

Breast Milk-Derived *Limosilactobacillus reuteri* Prevents Atopic Dermatitis in Mice via Activating Retinol Absorption and Metabolism in Peyer's Patches

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Scope: Supplementing *Limosilactobacillus reuteri* Fn041, a breast milk-derived probiotic from agricultural and pastoral areas, to maternal mice during late pregnancy and lactation prevents atopic dermatitis (AD) in offspring. This study aims to elucidate the molecular mechanism of Fn041-mediated immune regulation.

Methods and results: Fn041 is administered prenatal and postnatal to maternal mice, and to offspring after weaning. The ears are administered with calcipotriol to induce AD. Fn041 treatment significantly alleviates ear inflammation, and reduces mast cell infiltration. Fn041 treatment upregulates and downregulates intestinal ZO-1 and Claudin-2 mRNA expression, respectively. Transcriptome analysis of Peyer's patches reveals that pathways related to DNA damage repair are activated in AD mice, which is inhibited by Fn041 treatment. Fn041 activates pathways related to retinol absorption and metabolism. Untargeted metabolomic analysis reveals that Fn041 treatment increases plasma retinol and kynurenine. Fn041 treatment does not significantly alter the overall cecal microbiota profile, only increases the relative abundances of *Ligilactobacillus apodemi*, *Ligilactobacillus murinus*, *Akkermansia muciniphila*, and *Bacteroides thetaiotaomicron*.

Conclusions: Fn041 induces anti-AD immune responses directly by promoting the absorption and metabolism of retinol in Peyer's patches, and plays an indirect role by strengthening the mucosal barrier and increasing the abundance of specific anti-AD bacteria in the cecum.

1. Introduction

There has been a rise in the prevalence of allergic diseases including atopic dermatitis (AD) over recent decades of industrialization and urbanization while living on a farm is protective against allergic diseases.^[1] But the mechanism for this protective effect has not been fully elucidated. Accumulating evidence points to the importance of early colonization by a "healthy" microbiome as a key determinant of the prevention of AD.^[2] Children on farms consume unpasteurized farm milk and come into contact with animals and their stables, thereby continuously acquiring environmental bacteria, promoting the development of gut microbiota, and possibly preventing AD.^[1] At the same time, mothers living on farms during pregnancy also have a protective effect on offspring from developing AD.^[3,4] Human milk intake by infants, which is more widespread and lasts longer in farming communities, could be another source of maternal influence on infant health outcomes.^[5] Breast milk

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has a unique microbiome as well as various nutrients and other substances that strongly influence the growth of infant gut microbes.^[6] Our previous study found that the occurrence of *Limosilactobacillus reuteri* in the breast milk of mothers living on Tibetan farms in southern Gansu of China was much higher than that in samples from other urban areas.^[7] The potential probiotic strain isolated from the breast milk of a Tibetan farm, *Limosilactobacillus reuteri* Fn041 (Fn041), has a much higher ability to adhere to mucins than other typical probiotics, such as *Lactocaseibacillus rhamnosus* GG (LGG). When the maternal mice were given Fn041 during gestation/lactation, and the offspring were also given after weaning, the intestinal mucosal immune development of the offspring was promoted,^[8] and the offspring were prevented from developing calcein-induced AD.^[9] The mechanism by which Fn041 prevents AD in infant remains to be further elucidated.

Atopic susceptibility is partly due to abnormal intestinal mucosal barrier function and dysregulated immune responses to ubiquitous environmental antigens.^[10] The gut barrier functions are incompletely developed in early infancy, coupled with the inflammation caused by gut dysbiosis, which leads to increased antigen absorption and allergic immune response.^[11] Atopic dermatitis is immunologically characterized by increased systemic production of allergen-specific IgE and imbalance of T cell response. Although Th2 cells dominate in the acute phase of AD, Th1 cells take over in the chronic phase, resulting in AD.^[12] Impairment of immune tolerance is the root cause of this pathological response.^[13] Antigen transport across Peyer's patches is important in mucosal and systemic immune defense induction of immune tolerance. Orally administered probiotics could interact with Peyer's patches, where more than 70% of immune cells are located.^[14] They either induce inflammatory cytokines or immune tolerance responses, depending on the strain.^[15] Dendritic cells and macrophages can boost the formation of induced regulatory T (Treg) cells, which play a vital role in maintaining peripheral immunological tolerance by balancing the ratio of effector and Treg cells, if a strain induces an IL-10/TGF- β -rich cytokine milieu in Peyer's patches.^[16] Vitamin A and its metabolically active derivative, retinoic acid (RA), play an important role in the development and functions of the immune system. Dendritic cells (DCs) produced retinoic acid together with TGF- β 1 could induce Tregs.^[17] Furthermore, microbiota-produced butyrate may stimulate anti-inflammatory pathways by causing CD4⁺ T cells to differentiate into Treg cells.^[14]

The purpose of this study was to investigate the effect of Fn041 administration on parental mice on gene expression of Peyer's patches in offspring mice, as well as the effect on the intestinal mucosal barrier, plasma metabolites, and changes in the gut microbiome, to elucidate the mechanism of Fn041 preventing AD in offspring.

2. Experimental Section

2.1. Animals

All experiments were performed according to the National Guidelines for Experimental Animal Welfare and were approved by the Qingdao University Animal Ethics Committee (No.QYFYWZLL25893). BALB/C mice (24 6-week-old female mice and 12 6-week-old male mice) were obtained from Viton

Lever Co., Ltd (Beijing, China). Mice were housed under specific pathogen-free conditions. Animals (two to five mice per cage) were bred at a controlled temperature ($22 \pm 3^\circ\text{C}$, 40–70% humidity) and a 12-h light/12-h dark artificial light cycle, with food and water available ad libitum.

2.2. Oral Probiotics Supplementation and Experimental Atopic Dermatitis

Female mice were caged with males at $22 \pm 3^\circ\text{C}$ and 40–70% humidity with a light-to-dark cycle of 12:12 h under hygienic conditions overnight at a female/male ratio of 2/1 and examined for a vaginal plug the next morning. The males were removed from the cages after the dams were impregnated. Dams were randomly divided into four groups ($n = 5/\text{group}$ for dams; $n = 12/\text{group}$ for pups) (Figure 1A). The normal control group (CON) and atopic dermatitis model group were gavaged with PBS. The AD+Fn041 and the AD+LGG group were gavaged with *L. reuteri* Fn041 and LGG for 1×10^9 CFU day⁻¹ suspended in PBS (once a day, 100 μL each time), respectively. The dams were gavaged daily from 1 week before parturition until weaning. The pups were also treated by gavage in the same way for 1 week after weaning. After weaning (3 weeks after delivery), the right ears of the pups were smeared with calcipotriol (20 μL per ear, atopic dermatitis model) (MC903, Sigma-Aldrich, St. Louis, MO, USA) or absolute ethanol (normal control group) daily for 1 week. Pups were executed on day 8, after fasting for 6 h before an anesthetic.

The degree of AD symptoms in the ears based on Eczema Area and Severity Index^[18] was assessed on the 1st, 3rd, 5th, and 7th day of modeling, respectively. The scoring criteria include: 1) the ratio of erythema/bleeding area to the total ear skin of >80, 50–80%, 20–50%, <20%, and normal was scored 4, 3, 2, 1, and 0, respectively. 2) The area of scratches or erosions area to the total ear skin of >80%, 50–80%, 20–50%, <20%, and normal was scored as 4, 3, 2, 1, and 0, respectively. 3) For the condition of skin edema, dark red, moist, bumpy, and swollen was scored as 4; light red, slightly dry, bumpy, edema reduction was scored as 3; pink, the uneven surface was scored as 2; smooth and color close to that of healthy mice were scored as 1, and no above symptoms were scored 0. 4) The area of scaly degeneration of the skin accounts for >80%, 50–80%, 20–50%, <20%, and normal of the total ear skin area as 4, 3, 2, 1, and 0, respectively. The total score was the sum of the above scores.

2.3. Histological Analysis

A portion of the ear from the same site was taken. Paraformaldehyde-fixed mouse ears were embedded in paraffin, cut into 5 μm thick sections, and attached to detachment-proof glass slides. Sections were then sequentially dehydrated with different concentrations of alcohol, stained, destained, cleared with xylene, and mounted. Hematoxylin-eosin (H&E) staining was used to assess edema and inflammatory cell infiltration in mouse ears. Toluidine blue (Sembega Biotechnology Co., Ltd., Nanjing, China) staining was used to assess mast cell infiltration. Mast cells were counted in 10 random fields of pathological sections under a light microscope.

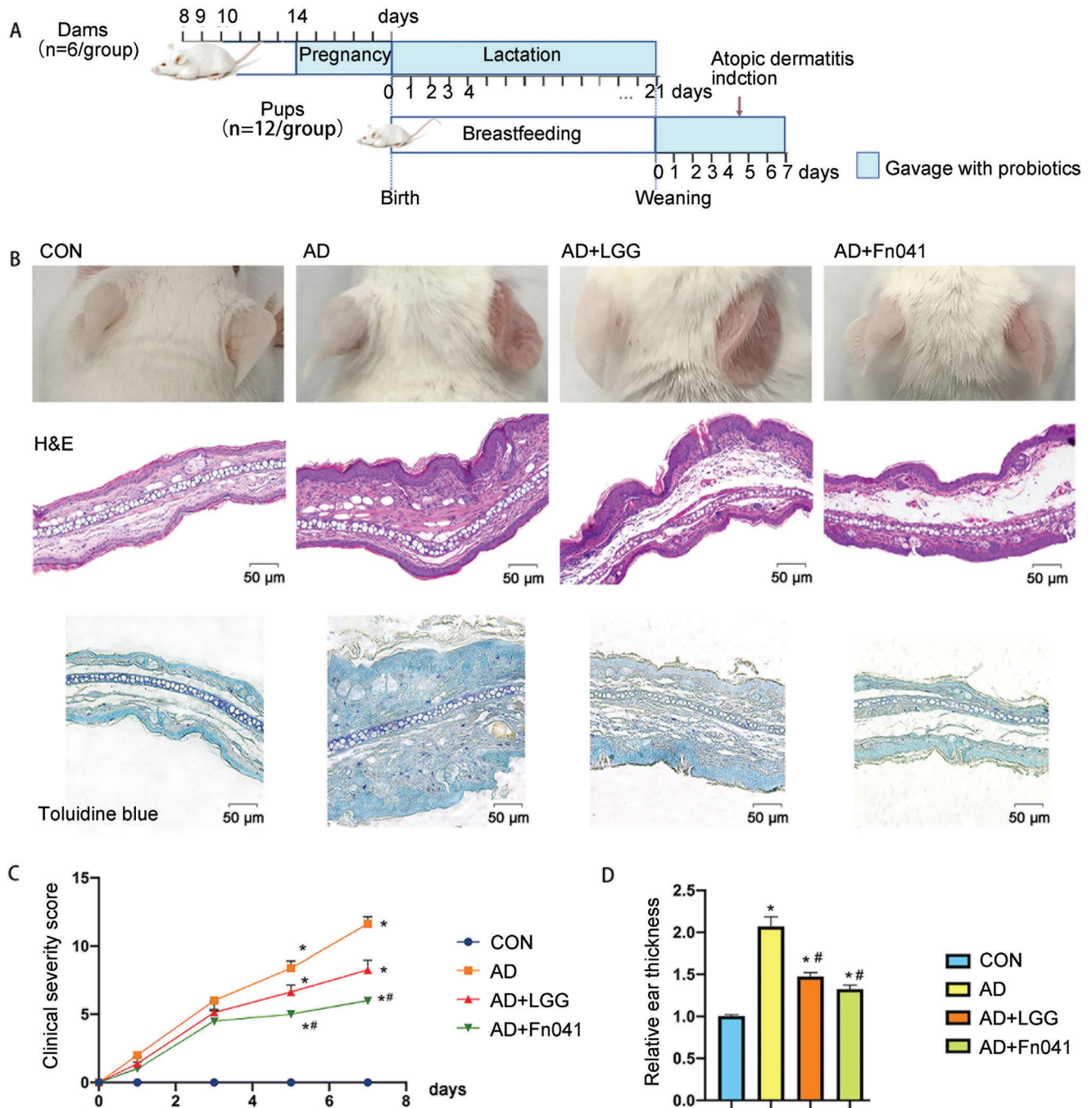


Figure 1. Dams and pups intake of *Limosilactobacillus reuteri* Fn041 (Fn041) or *Lactaseibacillus rhamnosus* GG (LGG) ameliorates MC903-induced atopic dermatitis (AD) symptoms in pups. Schematic presentation of the experimental design A). Female BALB/c mice were gavaged with PBS ($n = 6$), Fn041 ($n = 6$), or LGG ($n = 6$) during late pregnancy and lactation, respectively. After weaning at age 21 days, pups were gavaged with PBS for control (CON, $n = 12$) and AD model control ($n = 12$) mice, Fn041 ($n = 12$) or LGG ($n = 12$) for 7 days, respectively. The probiotic dose was 1×10^9 CFU day $^{-1}$. Pups in the AD and the probiotic treated group were topically treated with MC903 for 7 days after weaning for right ears. Gross appearance of the pups' ears on day 7 after modeling B). Hematoxylin and eosin (H&E) and toluidine blue skin staining show the thickness of the epidermis (EM) and mast cell (original magnification $\times 100$, scale bar = 50 μ m). Clinical severity score C). Relative ear thickness (the ear thickness of the normal control group is defined as 1) D). Data are shown as mean $\pm$ SD, and representative of data from three independent experiments. Student's t -test (unpaired; $n = 12$); * $p < 0.05$ versus CON group; # $p < 0.05$ versus AD group. AD, atopic dermatitis model control; AD+Fn041 group, atopic dermatitis mice gavaged with *Limosilactobacillus reuteri* Fn041; AD+LGG group, atopic dermatitis mice gavaged with *Lactaseibacillus rhamnose* GG. CON, the normal control group.

2.4. RNA Extraction and Reverse Transcriptase-Polymerase Chain Reaction

Total RNA from the ear and ileum was extracted using an RNeasy kit (Qiagen Inc., USA) according to the instruction of the manufacturer (Qiagen Inc.) RNA was reverse transcribed to cDNA using MonScript RTIII All-in-One Mix with dsDNase (Monad Biotech Co., Ltd., Suzhou, China). Then a qRT-PCR reaction was performed for mRNA expression of thymic stromal lymphopoietin (TSLP), interleukin 5 (IL-5), interleukin 13 (IL-13), and interleukin 22 (IL-22) of ears, and zonula occludens 1 (ZO-1) Occludin and Claudin-2 of ileum. The 20 μ L reaction system includes 5 μ L of cDNA, 0.4 μ L of both primers (Table S1, Supporting Information), 10 μ L of 2 $\times$ Quantinova SYBR Green PCR Master Mix (Vazyme Biotech Co., Ltd., Nanjing, China), and 4.2 μ L of RNase-free water. All reactions were tested with the Applied Biosystems Prism 7700 Sequence Detector (Perkin-Elmer Applied Biosystems). The thermal cycler conditions for these experiments were: initial activation step for 15 min at 95 $^{\circ}$ C, followed by three-step cycling repeated 40 times; denaturation for 2 min at 95 $^{\circ}$ C, annealing for 10 s at 60 $^{\circ}$ C, and extension for 30 s at 76 $^{\circ}$ C. The corresponding Ct (Threshold cycle) values of each well were collected. The expression was calculated according to the $2^{-\Delta\Delta C_t}$ method considering a pull of RNA from control samples.

2.5. Determination of Secretory Immunoglobulin A (sIgA)

Ileum sIgA was analyzed using a ELISA kit according to the instruction of the manufacturer (Jingmei Biotech Co., Beijing, China).

2.6. RNA Sequencing and Gene Set Enrichment Analysis

RNA sequencing for Peyer's patches as described in Supplementary Methods. The R package "limma" was utilized for the normalization of RNA expression profiles. Differentially expressed genes (DEGs) between AD and CON, AD+Fn041 and AD, and AD+LGG and AD were tested using the bayesian adjusted *t*-statistics from the linear models of limma.^[19] A multiple testing correction based on the false discovery rate (FDR) was performed. Log2 (fold change) (log2FC) > 1.5 and a FDR < 0.05 were used as the cut-off criteria of DEGs samples. DEGs that met this criterion were used to perform gene set enrichment analysis for biological process themes of Gene Ontology (GO) and Kyoto Encyclopedia of Genes and Genomes (KEGG) pathway using the R package "ClusterProfiler 4.0."^[20] A corrected *p* < 0.05 was the cut-off criterion. KEGG pathways were visualized with Pathview. Next, an online tool named ImmuCellAI-mouse^[21] was used to estimate the relative abundance of 36 immune cells based on gene expression profiles from RNA-Seq.

To acquire protein-protein interaction (PPI) networks, DEGs were mapped to the search tool for retrieval of interacting genes (STRING) (version 11.5) online database.^[22] The highest confidence of the argument of interactions was set at >0.4.

2.7. Untargeted Metabolomic Analysis of Plasma

Each 100 μ L plasma was mixed well with 750 μ L buffer (methanol: ultrapure water = 1:1) and centrifuged for 15 min (12 000 rpm and 4 $^{\circ}$ C). The supernatant was filtered through a 0.45 μ m pore size filter. Each sample was taken 10- μ L and pooled as quality control. The plasma extraction was analyzed by LC-MS/MS using an Agilent 1290-UPLC system coupled with a 6530B Quadrupole Time-of-Flight as described in the Supplementary method. Integrated mass spectrometry-based untargeted metabolomics data mining (IP4M) software^[23] was used for peak table preprocessing and orthogonal partial least squares-discriminant analysis (OPLS-DA). According to the variable importance in the projection (VIP) threshold of 1 from the OPLS-DA model, differential metabolites in the metabolic profiles between AD and CON, AD+Fn041 and AD, and AD+LGG and AD were obtained.

2.8. 16S rRNA Sequencing Amplicon of the Cecum

The bacterial DNA was extracted from fecal samples of the cecum with a QIAamp Fast DNA stool Mini Kit (Qiagen, Cat# 51604) and PCR amplification was conducted with barcoded specific bacterial primers targeting the variable region 3–4 (V3–V4) of the 16S rRNA gene: forward primer 5'-ACTCCTACGGGAGGCAGCA-3' and reverse primer 5'-GGACTACHVGGGTWTCTAAT-3'. Construction of sequencing libraries and paired-end sequencing was performed on an Illumina MiSeq platform at Biomarker Technologies Co, Ltd. (Beijing, China) according to standard protocols. EasyAmplicon v1.14^[24] was used to perform downstream amplicon bioinformatic analysis, by which the -unoise3 command of USEARCH (v10.0) was then used to denoise non-redundant sequences into amplicon sequence variations (ASVs). The ASVs were taxonomically classified using the BLCA algorithm, which was based on the NCBI 16S Microbial database and processed using 1. sub-set db acc.py.^[25] PICURSt2 software package (v2.2.0-b) was used to predict the functional content of microbial communities as KEGG ortholog profiles.

2.9. Determination of Cecal Short-Chain Fatty Acids

Cecal acetic, propionic, and butyric acids were determined by gas chromatography-mass spectrometry according to the methods in the supplementary material.

2.10. Statistical Analysis

Results were expressed as the mean $\pm$ SD as indicated and were analyzed by a two-tailed Student's *t*-test or one-way ANOVA followed by Bonferroni's test. *p* < 0.05 was considered statistically significant. Analyses were conducted using GraphPad 8.0 software. Hierarchical clustering of the samples based on microbiota ASVs was performed on Bray-Curtis dissimilarity and clustered using Ward's hierarchical clustering method. Hierarchical

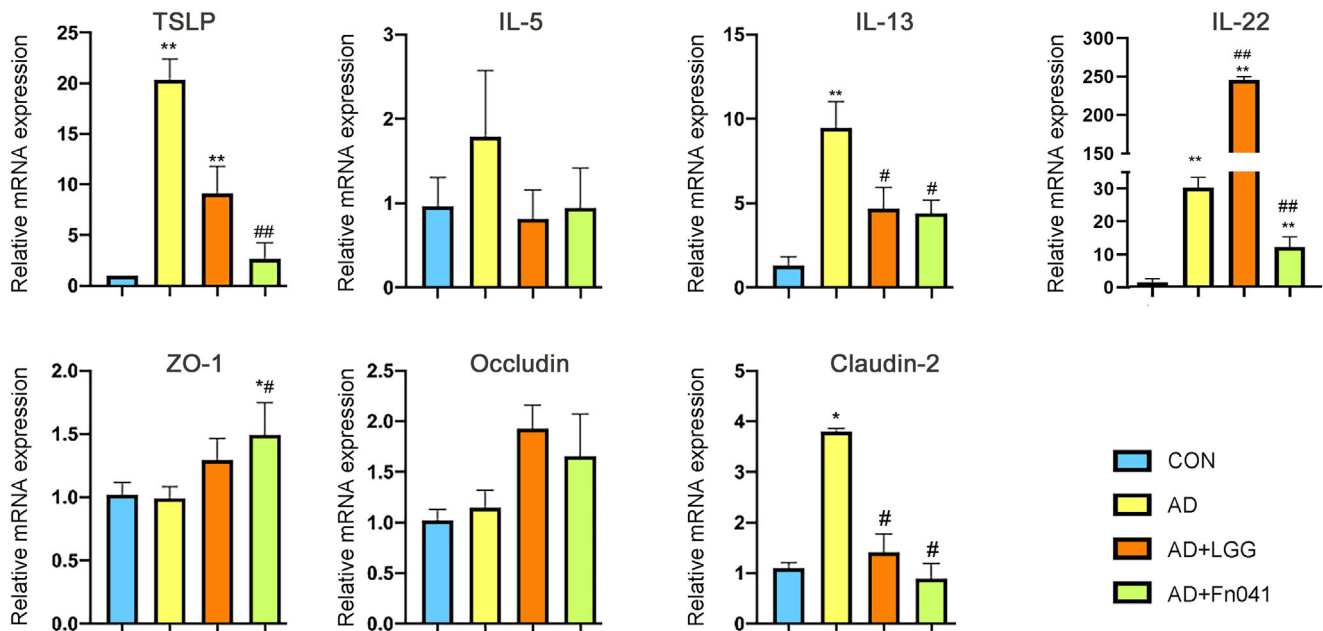


Figure 2. Relative expression of TSLP, IL-5, IL-13, and IL-22 gene mRNA in the ear and tight junction related gene, ZO-1, Occludin, and Claudin-2 of the ileum. Data are shown as mean $\pm$ SD and compared by student's *t*-test (unpaired; $n = 12$ /group); * $p < 0.05$, ** $p < 0.01$ versus CON group; # $p < 0.05$, ## $p < 0.01$ versus AD group. AD, atopic dermatitis model control; AD+Fn041 group, atopic dermatitis mice gavaged with *Limosilactobacillus reuteri* Fn041; AD+LGG group, atopic dermatitis mice gavaged with *Lactocaseibacillus rhamnose* GG. CON, the normal control group. IL-13, interleukin 13; IL-22, interleukin 22; IL-5, interleukin 5; TSLP, thymic stromal lymphopoietin; ZO-1, zonula occludens 1.

clustering at the species level was carried out for distance measured by Eudclidean using the Ward clustering algorithm. The difference of PICURSt2 predicted KEGG pathway of microbiota was analyzed by two-sided Welch's *t*-test and the result was filtered for *Q* value less than 0.05 and affect less than 0.30 using STAMP 2.0 software. The relative proportion of Peyer's patches immune cells was compared between two groups by two-sided Welch's *t*-test with Benjamini-Hochberg FDR correction.

3. Results

3.1. *L. reuteri* Fn041 Attenuates MC903-Induced Atopic Dermatitis in Mice

Dams were administrated with probiotic from 1 week before parturition until weaning, the pups were also treated to the end of AD induction (Figure 1A). (Figure 1A). At the end of the experiment, Fn041 significantly inhibited MC903-induced ear swelling and congestion (Figure 1B). Histological analysis showed that Fn041 treatment reduced MC903-induced ear epidermal and dermal thickening, as well as inflammatory cell infiltration, better than LGG, as determined by H&E staining (Figure 1B). The relative ear thickness of Fn041- and LGG-treated mice was also significantly lower than that of AD model control mice when measured with a pachymeter ($p < 0.05$, Figure 1D). Fn041 treatment also inhibited mast cell accumulation in the areas determined by toluidine blue staining (Figure 2B). From the 5th day of modeling, the ear pathological score of MC903-treated mice was significantly higher than that of normal control mice ($p < 0.05$). The pathological score of the Fn041-treated group was significantly

lower than that of the AD model control ($p < 0.05$) until the experiment's end (Figure 1C).

3.2. *L. reuteri* Fn041 Promotes sIgA Production and Modulates Gene Expression of Peyer's Patches

The mRNA expression of TSLP gene in the ears of AD mice was 20 times higher than that of healthy controls and was significantly decreased after the Fn041 treatment ($p < 0.05$). Both Fn041 and LGG significantly alleviated the elevated expression of the IL-13 gene in ears caused by MC903 ($p < 0.05$). Fn041 significantly down-regulated IL-22 mRNA in the ears of AD mice, but LGG increased the expression by eight times ($p < 0.01$). The mRNA expression of Claudin-2 was significantly increased in the ileum of AD mice ($p < 0.05$), which was significantly inhibited by both LGG and Fn041 ($p < 0.05$). Induction of AD had no significant effect on the number of intestinal Peyer's patches in mice, however, LGG treatment reduced the number to half that of normal mice ($p < 0.05$, Figure 3A). Fn041 treatment doubled the levels of ileal sIgA, the product of the Peyer's patches immune-induced response ($p < 0.05$, Figure 3B). We further pooled Peyer's patches of each mouse and performed RNA sequencing analysis. Genes with a fold change exceeding 1.5 were defined as DGEs (Figure 3C). In the AD model control group, 95 genes were significantly down-regulated, of which four genes with fold change lower than -3 were all retinol-inducible genes, including Defa3, Rsad2, Ifit3, Ifit3b, and Ifit1bl1, while only two genes were expressed significantly increased. Compared with the AD group, 384 genes were significantly up-regulated and 26 genes were significantly down-regulated in Fn041-treated

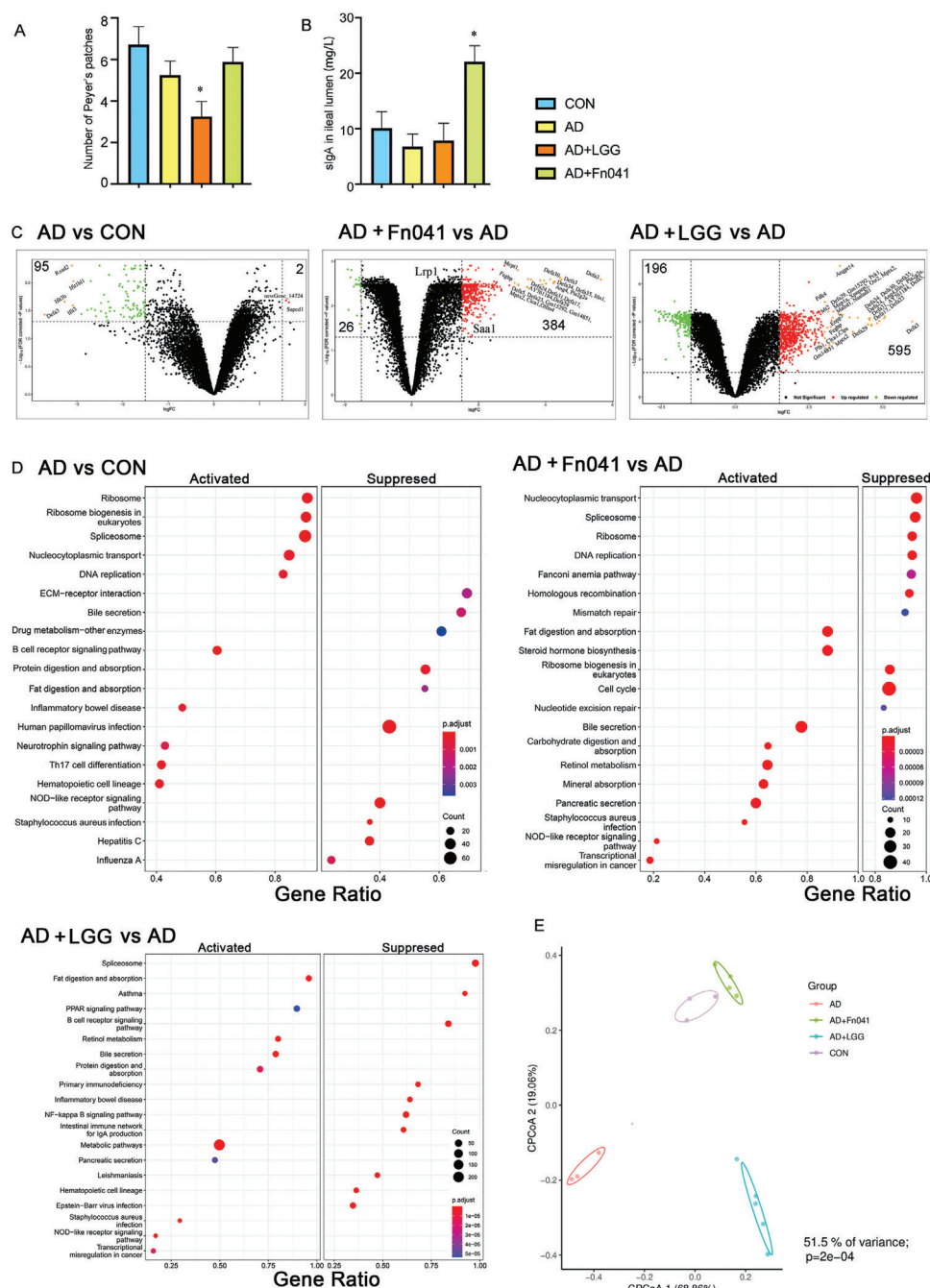


Figure 3. The number of Peyer's patches ($n = 12/\text{group}$) A); ileal free secretory immunoglobulin A (sIgA) levels B); Volcano plot C), KEGG enrichment scatter diagram and CPCoA D) of differently expressed genes for transcriptome analysis of Peyer's patches of offspring mice. For AD versus CON, down genes, log2 fold change more than 2 was labeled, AD+Fn041 up genes log2 fold change less than 3 was labeled. Data are shown as mean $\pm$ SD and compared by student's t -test; $*p < 0.05$ versus CON group. AD, atopic dermatitis model control ($n = 3$); AD+Fn041 group, atopic dermatitis mice gavaged with *Limosilactobacillus reuteri* Fn041 ($n = 4$); AD+LGG group, atopic dermatitis mice gavaged with *Lactisacibacillus rhamnose* GG ($n = 5$). CON, the normal control group ($n = 3$). KEGG, Kyoto Encyclopedia of Genes and Genomes; TSLP, thymic stromal lymphopoietin; ZO-1, zonula occludens 1.

mice. DEGs with a fold change of more than three included genes related to bactericidal activity like 10 alpha defensin genes, Defa39 (AY76184), Defcr-rs (Gm14851), Defa40 (GM15292) and Pla2g2a, four mucin-interacting genes (Clca1, Fcgb, Itln1, and Olfn4), mucosal pentraxin 2 (Mptx2), and Mast cell protease 1 (Mcpt1) (Figure S1, Table S2, Supporting Information). LGG

treatment caused broader gene expression changes. The number of genes significantly up-regulated and down-regulated in LGG-treated mice was 595 and 196, respectively. Among the strongly up-regulated genes (fold change >3), 17 were consistent with Fn041-responsive genes. There are multiple unique genes at the same time, including Angiogenin-4 with bacteri-

dal activity (Ang4), genes involved in the regulation of glucose and lipid metabolism (Angptl4, Pdk4, Pck1, Enpp7, Plb1), metalloprotease related gene (Mt2, Mep1a, Xpnpep2), protein odd-skipped-related 2 (Osr2), fibroblast growth factor 15 (malrd1), Aminopeptidase Naalad1, and E3 ubiquitin-protein ligase UBR1 (Clps).

Next, DRGs were subjected to Kegg pathway enrichment analysis, and Figure 3D shows the top 20 pathways significantly affected by different treatments. The top 10 pathways activated by AD modeling included the B cell receptor signaling pathway, Inflammatory bowel disease, and Th17 cell differentiation, suggesting that pups had inflammatory response activation and Th2-type hyperimmunity. Conversely, the inhibited pathways included protein and lipid digestion, absorption pathway, antibacterial innate immunity-related pathways, NOD-like receptor signaling pathway, and *Staphylococcus aureus* infection, suggesting a decrease in immune defense. These AD-inhibited pathways were significantly activated by Fn041 and LGG treatment, as well as multiple pathways related to other nutrient digestion, absorption, and metabolism, including carbohydrate digestion and absorption, mineral absorption, bile, and pancreatic juice secretion, retinol (vitamin A) metabolism pathways, and steroid hormone biosynthesis. LGG treatment inhibits inflammation-related pathways, such as Asthma, Inflammatory bowel disease, and NF-kappa B signaling pathway, but also inhibits the Intestinal immune network for IgA production (Figure S2, Supporting Information). LGG and Fn041 treatment shifted the overall gene expression of Peyer's patch in pup away from the AD phenotype, toward that of the healthy control group according to the CPcoA plot (Figure 3E).

3.3. *L. reuteri* Fn041 Treatment Activated the Expression of Major Genes in Retinol Absorption and Metabolism Pathways

As a fat-soluble vitamin, retinol and carotenoid are absorbed in the small intestine along with mixed micelle with lipids. Fn041 treatment activated almost all lipid digestion and absorption pathway genes in the pup's small intestine (Figure 4A). Among them, CD36 and niemann pick C1 like 1 are involved in the absorption of retinol precursor, carotene.^[26] Expression of SR-BI was not activated, which was not involved in the uptake of micellar preformed retinol expression of diacylglycerol acyltransferase 1 gene was highly activated which displays an acyl-CoA: retinol acyltransferase activity and plays a role in esterifying retinol into retinyl esters^[27] (Figure 4B). Retinyl-esters and carotenoids would be secreted in the lymph into chylomicrons,^[26] which was demonstrated by activation of genes involved in the formation of prechylomicron and chylomicron like MItp, ApoB-48, ApoA-IV, and ApoA-I (Figure 4A). Significantly up-regulated genes include plasma amyloid A gene and low-density lipoprotein receptor-related protein 1 gen (Figure 3C), which encode proteins involved in retinol uptake and transport into dendritic cells.^[28] The activation of these two genes may mediate the direct transport of retinol to dendritic cells after absorption, without entering the lymphatic circulation. In addition, Fn041 also activates a wide range of other nutrient absorption-related pathways, including mineral absorption (Figure S3, Supporting Information), vitamin digestion and absorption (Figure S4, Supporting

Information), and protein digestion and absorption (Figure S5, Supporting Information).

Fn041 treatment activated expression of alcohol dehydrogenase, retinol dehydrogenase, dehydrogenase/reductase-, and all-trans-retinol dehydrogenase (NAD⁺ (Figure 4B). They are enzymes that convert retinol to retinal. Retinal dehydrogenase gene expression was also significantly up-regulated by Fn041 treatment, this enzyme can further convert all-trans-retinal to all-trans retinoic acid/salt. Fn041 treatment also activated mRNA expression of multiple enzymes that metabolize retinoic acid to inactive secondary products, such as cytochrome P450 family 2 subfamily A6), cytochrome P450 family 3 subfamily A4, P cytochrome P450 family 3 subfamily A, and Glucuronosyltransferase. In addition to alcohol dehydrogenase, LGG treatment also up-regulated the same retinol-converting gene profile.

3.4. *L. reuteri* Fn041 Selectively Activates the Specific Immunity of Peyer's Patches and Inhibits the GO Biological Process Related to the Th2 Immune Response

GO biological process enrichment analysis was performed on the limma normalized RNA-sequencing data (Figure 5). Ten biological processes related to DNA damage response were activated in the AD group, which is the counter-response of the host cells to chronic inflammation.^[29] However, the biological processes related to mucosal innate immunity were inhibited (Figure 5A). Fn041 treatment inhibited DNA damage-related, Th2-type immune (B cell differentiation) and inflammation (mononuclear cell proliferation)-related biological processes, and specially activated metabolic process of fatty acid, lipid, organic acid, steroid, and those related to mucosal innate immune response (Figure 5B). LGG treatment showed a similar effect on activation of innate immune response and metabolic processes but inhibited more processes related to lymphocyte activation (Figure 5C).

3.5. *L. reuteri* Fn041 and LGG Treatment Differentially Affected Peyer's Patch Immune Cell Repertoire

The relative abundance of 36 immune cells in pup's Peyer's patches was estimated based on gene expression profile from RNA-Seq using ImmuCellAI-mouse.^[30] Compared with healthy mice, the M2 macrophage cell group of Peyer's patches in AD mice was significantly reduced, while the relative proportions of CD8⁺ T cells and "effector memory" T cells were significantly increased ($p < 0.05$, Figure 6A). M2 macrophages mainly secrete Arginase-I, IL-10, and transforming growth factor- β and other anti-inflammatory cytokines. Compared with the AD group, Fn041 treatment significantly increased the relation proportion of B cell-like follicular B cells and memory B cells, T cells including Treg, gamma delta T cells cells, Naïve CD4⁺ T cells, T helper cells, and Treg, and granulocytes composed by eosinophil, neutrophils, basophil, and mast cell ($p < 0.05$, Figure 6B). LGG treatment mainly upregulated follicular B, memory B, monocytes, eosinophils, and mast cells ($p < 0.05$, Figure 6C).

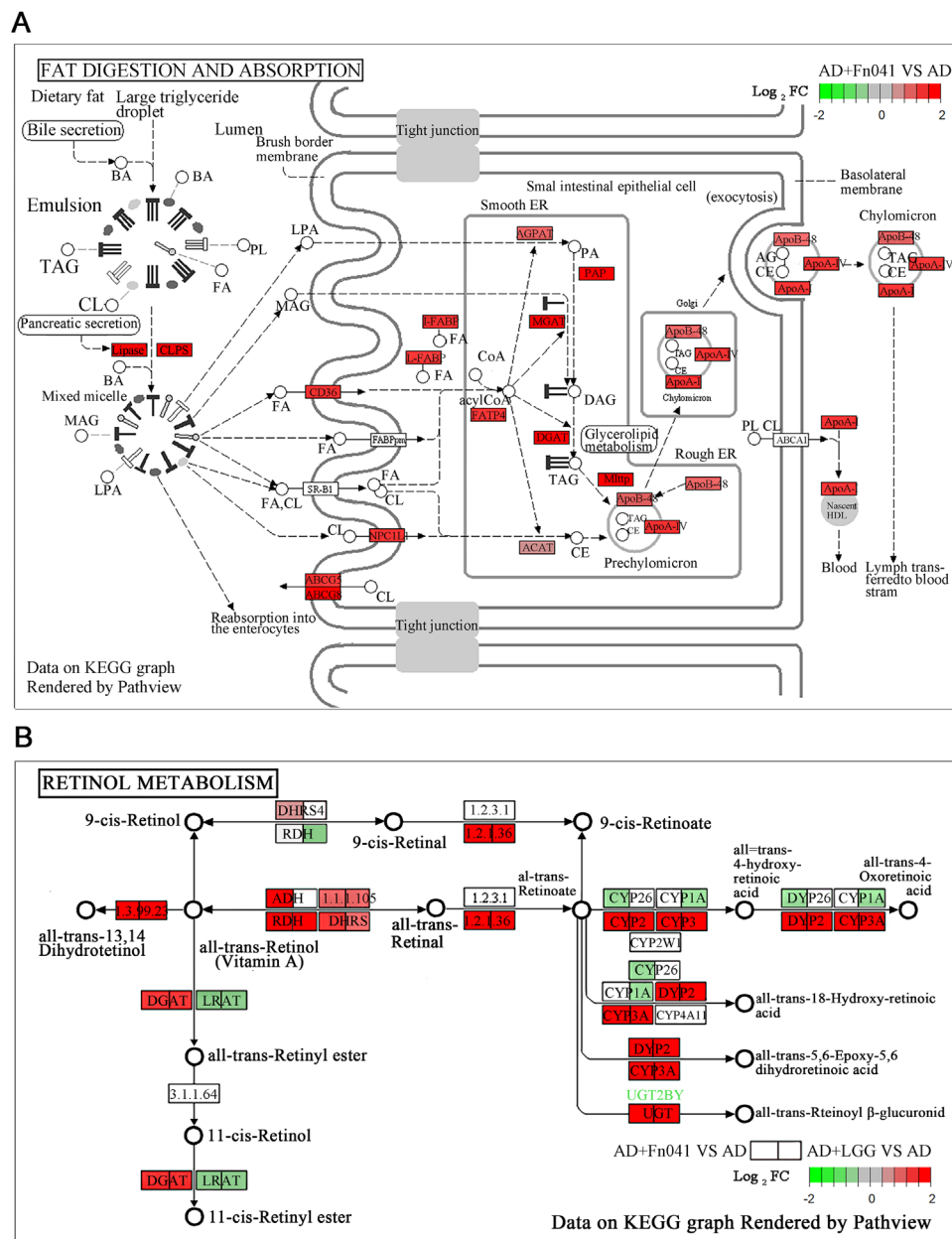


Figure 4. KEGG pathway graph of fat digestion and absorption A) and retinol metabolism B) rendered by Pathview. AD, atopic dermatitis model control ($n = 3$); AD+Fn041 group, atopic dermatitis mice gavaged with *Limosilactobacillus reuteri* Fn041 ($n = 4$); AD+LGG group, atopic dermatitis mice gavaged with *Lactocaseibacillus rhamnose* GG ($n = 5$). CON, the normal control group ($n = 3$). Gene information: <https://kegg.jp/pathway/map00830>; <https://kegg.jp/pathway/map04975>.

3.6. *L. reuteri* Fn041 Treatment Caused Significant Changes in the Plasma Metabolite Composition of Pups

Untargeted metabolomic analysis of plasma revealed that metabolite profiles of the AD group deviated from the CON group according to PLS-DA, and migrated toward the CON group after Fn041 and LGG treatment ($R^2Y = 0.947$, $Q^2 = 0.134$, Figure 7A). According to OPLS-DA, LGG (PI = 19.3%, Figure 7E) and Fn041 (PI = 10.8%, Figure 6H) treatments caused significant changes in plasma metabolic profiles, and the model was good (LGG: $R^2Y = 0.949$, $Q^2 = 0.737$, Figure 6F; Fn041:

$R^2Y = 0.991$, $Q^2 = 0.454$, Figure 7I). AD and CON are also clearly separated (Figure 7C), but the model is poorer according to Q2 ($Q^2Y = 0.134$, Figure 7C). OPLS-DA V-plot for AD versus CON (Figure 6D), AD+LGG (Figure 7F) versus AD, and AD+Fn041 versus AD (Figure 7I) were conducted to select the significant metabolites that were differentially expressed between every two groups, and variables with a VIP more than 1 were selected for subsequent analysis. Plasma retinol, L-Tryptophan, L-Phenylalanine, and 1-Methylnicotinamide were significantly downregulated in AD model mice. Plasma retinol, Kynurenine, and L-Phenylalanine were significantly upregulated after Fn041

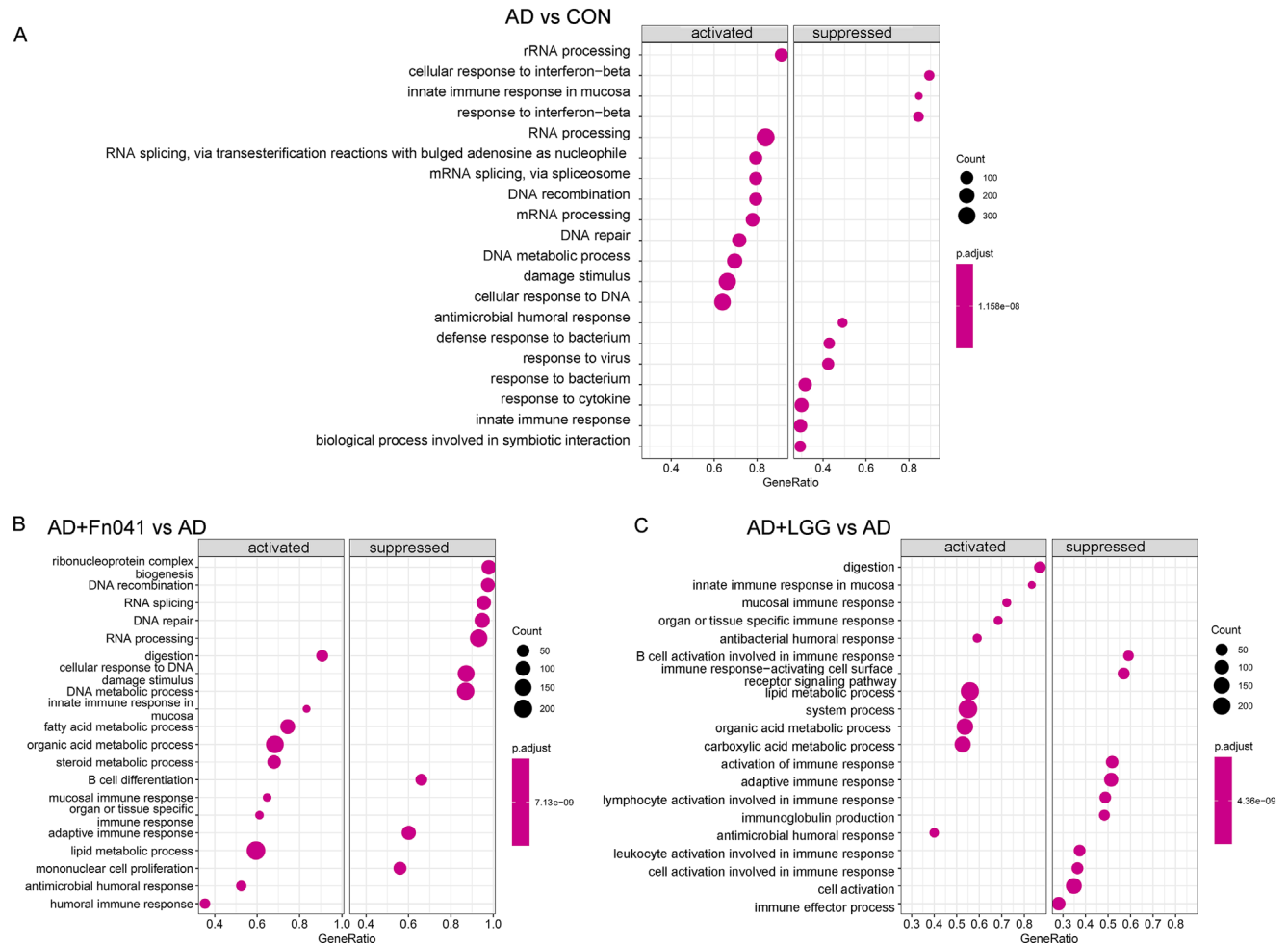


Figure 5. Gene ontology enrichment analysis of biological processes for differently expressed genes. AD versus CON A); AD+Fn041 versus AD B); AD+LGG versus AD C). AD, atopic dermatitis model control ($n = 3$); AD+Fn041 group, atopic dermatitis mice gavaged with *Limosilactobacillus reuteri* Fn041 ($n = 4$); AD+LGG group, atopic dermatitis mice gavaged with *Lactisecibacillus rhamnose* GG ($n = 5$). CON, the normal control group ($n = 3$).

treatment. However, after LGG treatment, kynurenine, indolelactic acid, and retinyl palmitate were significantly up-regulated ($p < 0.05$, Table 1).

We next analyzed whether Fn041 prevents AD by affecting the gut microbiota. According to the hierarchical clustering of ASVs of 16S rDNA amplicons, the AD group did not form an obvious cluster, and the distribution of mice in the probiotic intervention group deviates from the specific branch formed in the CON group. 5/8 of the LGG-treated mouse samples were in a separate clade, but no distinct clade was evident in the Fn041-treated group (Figure 8A). We then performed a cluster analysis on species (Figure 8B). It was found that *Bacteroides oleiciplenus* and *Bacteroides rodentium* were unique species of the AD group. Both Fn041 and LGG treated groups shared *Ligilactobacillus apodemi*, *Ligilactobacillus murinus*, *Akkermansia muciniphila*, *Bacteroides thetaiotaomicron*, and *Citrobacter amalonatigicus*. In addition, the LGG-treated groups were also specifically enriched with *Corynebacterium glutamicum*, *Psychrobacter submarines*, *Klebsiella oxytoca*, *Escherichia fergusonii*, and *Enterobacter cloacae*. According to the PICRUSt2-based gut microbiota function prediction (Figure 8C), 59 functions in the AD group were significantly

changed, and the mean proportion of several functions related to butyrate production was significantly reduced, including acetyl-CoA fermentation to butanoate II, succinate fermentation to butanoate, L-lysine fermentation to acetate and butanoate and glycolysis V. Compared with AD group, Fn041 treatment significantly increased adenosine nucleotides degradation II and superpathway of polyamine biosynthesis II, but decreased glyconeogenesis I. LGG treatment significantly changed 55 functions, of which the top 12 functions with the highest proportions were significantly improved, including the superpathway of lipopolysaccharide biosynthesis and norspermidine biosynthesis. Compared with the CON group, both LGG and Fn041 interventions significantly increased the level of cecal butyrate, but there was no significant change in the AD group (Figure 8D). Fn041 treatment increased propionic acid levels, which was not statistically significant.

4. Discussion

World Allergy Organization suggested routine use of probiotics as an intervention for preventing AD in high-risk infants.^[31]

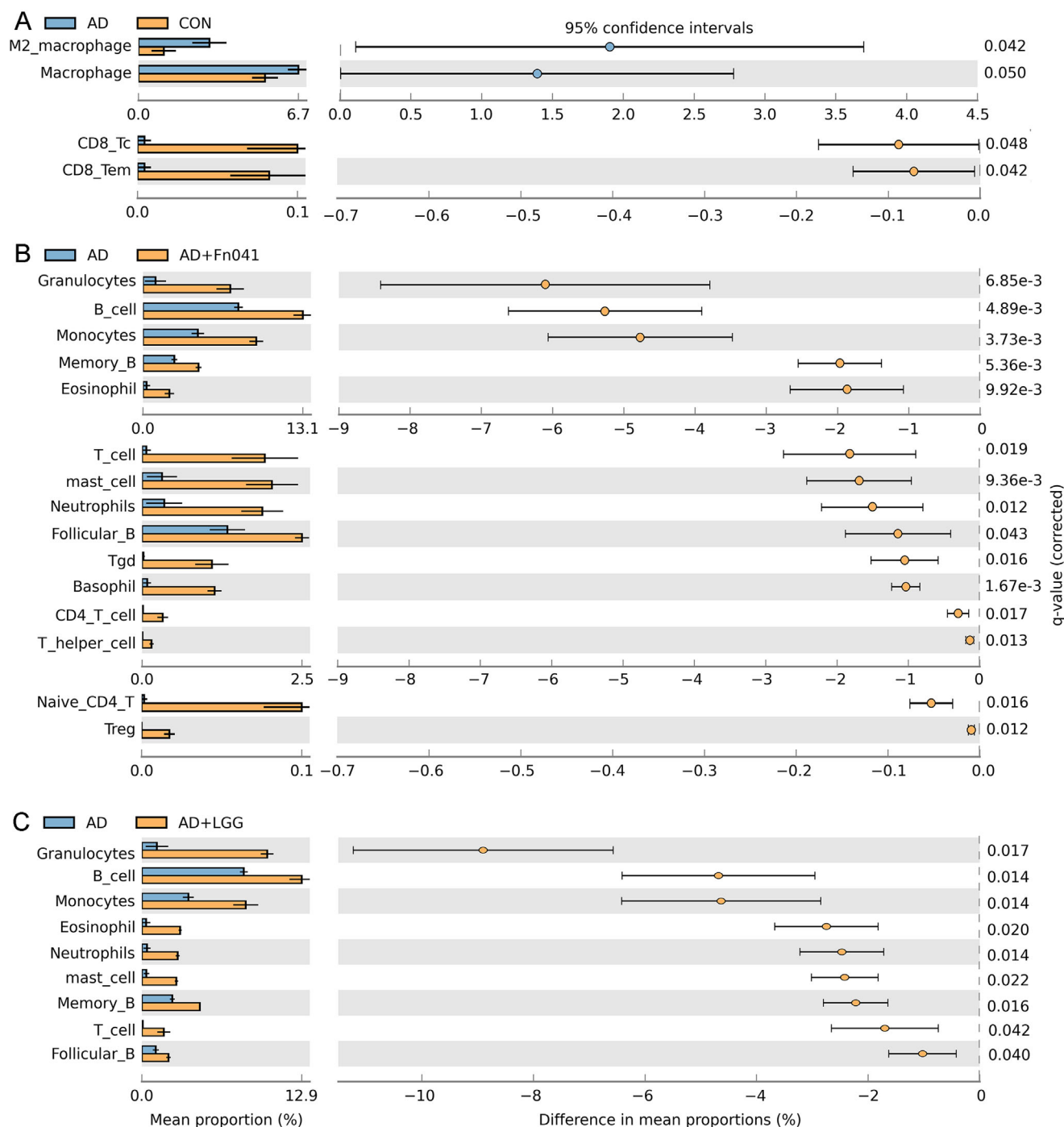


Figure 6. Change in the relative abundance of immune cells in Peyer's patches in AD mice A), that after Fn041 treatment B) and LGG treatment C) which was estimated using ImmuCellAI-mouse based on the gene signatures. AD, atopic dermatitis model control ($n = 3$); AD+Fn041 group, atopic dermatitis mice gavaged with *Limosilactobacillus reuteri* Fn041 ($n = 4$); AD+LGG group, atopic dermatitis mice gavaged with *Lactobacillus rhamnosus* GG ($n = 5$). CON, the normal control group ($n = 3$). Data were compared by two-sided Welch's t -test with Benjamini–Hochberg FDR correction.

However, most studies show significant heterogeneity in strains used for AD preventing.^[32] Therefore, finding suitable strains and elucidating their mechanism of action is still the primary task at present. This study demonstrated that supplementation of a farm breast milk-derived probiotic, *L. reuteri* Fn041, during late pregnancy and lactation in dams prevented AD in pups.

This effect was attributed to the fact that supplementation of Fn041-enhanced gut barrier function, increased the relative level of Treg in Peyer's patch of offspring mice, downregulation of DNA damage response-related pathway, and activated the expression of antimicrobial peptides and retinol absorption and metabolism pathways.

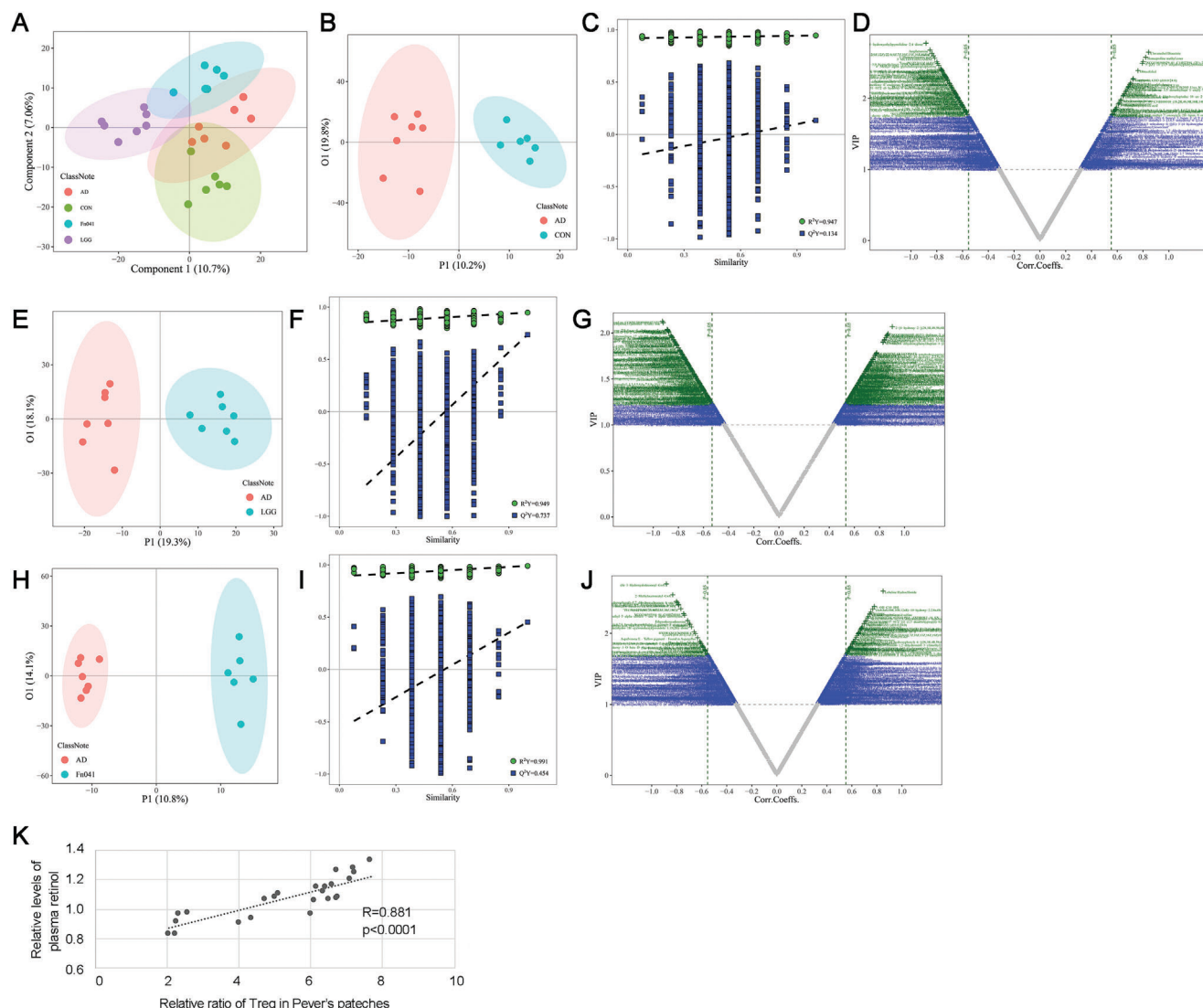


Figure 7. Differences in plasma metabolite profiles between groups by PLS-DA score plots (A: $R^2X = 0.134$, $R^2Y = 0.181$, $Q^2 = 0.101$), OPLS-DA score plot (B: $R^2X = 0.301$, $R^2Y = 0.947$, $Q^2 = 0.134$; E: $R^2X = 0.374$, $R^2Y = 0.949$, $Q^2 = 0.737$; H: $R^2X = 0.249$, $R^2Y = 0.991$, $Q^2 = 0.454$), the corresponding permutation plots C, F, I), v-plot D, G, J), and Pearson correlation between relative levels of plasma retinol and relative proportion of Peyer's junction regulatory T cells K). Untargeted metabolomic analysis was performed with UPLC-QTOF MS/MS. AD, atopic dermatitis model control ($n = 7$); AD+Fn041 group, atopic dermatitis mice gavaged with *Limosilactobacillus reuteri* Fn041 ($n = 6$); AD+LGG group, atopic dermatitis mice gavaged with *Lactisacibacillus rhamnase* GG ($n = 7$); CON, the normal control group ($n = 6$).

The induction of Treg cells is necessary for the control of AD, which suppresses mediator release from mast cells, conversion of conventional T cells into Th2 cells, and IgE production by B cells.^[33] Our previous study demonstrates that during late pregnancy and lactation Fn041 intervention induced proliferation of Treg in mesenteric lymph nodes of pups.^[9] The current study showed that Fn041 treatment upregulated Treg in pups Peyer's patch. Treg cells can be induced by tolerogenic DCs (tolDCs).^[34] The tolDCs were not defined based on specific phenotypic markers, therefore, we could not detect them by the RNA-seq prediction method. Diverse stimuli are reported to induce a tolDC phenotype, RA and aromatic hydrocarbon receptors ligands.^[34] In the present study, Fn041 treatment activated the retinol metabolism pathway which would result in

RA production and also significantly increased plasma retinol, a retinoic acid precursor, and kynurenine, and inhibited the decrease in L-tryptophan, precursors of kynurenine. L-kynurenine is an aryl hydrocarbon receptor agonist that activates aryl hydrocarbon receptor-directed polarization of naive T cells to an anti-inflammatory Treg phenotype.^[35] All these are favorable for tolDC and Treg induction.

It was found that lower plasma retinol levels were common in AD infants, and retinol deficiency worsened Th2 pathway-mediated inflammation in AD mice model.^[36] We found that plasma retinol was significantly reduced in AD mice, which was consistent with their finding. It was found that Treg and CD27⁺IgA⁺ B-cells are elevated in children with atopic disease to compensate for chronic inflammation.^[37] Both cells require

Table 1. Plasma metabolites significantly differentially expressed between AD and other groups.

Metabolites	AD versus CON			AD+LGG versus AD			AD+Fn041 versus AD		
	VIP	P	Change	VIP	P	Change	VIP	P	Change
Retinol	1.52	0.041	↓				2.00	0.014	↑
alpha-Tocopherol				1.53	0.009	↑			
Leucine	2.62	0.00	↓						
Kynurenine				1.43	0.017	↑	1.50	0.046	↑
Glycocholic acid				1.68	0.003	↑			
Glucosamine 6-phosphate	1.92	0.025	↓	1.34	0.027	↓			
Ganglioside GM3 (d18:0/24:0)	2.25	0.006	↑	1.22	0.048	↑	2.12	0.008	↑
Cholesterol				1.65	0.003	↑	1.57	0.070	↑
1-Methylnicotinamide	2.28	0.005	↓	1.51	0.010	↑			
(S)-3-Hydroxydecanoyl-CoA	1.87	0.031	↓	1.86	0.000	↓	2.68	0.000	↓
LysoPC(P-18:1(9Z))	1.85	0.033	↓						
LysoPE(0:0/15:0)				1.04	0.099	↑			
LysoPE(0:0/20:11Z)	2.35	0.003	↓	1.95	0.00	↓	1.98	0.016	↓
Menaquinone-4	2.25	0.006	↓	1.26	0.04	↓	1.47	0.095	↓
L-Tryptophan	1.85	0.033	↓						
Indolelactic acid				1.16	0.043	↑			
L-Phenylalanine	1.89	0.029	↓	1.52	0.009	↑	1.76	0.038	↑
L-Tyrosine	1.56	0.083	↓						
1-Methylnicotinamide	2.28	0.005	↓	1.51	0.010	↑			
Retinyl palmitate				1.15	0.066	↑			
Lactosylceramide (d18:1/12:0)				1.25	0.041	↓			

VIP scores were obtained from the OPLS-DA model. AD, atopic dermatitis model control ($n = 7$); AD+Fn041 group, atopic dermatitis mice gavaged with *Limosilactobacillus reuteri* Fn041 ($n = 6$); AD+LGG group, atopic dermatitis mice gavaged with *Lactocaseibacillus rhamnose* GG ($n = 7$); CON, the normal control group ($n = 6$); VIP, variable importance in projection.

RA induction and thus may cause retinol depletion. In this study, the promotion of retinol absorption by Fn041 may be the key to preventing AD inflammation. Further, Fn041 activated the lipid absorption pathway and retinol absorption pathway which may contribute to elevated plasma retinol. Fn041 also activated the expression of low density protein receptor related protein 1 and serum amyloid A1 (saa) genes. Recently, Lrp1 was identified as a myeloid cell surface receptor for retinol, which attaches to Saa-retinol complexes to promote endocytosis.^[28] The up-regulated expression of these two genes may suggest that the dendrite cells of Peyer's patch endocytosis retinol for transformation into RA, which needs to be further verified.

Atopic dermatitis is a systemic disorder characterized by abnormal barrier function across multiple organ sites including the gut.^[38] In the present study, Fn041 treatment activated the tight junction pathway of Peyer's patches (Figure S6, Supporting Information). The mRNA expression of ZO-1 and Claudin-2 (a pore-forming claudin) in the ileum was activated and inhibited, respectively. The expression of Claudin-2 enhances intestinal mucosal permeability through the formation of permeable paracellular channels, and is positively correlated with mucosal barrier damage.^[39] Fn041 inhibition of Claudin-2 gene expression may enhance intestinal mucosal barrier function. Fn041 activates the expression of genes that are key components in a variety of mucus (Cluster 1 in Figure S1, Supporting Information)

and the production of sIgA. Both are involved in the formation of the intestinal mucosal barrier.^[40,41] Increased sIgA production imply induction of Foxp3⁺ Tregs, which could differentiate into Foxp3⁺ T follicular helper cells in the PPs and promote the generation of IgA-producing B cells.^[42] SIgA plays an important role in the maintenance of tolerance by capturing foreign structures and commensal, which prevent them from access into the tissues beneath the mucosa.^[43]

Intestinal dysbiosis is one of the main features of AD.^[44] In this study, the gut microbiota of AD mice was significantly altered, and the functions related to butyrate production were significantly reduced. Fn041 treatment did not significantly increase cecal short chain fatty acids, but only increased the abundance of four species, *L. apodemi*, *L. murinus*, *Akkermansia muciniphila*, and *B. thetaiotaomicron*. RORγ⁺ Treg could be induced by specific pulmonary *L. murinus*.^[45] Mice gut *L. murinus* was reported to attenuate food-allergic responses by promoting Th1 polarization and modulated dendritic cell functions.^[46] Children with persistent AD showed a decreased gut *Akkermansia*.^[47] However, other research found that *A. muciniphila* was enriched in eczematous infants.^[48] Although whether *A. muciniphila* has a protective effect on AD is inconclusive. But its anti-inflammatory properties have been confirmed by other studies.^[49] There is no study on the association between *B. thetaiotaomicron* and AD, but the anti-inflammatory nature of this species has been proved.^[50] Fn041

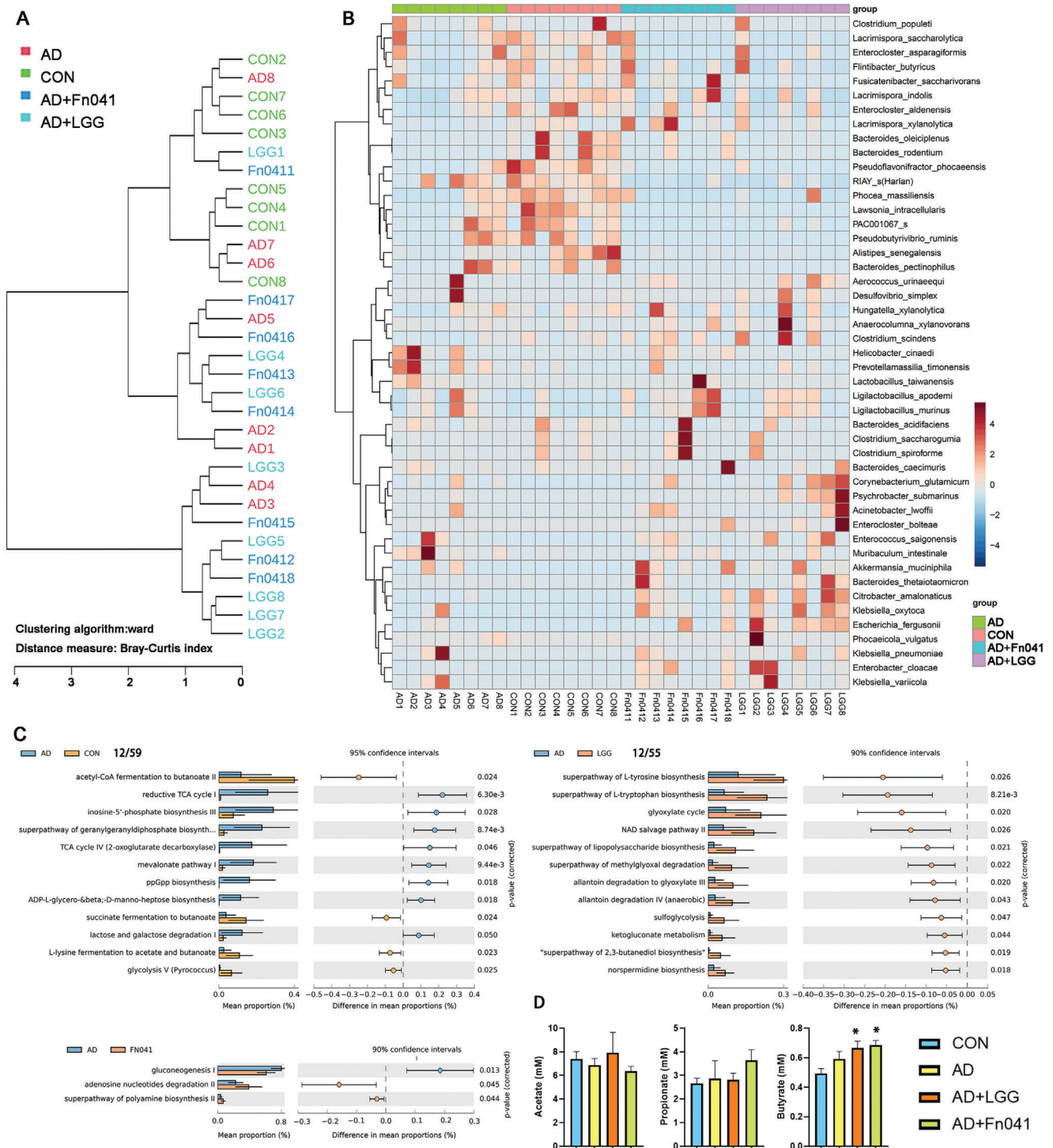


Figure 8. The shift of gut microbiota and function for *L. reuteri* Fn041 and *Lactobacillus rhamnose* GG treatment in AD mice ($n = 8/\text{group}$). Hierarchical clustering of the samples based on microbiota ASVs performed on Bray–Curtis dissimilarity and clustered using Ward's hierarchical clustering method A). Hierarchical clustering and Heatmap of species with distance measured by Eudclidean using Ward clustering algorithm B). The difference of PICURSt2 functions prediction of microbiota which was analyzed by two-sided Welch's *t*-test and the result was filtered for *Q* value less than 0.05 and affect less than 0.30 C). Concentration of short-chain fatty acids D). Data are shown as mean $\pm$ SD and compared by student's *t*-test (unpaired; $n = 12/\text{group}$); * $p < 0.05$ versus CON group. AD, atopic dermatitis model control; AD+Fn041 group, atopic dermatitis mice gavaged with *Limosilactobacillus reuteri* Fn041; AD+LGG group, atopic dermatitis mice gavaged with *Lactobacillus rhamnose* GG; CON, the normal control group.

treatment also increased superpathway of polyamine biosynthesis II of gut microbiota, for potential production of polyamine that could promote homeostatic differentiation of Treg cells.^[51]

LGG is currently the most widely studied strain for AD prevention.^[52] However, some researchers observed that administration of LGG increased the risk of AD.^[53] In this study, LGG showed limited efficacy in preventing AD. This may be attributed to the inhibition of Peyer's patches formation and its IgA production pathway, the up-regulation of the lipopolysaccharide production pathway, and the relative content of potential inflammatory bacteria in gut microbiota.

The shortcomings of this study mainly include: 1) Fn041 is more likely to colonize the ileum, and the ileal microbiota was not analyzed. 2) Fn041 may enter PPs to play a role, which requires further study.

5. Conclusion

During late pregnancy and lactation supplementation of Fn041 prevented AD development in pups. Fn041 enhances the intestinal mucosal barrier, promotes retinol absorption and metabolic pathways of Peyer's patches, and increases relative abundance of Treg in Peyer's patches, thereby inhibiting ear Th2 inflammation and mast cell granulation of skin, and ultimately inhibiting AD.

Supporting Information

Supporting Information is available from the Wiley Online Library or from the author.

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Conflict of Interest

The authors declare no conflict of interest.

Author Contributions

C.Q. and H.T. contributed by acquiring analyzing, interpreting the data, and drafting the work. C.Q. contributed by acquiring the data, data visualization, and by revising the work. Y.Z., J.Z., J.C., and H.H. contributed by acquiring, analyzing, and interpreting the data. J.S. contributed by conceiving and designing the work, analyzing and interpreting the data, and drafting and revising the work. R.Y. Obtain funding and participate in experimental design. All authors have approved the submitted version and have agreed to be personally accountable for the author's contributions.

Data Availability Statement

Raw sequencing data have been deposited in the National Center for Biotechnology Information Sequence Read Archive database with accession number PRJNA747168.

Keywords

atopic dermatitis, gut microbiota, *Limosilactobacillus reuteri*, regulatory T cells, retinol

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