-thick sections for staining with hematoxylin and eosin (HE)[69,70] and IHC-F4/80.[71]

### Evaluation of histological score and macrophages infiltration

The images of colon tissues stained with HE and IHC-F4/80 were captured by Automatic Digital Slice Scanning System (Guangzhou Betrue Technology Co., Ltd) at 40× magnification, and whole-slide images (WSIs) were obtained. The pathological score was calculated in a blind fashion based on the following comprehensive score criteria: (i) epithelial loss, (ii) crypt damage, (iii) depletion of goblet cells, and (iv) infiltration of inflammatory cells,[72] as shown in Table S2. The macrophage infiltration was defined as the presence of macrophages in more than 30% of the mucosal and submucosal area.

### Real-time quantitative polymerase chain reaction (qPCR)

Total RNA was extracted from the colon tissues using standard techniques by TRIzol reagent (lot 410404, Ambion). Reverse transcribe RNA into complementary DNA (cDNA) using reverse transcriptase. Design specific primers targeting the genes of anti-inflammatory cytokines (including IL-4, IL-10, IL-13, Tgf-β, CD163, CD206, and Ym1), and intestinal barrier related genes (including *Cav1*, *Hif2α*, *Vegf*, and *Ankrd37*). Then run qPCR cycles to amplify the target genes, and fluorescence was measured during each cycle to monitor DNA amplification. Calculate the cycle threshold (Ct) value for each gene and Rps18 was defined as reference genes. The expression levels were compared between different samples using the ΔΔCt methods. The design scheme of each primer was shown in Table S3.

### DNA extraction from feces, sequencing library construction, and 16S rRNA sequencing

DNA from mouse feces was extracted by utilizing a MoBio PowerSoil DNA extraction kit (Qiagen, USA), and the concentration was measured with a nanodrop (ThermoFisher, USA). Fifty nanograms of DNA was used for 16S rRNA sequencing library construction using Q5 high-fidelity DNA polymerase (NEB), targeting the V3-V4 region of the bacterial 16S rRNA gene (forward primer, 5′-CCTACGGGNGGCWGCAG-3′; reverse primer, 5′-GACTACHVGGGTATCTAATCC-3′), followed by purification with AMPure XP (Beckman).

### 16S rRNA amplicon sequencing and bioinformatics statistics

A standard QIIME 2 pipeline was approved, and high-quality amplicon sequence variants (ASVs) were obtained by the DADA2 algorithm.[73,74] The taxonomy profile analysis was performed against the Greengenes database[75] and transformed into relative abundance at the phylum, class, order, family, genus, and species levels. Microbial data indicating a relative abundance less than 0.001 or an attendance rate less than 70% in all groups were filtered to obtain core bacterial taxa for further analysis. α-diversity and β-diversity analyses, cooccurrence analysis, structure plot, random forest model and KEGG-based PICRUSt functional prediction were performed using the R package EasyMicroPlot.[76,77]

## QUANTIFICATION AND STATISTICAL ANALYSIS

The statistical details of all experiments and the sample numbers are described in the figure legend of each figure. The results are presented as the mean ± standard error of the mean (SEM). For significant comparisons between two groups, a two-tailed, unpaired Student's t-test or Mann-Whitney-Wilcoxon test was used. Statistical significance is described in the figure legends as follows: *, $p < 0.05$; **, $p < 0.01$; ***, $p < 0.001$.

## ADDITIONAL RESOURCES

All research-related protocols were approved by the Medical Ethics Committee of Zhujiang Hospital of Southern Medical University and registered at clinicalTrials.gov (NCT05462366).