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Ovotransferrin alleviates ulcerative colitis by mediating gut microbiota involvement in secondary bile acid biosynthesis

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ABSTRACT: Ovotransferrin (OVT) is a functionally active protein that has been demonstrated to alleviate ulcerative colitis (UC), yet its underlying mechanism of action has not yet been fully elucidated. In this context, this study employed a DSS-induced UC mouse model to confirm that OVT alleviates UC, as evidenced by reduced disease activity index (DAI; 40.63%), histopathological score (42.50%), and expression of TNF- α (38.50%), IL-1 β (41.73%), and IL-6 (41.73%). However, in the pseudo-germ-free mouse model, OVT failed to alleviate UC. Subsequently, after fecal microbiota transplantation, the alleviation of OVT on UC was restored, including reducing the DAI (46.38%), histopathological score (38.46%), and the expression of TNF- α (30.97%), IL-1 β (28.95%), and IL-6 (42.40%). These results indicated that OVT alleviates UC in a gut microbiota-dependent manner. The 16S rRNA gene sequencing was employed to investigate the changes in the gut microbiota of mice, and it was found that *g__Lactobacillus* might be a potential genus (highest LDA score: 4.77). Subsequently, broad-target metabolomics identified 206 differential metabolites (DMs) in the intestinal contents of the OVT vs DSS group. KEGG pathway analysis of the DMs revealed significant enrichment in the secondary bile acid biosynthesis pathway. Furthermore, 6 differential bile acids (DBAs) were identified, including ursodeoxycholic acid (UDCA), 3-epideoxycholic acid (3 β -DCA), deoxycholic acid (DCA), chenodeoxycholic acid (CDCA), tauroursodeoxycholic acid (TUDCA) and isochodeoxycholic acid (isoCDCA), which reduced paracellular flux of FITC-Dextran (16.80%-31.16%) and Lucifer yellow (31.61%-47.09%), the expressions of TNF- α (36.19%-59.30%), IL-1 β (41.30%-54.12%), and IL-6 (31.40%-53.15%), thereby exhibiting anti-inflammatory properties. Notably, UDCA, isoCDCA, and TUDCA exhibited stronger anti-inflammatory effects. In summary, this study established the connection among OVT, the gut microbiota, and bile acids, and elucidated the mechanism by which OVT alleviates UC through modulation of the gut microbiota and its involvement in secondary bile acid biosynthesis.

Keywords: Ovotransferrin; Ulcerative colitis; Gut microbiota; Pseudo-germ-free mouse model; Fecal microbiota transplantation; Bile acid

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1. Introduction

Ulcerative colitis (UC) is a subtype of inflammatory bowel disease (IBD). Clinical studies have shown that UC typically manifests initially in the rectum and may extend continuously upward to the proximal colon^[1]. Its clinical manifestations primarily include bloody diarrhea and abdominal pain. Recently, the global prevalence and incidence of UC have continued to rise, making it a significant public health challenge^[2]. Epidemiological data indicate that although incidence rates have stabilized or declined in some high-income countries, they continue to increase markedly in many low- and middle-income countries, and even persist in certain high-income regions^[2, 3]. Currently, UC is understood as a chronic inflammatory condition resulting from the interplay of multiple factors, including impaired intestinal barrier function, immune dysregulation, and alterations in the gut microbiota, which occur in genetically susceptible individuals triggered by environmental factors^[4]. Clinical management relies primarily on aminosalicylates, corticosteroids, and immunosuppressants^[5]. Although corticosteroids are effective in controlling inflammation, their systemic side effects and, in some cases, low oral bioavailability limit their applicability^[6]. Biologics such as anti-TNF- α agents and IL-12/23 inhibitors, as well as subsequently developed anti-integrin therapies and S1P receptor modulators, offer additional options for patients unresponsive to conventional treatments^[7, 8]. However, most of these drugs are costly, may increase risks such as infection, and none are capable of curing the disease^[5, 9, 10]. Thus, identifying safer and more effective therapeutic approaches remains an important objective in current UC studies.

The dysbiosis of gut microbiota is a central element in the progression of UC^[4]. Therefore, therapeutic strategies targeting the gut microbiota attracted considerable attention. Analysis of surgical specimens revealed a significant decrease in the abundance of commensal bacteria such as p__Bacteroidetes and p__Firmicutes in the intestines of UC patients^[11], particularly butyrate-producing strains including *Roseburia hominis* and *Faecalibacterium prausnitzii*^[12, 13]. These alterations lead to impaired epithelial barrier function, immune activation, and chronic inflammation^[14]. In clinical observations of UC, patients achieving mucosal healing following infliximab treatment exhibited partial restoration of beneficial bacteria (e.g., *Faecalibacterium prausnitzii* and *Bacteroides fragilis*) in fecal samples^[15]. Moreover, fecal microbiota transplantation (FMT) to reconstitute the gut microbiota resulted in significant alleviation of UC^[16]. Subsequently, researchers focused on the regulatory effects of natural food components on the gut microbiota as a means to ameliorate UC. Previous studies have shown that food components such as egg white protein^[17], enzymatically hydrolyzed wheat bran-derived zinc phytate^[18], carvacrol and thymol^[19] were demonstrated to modulate the gut microbiota, thereby alleviating UC. Notably, the gut microbiota not only participates in host immune responses through strain-host interactions but is also involved in host metabolic processes, including bile acid metabolism^[20], tryptophan metabolism^[21], and lipid metabolism^[22]. Recent studies demonstrated that microbial metabolites, such as short-chain fatty acids, tryptophan-derived indole compounds, succinate, secondary bile acids, and retinoic acid, can regulate the quiescence, proliferation, and differentiation of gastrointestinal stem cells through multiple dimensions^[23], highlighting their potential in alleviating UC.

However, given the unique pathophysiological context of UC and factors such as microbial diversity, intervention strategies targeting the gut microbiota still require further elucidation of the underlying biochemical processes, specific mechanisms of action, and strain-specific interactions to achieve dual optimization of efficacy and safety^[24]. Therefore, identifying specific bioactive proteins from natural food sources, particularly those with established antimicrobial activities and the capacity to promote beneficial microbial metabolite production, represents a promising avenue for developing targeted microbiota-based therapies for UC.

Ovotransferrin (OVT) is a member of the transferrin family. It is a glycoprotein composed of a single polypeptide chain containing 686 amino acid residues, with a molecular weight of approximately 77.9 kDa^[25]. The structure of OVT is similar to that of lactoferrin (LF), and OVT also possesses biological functions analogous to those of LF, including anti-inflammatory activity and multiple potential benefits for intestinal health^[26, 27]. Inflammation is a normal immune response; however, persistent or excessive inflammation drives the progression of various diseases, particularly chronic intestinal inflammation. In animal models, oral administration of OVT significantly alleviates symptoms of experimental colitis by regulating cytokines and improving epithelial function^[28]. Furthermore, various bioactive peptides derived from its degradation (e.g., CR, FL, HC, LL MK, IRW and IQW) have been confirmed to possess distinct anti-inflammatory properties^[29, 30]. These findings collectively reveal, from the intact protein level down to specific peptides, the potential of OVT and its hydrolysates in modulating inflammation. Additionally, researches indicate that OVT and its active peptide IRW can modulate the gut microbiota structure in model mice, increasing the ratio of beneficial to harmful bacteria, and improving intestinal morphology and the mucosal barrier^[31, 32]. However, the underlying mechanisms through which OVT interacts with the gut microbiota remain poorly understood, and the causal relationship between its microbiota-modulating effects and anti-inflammatory activity has yet to be fully elucidated.

Therefore, this study aims to elucidate the association between OVT, gut microbiota, and the ameliorative effect of OVT on UC. In this study, a DSS-induced UC mouse model was used to verify the alleviating effect of OVT on UC. Subsequently, the necessity of gut microbiota in OVT-mediated alleviation of UC was investigated using a pseudo-germ-free mouse model, while 16S rRNA sequencing was employed to clarify the impact of OVT on the gut microbiota. Subsequently, broad-targeted metabolomics was applied to analyze metabolite changes in colons, and an LPS-induced Caco-2 cell inflammation model was utilized to validate the anti-inflammatory effects of the identified metabolites. In summary, this study clarified the mechanism through which OVT alleviates UC by modulating gut microbiota and its metabolites, thereby providing scientific evidence for OVT as a novel dietary nutrient for promoting intestinal microecological homeostasis.

2. Materials and methods

2.1 Materials and reagents

OVT (purity $\geq$ 90.00%) was provided by Anhui Rongda Food Co., Ltd. (Guangde, China). Ampicillin, gentamicin, neomycin, metronidazole, vancomycin, lipopolysaccharides (LPS; from *Escherichia coli* O55:B5), dextran sulfate sodium salt (DSS; 40 kDa), ursodeoxycholic acid (UDCA), 3-epideoxycholic acid (3 β -DCA), deoxycholic acid (DCA), chenodeoxycholic acid (CDCA), tauroursodeoxycholic acid (TUDCA) and isochodeoxycholic acid (isoCDCA) were purchased from Shanghai Aladdin Biochemical Technology Co., Ltd. (Shanghai, China). The C57BL/6 mice (male, 5 weeks old, 22-25 g) were supplied by GemPharmatech Co., Ltd. (Nanjing, China). Caco-2 cells (CL-0050) were obtained from Procell Life Science & Technology Co., Ltd. (Wuhan, China).

2.2 Animals and treatment

All animals were housed in the Laboratory Animal Center of Hefei University of Technology under standard conditions. The ethics license number was HFUT20250405001M. All animals were subjected to adaptive feeding for 7 days and then randomly divided into three groups (Con, DSS and OVT) based on body weight, with 12 animals in each group. The Control group received free access to sterile deionized water, while the DSS and OVT groups were administered with water containing 3% (wt/vol) DSS for 7 days to induce UC. Subsequently, all mice were transitioned to normal drinking water. The Con and DSS groups received physiological saline for 7 days, while the OVT group was administered OVT. Based on the results of our previous study, OVT was administered at a daily dose of 200 mg kg⁻¹[33].

2.3 Disease activity index assessment

The disease activity index (DAI) was determined using established scoring criteria^[34]. Scoring followed the grading standards provided in Table S1, and the DAI was derived using the following formula:

$$DAI = \frac{\text{body weight loss} + \text{Hematochezia} + \text{Fecal condition}}{3} \quad (1)$$

2.4 Histopathological evaluation and immunofluorescence staining

Colonic tissues were fixed with 4% paraformaldehyde, followed by paraffin embedding and sectioning at a thickness of 5 μ m. Sections were stained with hematoxylin and eosin (H&E), periodic acid-Schiff (PAS), and immunofluorescence (IF; antibody details are provided in Table S2). Images were acquired using slide scanners (PANNORAMIC MIDI II, 3DHISTECH for H&E and PAS; KF-PRO-040-HI, KONFOONG BIOINFORMATION TECH for IF). Histopathological evaluation was conducted in accordance with the established criteria detailed in Table S3^[35], with a maximum score of 11. Goblet cells were quantified as described previously^[36].

2.5 Quantitative Real-Time PCR

RNA was extracted from samples using TRIzol (Thermo, Waltham, MA, USA), checked on a 1% agarose gel, and reverse-transcribed into cDNA with a PrimeScript RT kit (Thermo, Waltham, MA, USA). RT-qPCR was performed on a 7500 Real-Time PCR System (ABI, California, USA) with SYBR Green Mix (Yifei Xue

Biotech, China) under standard cycling conditions (95°C/15 min; 40 cycles: 95°C/10 s, 55°C/20 s, 72°C/32 s). Relative expression levels were normalized with reference to GAPDH and calculated using the $2^{-\Delta\Delta CT}$ method. The sequences of the primers employed in this study were as detailed in Table S4.

2.6 Intestinal permeability

The mice (Fasting for 24 h) were orally gavaged with FITC-dextran (4 kDa, 60 mg kg⁻¹). After 4 h, serum was collected, and concentration of FITC-dextran (Ex/Em: 490/520 nm) was measured using a microplate reader (Varioskan LUX; Thermo Scientific, MA, USA).

2.7 Cytokine and oxidative stress assays

TNF- α , IL-1 β , IL-6 and IL-10 were assayed using ELISA kits (Solarbio, Beijing, China). The CAT, GSH-Px, SOD activities and MDA concentration were assayed using biochemical kits (Nanjing Jiancheng, Nanjing, China).

2.8 Establishment of pseudo-germ-free mouse model

The housing conditions for the mice and the ethical approval number were the same as those described in “2.2 Animals and treatment”. The mice were randomly divided into three groups (Ab, Ab-OVT, and OVT-FMT) based on body weight, with 12 mice in each group. Subsequently, All mice were free to drink antibiotic solution containing ampicillin (1 mg mL⁻¹), gentamicin (1 mg mL⁻¹), neomycin (1 mg mL⁻¹), metronidazole (0.5 mg mL⁻¹), and vancomycin (0.5 mg mL⁻¹) for 14 days^[37]. Following antibiotic treatment, UC was induced using DSS (3% wt/vol) for 7 days and subsequent experiments were conducted. The Ab group received normal saline for 7 days; the Ab-OVT group received 200 mg kg⁻¹ of OVT; and the Ab-FMT group received FMT and 200 mg kg⁻¹ of OVT.

2.9 Fecal microbiota transplantation

Fresh fecal samples from healthy mice were suspended in sterile PBS (0.1 μ mol L⁻¹, pH=7.4, Biosharp, Beijing, China) to obtain a 200 mg mL⁻¹ homogenate, and centrifuged (4°C, 1000 rpm, 5 min) to prepare the fecal microbiota suspension. OVT was subsequently dissolved in this suspension (5 mg mL⁻¹) and administered to pseudo-germ-free mice at a daily dose of 200 mg kg⁻¹ OVT.

2.10 16S rRNA sequencing

DNA was extracted from mouse colonic contents using the E.Z.N.A.® Stool DNA Kit (Omega Bio-Tek, USA). The quality, concentration, and purity of DNA were assessed by 1% agarose gel electrophoresis and a NanoDrop 2000 spectrophotometer (Thermo Scientific, Wilmington, USA), respectively. The V3-V4 region of the bacterial 16S rRNA gene was amplified with primers 338F/806R (338F: 5'-ACTCCTACGGGAGGCAGCAG-3', 806R: 5'-GGACTACHVGGGTWTCTAAT-3') on a GeneAmp 9700 thermal cycler (ABI, USA). Amplified products were used to construct libraries with the TruSeq® DNA PCR-

Free Sample Preparation Kit (Illumina, San Diego, USA). Libraries were quantified using Qubit® and qPCR, and qualified ones were sequenced on an Illumina MiSeq platform (Illumina, San Diego, USA) following the standard protocols provided by Jiangsu Sanshu Biotechnology Co., Ltd. (Jiangsu, China). Taxonomic annotation was performed using QIIME2 and the SILVA database.

2.11 Measurement of metabolites using LC-MS/MS

Broad-targeted and analyses were conducted by Metware Biotechnology Co., Ltd. (Wuhan, China). Metabolites were extracted from samples using 70% (vol/vol) aqueous methanol (for broad-targeted) or 99% (vol/vol) methanol (for targeted). After homogenization, samples were centrifuged (4°C, 12000 rpm, 10 min), and the supernatant was collected for LC-MS/MS to identify DMs (VIP>1; $P < 0.05$, Student's t-test). Subsequently, DMs were annotated and subjected to pathway mapping using the KEGG database.

2.12 Cell culture

Caco-2 cells were cultured in DMEM supplemented with 10% FBS and 1% penicillin-streptomycin at 37°C under 5% CO₂. Cells were treated with LPS (1.0 µg mL⁻¹, 24 h) to induce inflammation, followed by treatment with bile acids (BAs; 0 mM, 25 mM, 50 mM, 100 mM, 200 mM) at appropriate concentrations for another 24 h before being collected for subsequent assays. Cytotoxicity was assessed by CCK-8 method.

2.13 Measurement of paracellular permeability

Cells were seeded at a density of 2×10^5 mL⁻¹ onto polycarbonate membrane in Transwell inserts (pore size 0.4 µm; Corning, Lowell, MA) and cultured for 14-21 days to form a single cell layer. Transepithelial electrical resistance (TEER) was monitored regularly using Millicell ERS-2 (Millipore, Billerica, MA). When TEER exceeded 400 Ω·cm², the cells were used for subsequent permeability assays^[38]. The 100 µg mL⁻¹ FITC-dextran (4 kDa; Ex/Em: 490/520 nm; Aladdin, Shanghai, China) and Lucifer yellow (Ex/Em: 410/520 nm; Aladdin, Shanghai, China) were added to the top chamber of the Transwell system and incubated for 4 h. Fluorescence intensity of the solution from basolateral compartment was measured using a microplate reader (Varioskan LUX; Thermo Scientific, MA, USA).

2.14 Statistical analysis

Data were presented as mean ± SD. GraphPad Prism (version 10) was used for figure preparation. Statistical significance was analyzed using IBM SPSS Statistics (R27.0.1.0). Group comparisons were made using Student's t-test or one-way ANOVA, with statistical significance defined as $P < 0.05$. Significance was indicated using lowercase letters.

3. Results

3.1 Oral OVT alleviated DSS-induced UC

To investigate the beneficial effects of OVT on UC, the experimental procedure shown in Figure 1A was conducted. OVT significantly ameliorated DSS-induced UC, as evidenced by attenuation of body weight loss, reduction in DAI, prevention of colon shortening, and decreased intestinal permeability (Figure 1B-F). Moreover, OVT downregulated the levels of TNF- α , IL-1 β , and IL-6, while elevating the level of IL-10 (Figure 1G-J). Additionally, OVT reduced oxidative stress in colonic tissues by increasing the activities of CAT, GSH-Px, and SOD, and decreasing the concentration of MDA (Figure 1K-N). Histological analysis further revealed that OVT alleviated tissue damage, inflammatory infiltration, and lowered the histopathological score (Figure 1O&P). PAS staining showed that OVT restored the number of goblet cells per crypt (Figure 1O&Q). Concurrently, IF and RT-qPCR analysis of colons showed that OVT enhanced the expression of MUC2, Claudin-1, Occludin, and ZO-1, thereby improving intestinal barrier dysfunction (Figure 1O&R-U).

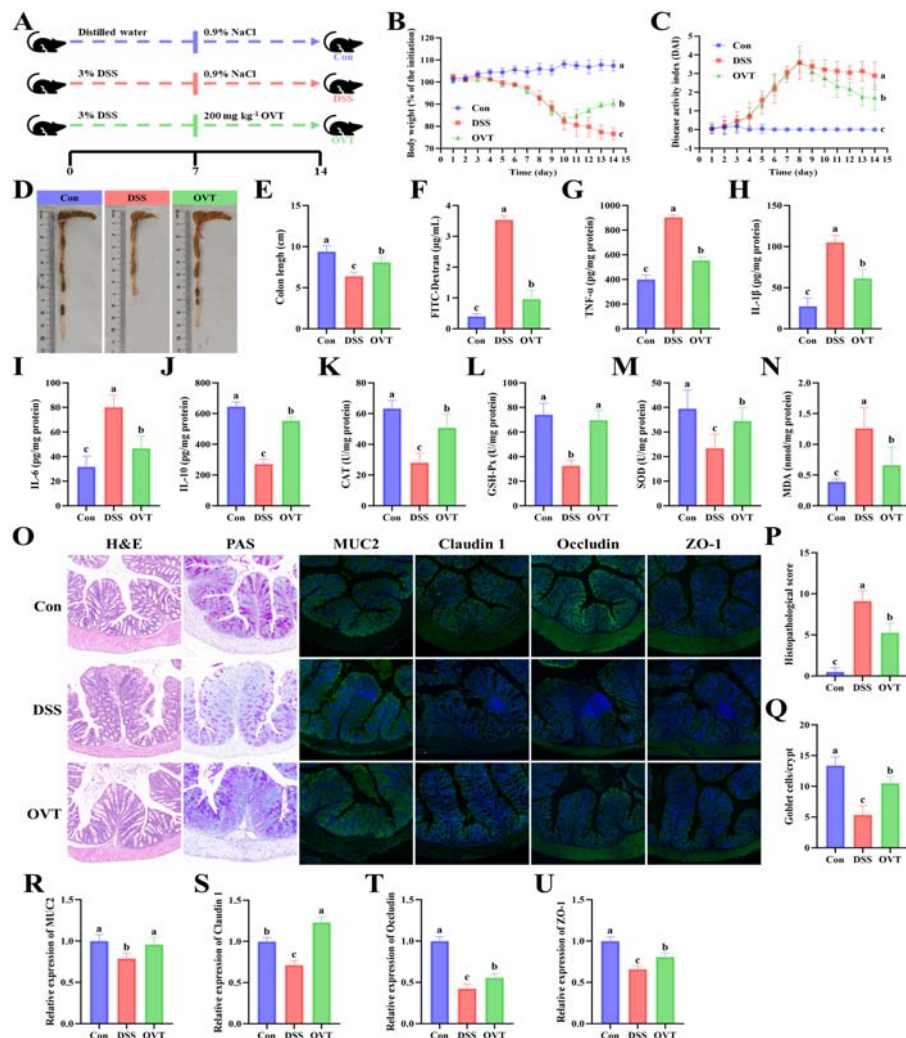


Figure 1 OVT ameliorated DSS-induced UC in mice. (A) Schematic of the experimental design. (B) Body weight changes. (C) DAI. (D) Representative macroscopic images of colons. (E) Colon length. (F) Intestinal permeability. (G-J) Concentrations of TNF- α , IL-1 β , IL-6, and IL-10 in colonic tissues. (K-N) Activities of CAT, GSH-Px, SOD and the concentrations of MDA in colonic tissues. (O) Representative images of H&E staining, PAS staining, and IF staining of colons. (P) Histopathological scores. (Q) Goblet cell counts per crypt. (R-U) Relative mRNA expression levels of MUC2, Claudin-1, Occludin, and ZO-1 in colonic tissues. Data are presented as mean $\pm$ SD (n=5-8 per group).

3.2 OVT alleviated DSS-induced UC in a gut microbiota-dependent manner

To investigate the necessity of the gut microbiota in the alleviation of UC by OVT, the experimental procedure shown in Figure 2A was conducted. The gut microbiota of the mice was analyzed using 16S rRNA sequencing, and it was found that treatment with the antibiotic mixture reduced the diversity of the gut microbiota (Figures 2B-G), indicating the successful establishment of a pseudo-germ-free mouse model. In the pseudo-germ-free model, OVT failed to ameliorate DSS-induced UC (Figure 3A-T). However, transplantation of fecal microbiota from healthy mice into the pseudo-germ-free model significantly alleviated UC symptoms, as evidenced by attenuated weight loss, reduced DAI, improved colon shortening, and decreased intestinal permeability (Figure 3A-E); concurrently, the balances of cytokine expression and redox reaction were restored (Figure 3F-M). RT-qPCR analysis of colons showed that OVT-FMT treatment increased the expression of MUC2, Claudin-1, Occludin, and ZO-1, consistent with IF results (Figure 3N-R). Furthermore, H&E staining revealed that OVT-FMT alleviated tissue damage and inflammatory infiltration, reducing the histopathological score from 10.13 to 6.00 (Figure 3R&S). PAS staining indicated an increase in goblet cell count from 3.38 to 8.75 cells per crypt (Figure 3R&T). These findings demonstrated that the ameliorative effect of OVT on DSS-induced UC was dependent on the gut microbiota.

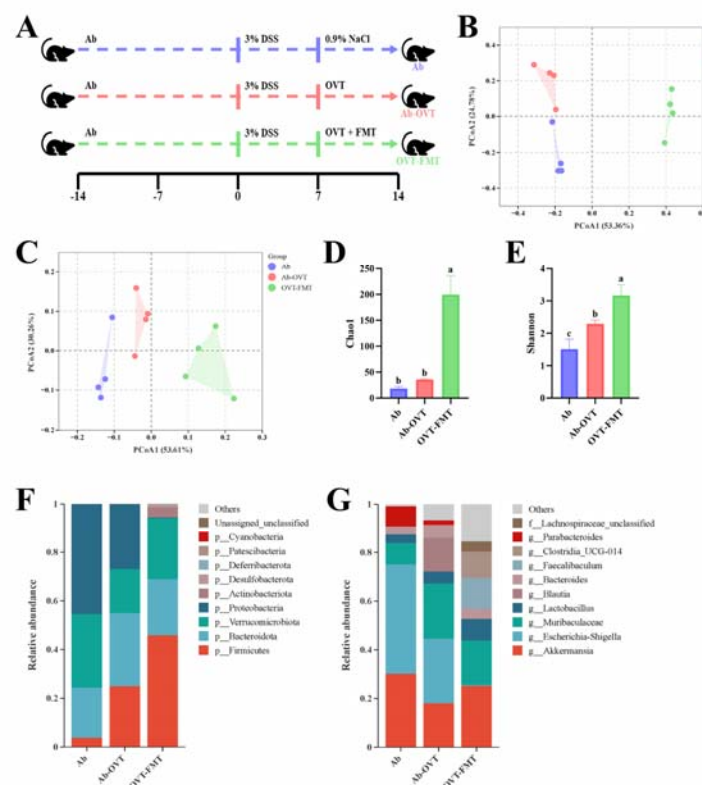


Figure 2 Effects of OVT on gut microbiota in pseudo-germ-free mice. (A) Schematic of the experimental design. (B) PCoA based on Bray-Curtis. (C) PCoA based on weighted UniFrac distances. (D) Chao1 index. (E) Shannon index. (F) Relative abundance of gut microbiota at the phylum level. (G) Relative abundance of gut microbiota at the genus level. Data are presented as mean $\pm$ SD (n=4 per group).

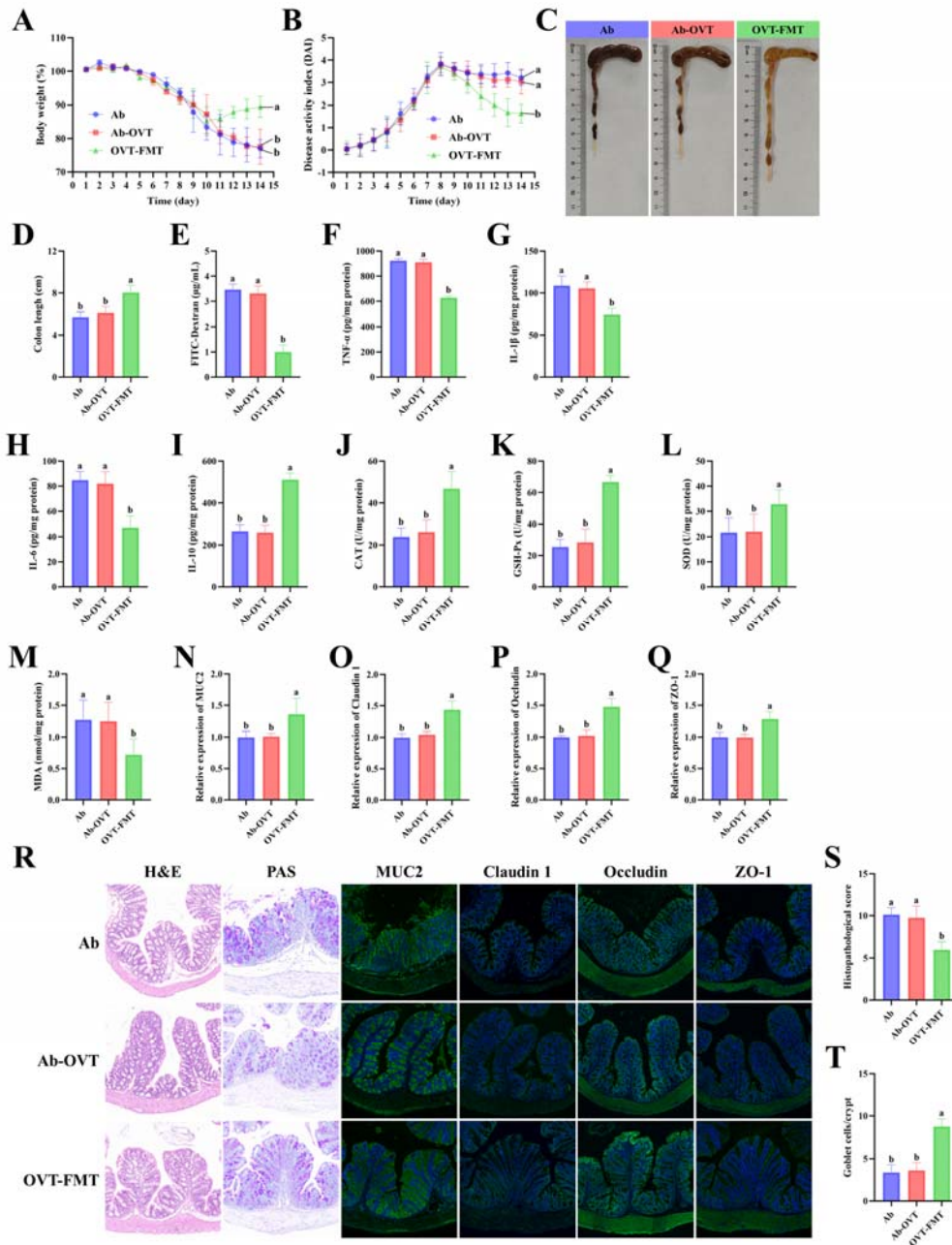


Figure 3 OVT alleviated DSS-induced UC in a gut microbiota-dependent manner. (A) Body weight changes. (B) DAI. (C) Representative macroscopic images of colons. (D) Colon length. (E) Intestinal permeability. (F–I) Concentrations of TNF- α , IL-1 β , IL-6, and IL-10 in colonic tissues. (J–M) Activities of CAT, GSH-Px, SOD and the concentrations of MDA in colonic tissues. (N–Q) Relative mRNA expression levels of MUC2, Claudin-1, Occludin, and ZO-1 in colonic tissues. (R) Representative images of H&E staining, PAS staining, and IF staining of colons. (S) Histopathological scores. (T) Goblet cell counts per crypt. Data are presented as mean $\pm$ SD (n=5–8 per group).

3.3 Effects of OVT on gut microbiota

To identify the key microbiota mediating the beneficial effects of OVT on clinical symptoms and colonic injury in UC, 16S rRNA sequencing was employed to assess the influence of OVT on gut microbial diversity and composition. Principal coordinate analysis (PCoA) revealed that there was a clear clustering of microbiota among groups (Figure 4A&B). The α -diversity analysis indicated that OVT mitigated the DSS-induced decline in gut microbial diversity and richness (Figure 4C&D). To explore the overall structure and composition of the gut microbiota, taxonomic similarities were analyzed. At the phylum level, OVT

significantly reversed the DSS-induced reduction in the abundance of p__Firmicutes (Figure 4E). At the genus level, OVT increased the abundances of g__Lactobacillus, g__Faecalibaculum, g__Muribaculaceae, g__Akkermansia, g__Desulfovibrio, and others (Figure 4F). LEfSe analysis identified 6 bacterial genera enriched in the OVT group, among which g__Lactobacillus showed the highest LDA score (4.77), suggesting it may be a key genus involved in OVT-mediated alleviation of UC (Figure 4G).

Additionally, OVT exerted a discernible influence on the gut microbiota of pseudo-germ-free mice (Figure 2B&C). While OVT did not significantly affect the Chao1 index in pseudo-germ-free mice, it increased their Shannon index (Figure 2D&E). Notably, although OVT treatment increased the Shannon index in pseudo-germ-free mice, it remained lower than that in SPF mice treated with OVT (Ab-OVT: 2.30 ± 0.11 , OVT: 4.79 ± 0.04 ; Figure 2E and Figure 4D). Following FMT, both the Chao1 and Shannon indices increased significantly (Figure 2D&E). Subsequent taxonomic analysis showed that the abundances of p__Firmicutes and g__Lactobacillus increased after FMT (Figure 2F&G).

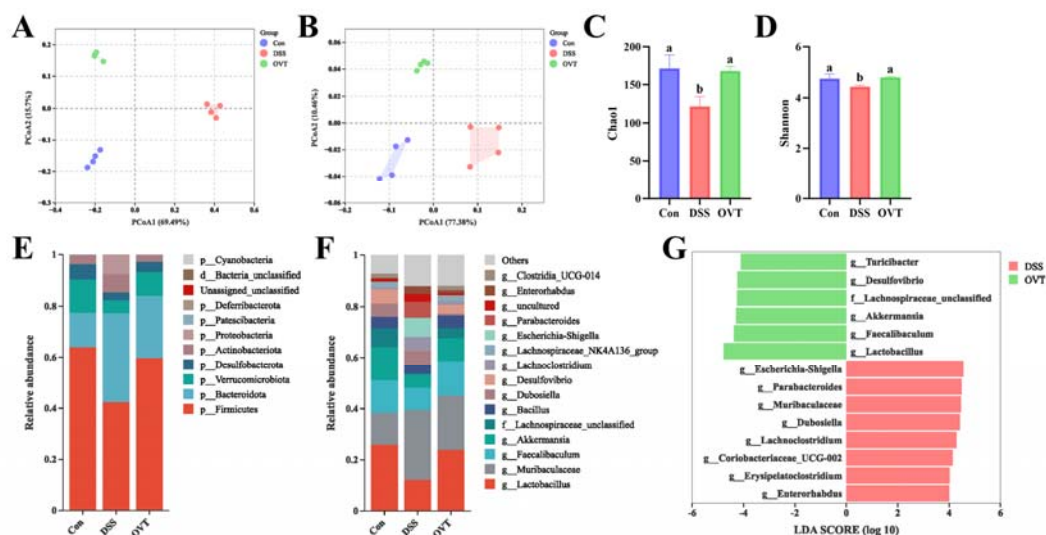
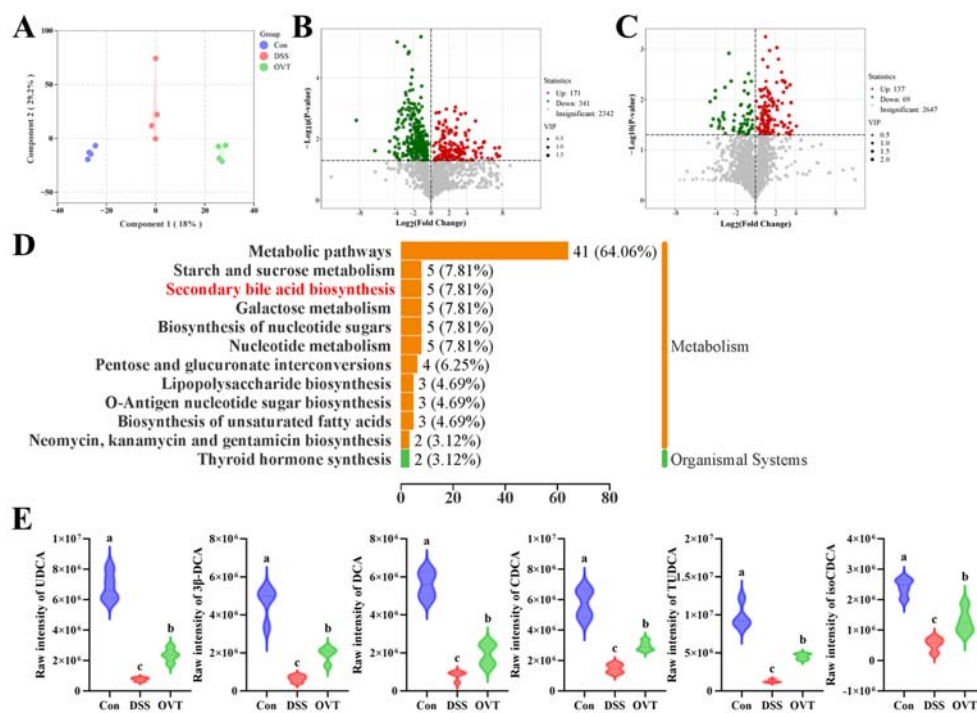


Figure 4 Effects of OVT on gut microbiota composition. (A) PCoA based on Bray-Curtis. (B) PCoA based on weighted UniFrac distances. (C) Chao1 index. (D) Shannon index. (E) Relative abundance of gut microbiota at the phylum level. (F) Relative abundance of gut microbiota at the genus level. (G) LEfSe analysis. Data are presented as mean $\pm$ SD (n=4 per group).

3.4 Effects of OVT on gut metabolites

The involvement of gut microbiota in host metabolic activities was well established by performing broad-target metabolomics on colonic contents. Orthogonal partial least squares-discriminant analysis (OPLS-DA) of the overall metabolome revealed clear separation among the Con, DSS, and OVT groups (Figure 5A). The 512 differential metabolites (DMs; up-regulated: 171, down-regulated: 341) were identified in the DSS vs Con group. In contrast, 206 DMs (up-regulated: 137, down-regulated: 69) were identified in OVT vs DSS group (Figure 5B&C). To elucidate the metabolic pathways associated with these DMs, KEGG enrichment analysis was conducted, and metabolic pathways, starch and sucrose metabolism, secondary bile acid biosynthesis, galactose metabolism, biosynthesis of nucleotide sugars, and nucleotide metabolism were

enriched (Figure 5D). The results of 16S rRNA sequencing indicated that *g__Lactobacillus* was a potentially key genus. Additionally, Ziwei Song et al. demonstrated that *g__Lactobacillus* harbors genes encoding bile salt hydrolase (BSH) and is capable of participating in host bile acid metabolism^[39-42]. Furthermore, in our previous study, metabolomic analysis of colonic tissues from mice orally administered with OVT revealed enrichment of the bile secretion pathway^[33]. Based on these findings, secondary bile acid biosynthesis was suggested as a potential pathway through which OVT mediates *g__Lactobacillus* to alleviate UC. Furthermore, 6 BAs were found to be increased in the OVT group, including UDCA, 3 β -DCA, DCA, CDCA, TUDCA and isoCDCA (Figure 5E). Pearson correlation analysis showed that UDCA, 3 β -DCA, CDCA, and TUDCA were positively correlated with colon length, oxidative stress-related enzyme activities, IL-10 levels, goblet cell count, and tight junction (TJ) protein expression, and negatively correlated with intestinal permeability, pro-inflammatory cytokine levels, MDA concentration, and histopathological score (Figure 5F). Although DCA showed no correlation with MUC2 and ZO-1 expression, and isoCDCA showed no correlation with MUC2 expression, their correlations with other indicators were consistent with those of UDCA, 3 β -DCA, CDCA, and TUDCA (Figure 5F). Additionally, all of 6 BAs were positively correlated with the abundance of *g__Lactobacillus*. In brief, Pearson correlation analysis statistically demonstrated the levels of the 6 BAs correlated with the severity of UC as well as the abundance of *g__Lactobacillus* (Figure 5F). These findings suggested that OVT may alleviate DSS-induced UC by mediating the involvement of *g__Lactobacillus* in secondary bile acid biosynthesis.



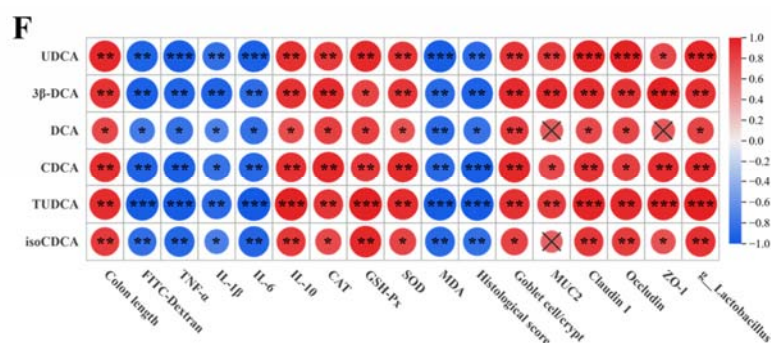


Figure 5 Effects of OVT on gut metabolites. (A) OPLS-DA of overall metabolomic profiles. (B) Volcano plots of DMs in DSS vs Con. (C) Volcano plots of DMs in OVT vs DSS. (D) KEGG pathway enrichment analysis of DMs in OVT vs DSS comparison. (E) Concentrations of UDCA, 3β-DCA, DCA, CDCA, TUDCA and isoCDCA. (F) Pearson correlation analysis among the 6 BAs, the severity of UC, and the abundance of *g__Lactobacillus*: * $P < 0.05$, ** $P < 0.01$, *** $P < 0.001$. Data are presented as mean $\pm$ SD (n=4 per group).

3.5 Effects of BAs on LPS-Induced Caco-2 inflammation

The anti-inflammatory effects of UDCA, 3β-DCA, DCA, CDCA, TUDCA, and isoCDCA were validated using an LPS-induced Caco-2 cell inflammation model. To determine the optimal concentration of BAs for cell experiments, the effect of BAs on Caco-2 cell viability was assessed using the CCK-8 assay, and the results were shown in Supplementary Figure S1. The cell viability results showed that BAs at low concentrations (≤ 50 mM), including UDCA, 3β-DCA, DCA, CDCA, TUDCA, and isoCDCA, did not exhibit cytotoxicity (supplementary Figure S1). However, when the concentration exceeded 50 mM, cell viability began to decrease (supplementary Figure S1). Therefore, 50 mM was selected as the concentration for subsequent experiments. As shown in Figure 6A-K, LPS reduced the TEER of Caco-2 cells and increased paracellular permeability (Figure 6A-C). Furthermore, LPS increased the expression of TNF-α, IL-1β, IL-6 and elevated oxidative stress levels, while reducing the expression of IL-10. (Figure 6D-K). In contrast, UDCA, 3β-DCA, DCA, CDCA, TUDCA, and isoCDCA reversed the adverse effects induced by LPS, with UDCA, isoCDCA, and TUDCA demonstrating the most significant effects (Figure 6A-K). These results indicated that UDCA, 3β-DCA, DCA, CDCA, TUDCA, and isoCDCA exhibited favorable anti-inflammatory activity. Notably, the anti-inflammatory effects of UDCA, isoCDCA, and TUDCA were stronger than those of 3β-DCA, DCA, and CDCA.

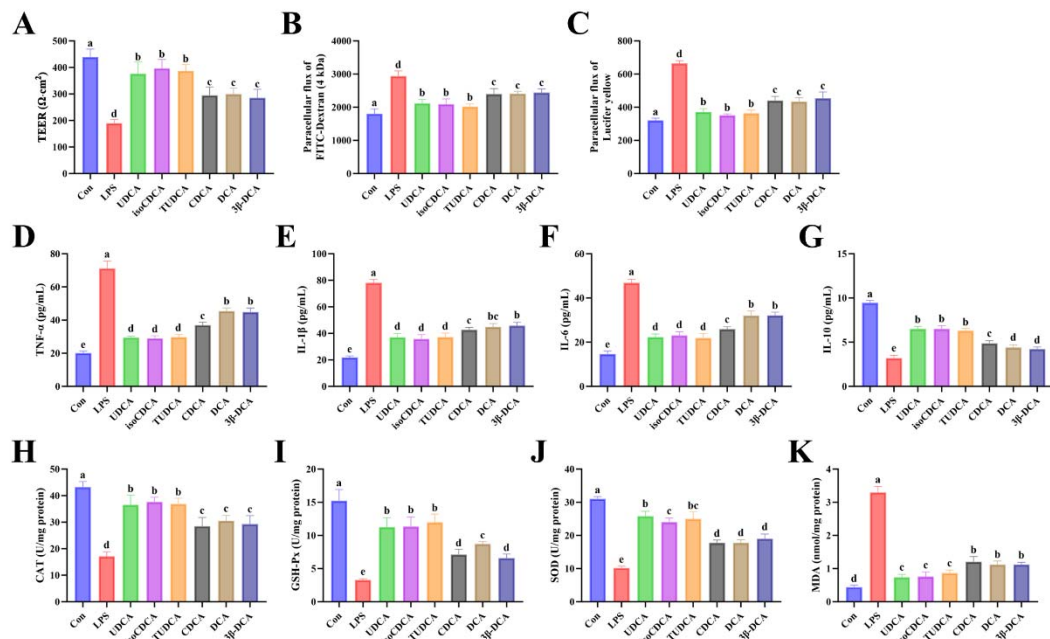


Figure 6 BAs suppressed LPS-induced inflammation in Caco-2 cells. (A) TEER. (B) Paracellular flux of FITC-dextran (4 kDa). (C) Paracellular flux of Lucifer yellow. (D-G) Concentrations of TNF- α , IL-1 β , IL-6 and IL-10. (H-K) Activities of CAT, GSH-Px, SOD and concentrations of MDA. Data are presented as mean $\pm$ SD (n=5-8 per group).

4. Discussion

The intestine is not only the largest immune organ in the human body in terms of surface area and the organ with the highest antigen load, but it is also a complex ecosystem^[43]. It harbors trillions of microorganisms (including viruses, bacteria, fungi, and other microbes) that exist in a state of dynamic balance and participate in maintaining host immune homeostasis^[44]. Studies have confirmed that the dysbiosis of gut microbiota is one of the key features of UC, and modulating the gut microbiota represents a potential therapeutic strategy for UC^[4]. Subsequent studies have confirmed that reconstitution of the gut microbiota significantly ameliorates UC^[16, 45]. Compared with commonly used drugs in the clinical treatment of UC, such as aminosalicylates, corticosteroids, and immunosuppressants, therapeutic strategies targeting the gut microbiota offer a better safety profile and lower toxicity^[5]. Notably, these gut microbiota-targeted approaches highlight the role of nutritional intervention in the treatment of UC^[20]. In recent years, researchers identified dietary components capable of alleviating UC by modulating the gut microbiota^[46]. As a functional dietary protein, OVT has been demonstrated to ameliorate UC^[28]. However, it remains unclear whether OVT alleviates UC by modulating immune responses through the gut microbiota and its metabolites. Therefore, this study employed a DSS-induced colitis model and a pseudo-germ-free mouse model to investigate whether OVT alleviates UC by modulating the gut microbiota, and metabolomics was used to elucidate the metabolic changes in intestinal contents.

The pathogenesis of UC remains incompletely understood, however, dysbiosis of the gut microbiota with notable reductions in Bacteroidetes and Firmicutes has been established as closely associated with both the onset and exacerbation of UC^[4, 11]. In DSS-induced UC model, oral administration of OVT significantly ameliorated disease severity, as evidenced by attenuated weight loss, reduced DAI, decreased colon

shortening, and reduced intestinal permeability (Figure 1B-F). These clinical improvements were paralleled by restored cytokine balance, alleviation of oxidative stress, promotion of colonic mucosal repair, and enhanced expression of TJ proteins (Figure 1G-U). Subsequently, to determine whether these protective effects were dependent on the gut microbiota, a pseudo-germ-free mouse model was established using a broad-spectrum antibiotic cocktail. Strikingly, OVT failed to mitigate the adverse effects induced by DSS in the pseudo-germ-free model (Figure 2A-T), indicating that OVT alleviates UC in a gut microbiota-dependent manner. Further analysis via 16S rRNA gene sequencing revealed that OVT significantly enriched specific bacterial genera in the gut microbiota of UC mice, including *g__Lactobacillus*, *g__Faecalibaculum*, *g__Muribaculaceae*, *g__Akkermansia*, and *g__Desulfovibrio* (Figure 4F). Among these, *g__Lactobacillus* exhibited the highest LDA score (4.77, Figure 4G). This genus is widely recognized as a probiotic and can inhibit UC progression by modulating epithelial homeostasis and inflammatory responses^[47]. Therefore, it represents a potential key microbial mediator in the amelioration of UC by OVT.

Generally, the gut microbiota does not directly act on intestinal tissues but rather contributes to the maintenance of intestinal homeostasis by participating in host metabolic processes^[48]. Therefore, this study utilized broad-targeted metabolomics to analyze metabolic differences in intestinal contents, identifying a total of 206 differential metabolites (Figure 5C). KEGG pathway analysis revealed significant enrichment in the metabolic pathways, starch and sucrose metabolism, secondary bile acid biosynthesis, galactose metabolism, biosynthesis of nucleotide sugars, and nucleotide metabolism pathways (Figure 5D). Ziwei Song et al. analyzed metagenomic data from public human microbiome databases and demonstrated that *g__Lactobacillus* harbors genes encoding BSH and is involved in host bile acid metabolism^[42]. Chen Liu et al. confirmed that *Lactobacillus johnsonii* is capable of converting primary bile acids into secondary bile acids, thereby inhibiting tumor development^[49]. Furthermore, Liansheng Zhang et al. demonstrated that the enzymatically hydrolyzed wheat bran-derived zinc phytate modulates the host's bile acid profile through *Lactobacillus vaginalis*, thereby alleviating UC^[18]. In our previous study, the bile secretion pathway was significantly enriched in the colonic tissue of UC mice orally administered OVT^[33]. Collectively, these findings suggested that secondary bile acid biosynthesis represents a potential pathway through which OVT mediates the alleviation of UC via *g__Lactobacillus*. Subsequently, six differential metabolites annotated within this pathway were further screened: UDCA, 3 β -DCA, DCA, CDCA, TUDCA, and isoCDCA (Figure 5E). Pearson correlation analysis showed that, statistically, UDCA, 3 β -DCA, DCA, CDCA, TUDCA, and isoCDCA were associated with the severity of UC as well as the abundance of *g__Lactobacillus* (Figure 5F). Among them, UDCA, DCA, CDCA, and TUDCA have been shown to regulate the NF- κ B signaling pathway through receptors such as PXR, FXR, and TGR5, thereby suppressing inflammatory responses^[50-52]. Although 3 β -DCA and isoCDCA have not been demonstrated to have significant therapeutic efficacy in colitis models or clinical trials, it can activate FXR to exert immunomodulatory effects^[53, 54].

To validate the anti-inflammatory properties of these metabolites, an LPS-induced Caco-2 cell inflammation model was employed. Treatment with UDCA, 3 β -DCA, DCA, CDCA, TUDCA, and isoCDCA

reversed the LPS-induced increase in paracellular permeability (Figure 6A-C). Furthermore, these compounds reduced the concentrations of TNF- α , IL-1 β , IL-6, and MDA, while increasing the concentration of IL-10 and enhancing the activities of CAT, GSH-Px, and SOD (Figure 6D-K). These results demonstrated that UDCA, 3 β -DCA, DCA, CDCA, TUDCA, and isoCDCA possess significant anti-inflammatory effects. Notably, the anti-inflammatory effects of UDCA, isoCDCA, and TUDCA were stronger than those of 3 β -DCA, DCA, and CDCA. A review study by Yinping Pan et al. indicated that host bile acid metabolism is a complex process involving multiple BAs, and listed several BAs capable of alleviating IBD, including isolithocholic acid (isoLCA), allolithocholic acid (alloLCA), 3-oxolithocholic acid (3-oxoLCA), 3-oxoalloithocholic acid (3-oxoalloLCA), and isodeoxycholic acid (isoDCA)^[20]. Although there are no reports comparing the anti-inflammatory effects of different BAs, given the fact that the host's bile acid profile is relatively complex, the beneficial role of any individual component within the complex gut microecology should not be overlooked. Based on these findings, OVT mediates the gut microbiota to improve secondary bile acid biosynthesis, thereby ameliorating UC. Furthermore, *g__Lactobacillus* may represent a potential key genus in this process.

5. Conclusions

In summary, this study utilized a DSS-induced UC model to confirm the therapeutic efficacy of OVT in ameliorating UC. By constructing a pseudo-germ-free mouse model, it was demonstrated that OVT alleviates UC in a gut microbiota-dependent manner. Subsequently, 16S rRNA gene sequencing analysis revealed that OVT increased the abundance of *g__Lactobacillus* in the intestinal contents. Further, broad-targeted metabolomics identified 6 DBAs (UDCA, 3 β -DCA, DCA, CDCA, TUDCA, and isoCDCA) in the intestinal contents. Using an LPS-induced Caco-2 cell inflammation model, the anti-inflammatory properties of these 6 DBAs were validated. The results indicated that UDCA, 3 β -DCA, DCA, CDCA, TUDCA, and isoCDCA exhibit significant anti-inflammatory effects, particularly UDCA, isoCDCA, and TUDCA. Collectively, this study established a link among OVT, gut microbiota, and BAs in the mitigation of UC, demonstrating that OVT mediates the gut microbiota to improve secondary bile acid biosynthesis, thereby ameliorating UC. Furthermore, *g__Lactobacillus* may represent a potential key genus in this process.

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Conflict of interest

The authors report there are no competing interests to declare.

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