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Original Article

# Atractylenolide III Relieves Diphenoxylate-Induced Constipation *via* Gut Microbiota and Bile Acid Pathway Modulation

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## ABSTRACT

**Aim/Background:** An imbalance of intestinal flora and its fermentation products can disrupt intestinal peristalsis and lead to constipation. This study aimed to investigate how Atractylenolide III (ATL-III) may enhance constipation treatment by regulating the gut microbiota-bile acid metabolism pathway. **Materials and Methods:** In this study, an experimental mouse model of constipation was established and intestinal motility, colonic neurotransmitter release, and bile acid metabolism were measured. By 16S rDNA gene sequencing, the character of the balance of gut microbiota was observed in constipated mice, and the relationship between intestinal flora, bile acid, and neurotransmitter secretion was analyzed. **Results:** Our findings proved that ATL-III treatment profoundly boosted intestinal motility, promoted colonic neurotransmitter release, and prevented gut barrier damage in constipated mice induced by diphenoxylate. Liquid chromatography-tandem mass spectrometry showed that ATL-III also regulated bile acid metabolism in constipated mice. Several intestinal bacteria at phylum, family, and genus levels were statistically reversed when atractylenolide III was administered to constipated mice, as revealed by 16S rDNA gene sequencing. This partly led to a balance in the production of intestinal metabolites, including bile acids. ATL-III-initiated constipation prevention confirms the importance of gut microbiota. **Conclusion:** ATL-III may ameliorate the development of compound diphenoxylate-induced constipation in mice by remodeling the structure of the gut microbial community.

**Keywords:** Atractylenolide III (ATL-III), Constipation, Intestinal barrier, Gut microbiota, Bile acids (BAs).

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## INTRODUCTION

Constipation is a common clinical condition with a high prevalence rate of about 16% among adults nationwide, leading to dryness and difficulty in defecation.[1] Prolonged constipation can lead to many anal and intestinal complications such as hemorrhoids, anal fissure, anterior rectal protrusion, etc., the more serious may also cause and exacerbate all kinds of cardiovascular and cerebral vascular diseases, and even upper and lower gastrointestinal tract cancers, which seriously affects the patient's normal work and quality of life standard.[2] Constipation belongs to the global high morbidity,[3] in China; the incidence of constipation is also increasing year by year, and is easily affected by age, gender, region, and other factors.[4] Currently, patients with mild constipation can be relieved by adjusting their diets and lifestyles, while patients with more severe constipation need medication or even surgery. Among them, medication is the most commonly used treatment, but long-term use of medication may cause adverse reactions, and resistance to medication is easy to emerge. Surgery is highly invasive and carries the risk of recurrence and complications.[5,6] Despite the high annual cost of treating constipation globally, more than 50% of patients with constipation are not cured.[7] Therefore, it is necessary to find a treatment method with certain efficacy, few side effects, and high patient satisfaction.

In recent years, studies have confirmed that Traditional Chinese Medicine (TCM) has unique medical advantages due to its multi-targets, good efficacy, and few side effects. Therefore, it is of great clinical value and positive significance to search for efficient, safe, and widely applicable diagnostic and therapeutic solutions in TCM.[8] *Atractylodis maceocephalae* koidz. (AM), an herb of traditional Chinese medicine, is well-known and widely

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used for anti-oxidant, anti-tumor, liver protection and immune regulation potential.[9,10] Treatment of intestinal inflammation with AM is common in clinical practice, such as polysaccharide from *Atractylodes macrocephala* Koidz. improvement of Th17/Treg cell balance leads to alleviation of dextran sodium sulfate-induced colitis.[11] Atractylenolide I inhibits NLRP3 inflammasome activation in colitis-associated colorectal cancer by inhibiting Drp1-mediated mitochondrial fission.[12] Moreover, Atractylenolide III (ATL-III) is the main active ingredient of AM and it has been demonstrated that ATL-III has pharmacological properties including anti-inflammatory, antibacterial, anti-tumor, and anti-angiogenesis properties.[13,14] However, the efficacy and mechanism of Atractylenolide III on constipation is unknown.

It has been suggested that intestinal motility disorders may be the main pathophysiologic mechanism of constipation.[15] The movement of the intestines is controlled by the interplay of the immune system in the intestines, secretions in the intestines, the flora in the intestines, and the products of fermentation. A disruption in intestinal movement can result in constipation.[16,17] The role of gut microbiota in Gastrointestinal tract (GI) motility has been studied in Germ-Free (GF) and antibiotic-treated animals. In addition, GF mice exhibit a significant delay in gastrointestinal motility and a decreased number of nitrergic neurons.[18] By colonizing GF rats with *Lactobacillus acidophilus* and *Bifidobacterium bifidum*, intestinal transit and contractility are partially reversed.[19] Moreover, microbial dysfunction can cause constipation by altering gut microbial metabolic output, such as Bile Acids (BAs).[20] BAs play an important role in inhibiting intestinal bacterial overgrowth and translocation, as well as promoting intestinal peristalsis and intestinal secretion. Decreases in the rate of BA synthesis and bile acid secretion in the colon are associated with the development of constipation. In addition, the gut microbiota can modify BAs through esterification, desulfuration, and differential isomerization reactions, increasing the diversity of the BA pool within the host. Therefore, regulating BA metabolism by maintaining the diversity of gut microbial communities may be an important target for the prevention or treatment of constipation. Studies have also reported that AM volatile oil relieves acute ulcerative colitis by modulating gut microbiota and gut microbiota metabolism.[21] However, it is unclear whether ATL-III alleviates constipation by regulating gut microbiota-BA metabolism.

Therefore, in this study, we investigated the protective effects of ATL-III on gut microbiome imbalance and constipation-related physical measurements in a mouse model induced by a combination of diphenoxylate. Furthermore, we assessed the changed concentrations of gut metabolites through the use of Liquid Chromatography-Mass Spectrometry (LC-MS). Further, 16s RNA sequencing was conducted to study the important role that gut microbiota play in ATL-III treatment of constipation.

## MATERIALS AND METHODS

### Reagents

Compound diphenoxylate (Diphenoxylate hydrochloride 2.5 mg, atropine sulfate 25 μg) purchased from Shenzhen Rock Biochemical Technology Co., Ltd. ATL-III (#73030-71-4) was purchased from Shanghai Baishikai Chemical Technology Co., Ltd. 5-hydroxytryptamine (5-HT, #15619), substance P (SP, #1445), Nitric oxide (NO, #5962), and vasoactive intestinal peptide (VIP, #1447) ELISA kit purchased from Jiangsu Enzyme Immunoassay Industrial Co., Ltd. (Yancheng, China). Indole and BA standards including Cholic Acid (CA), Taurocholic Acid (TCA), Deoxycholic Acid (DCA), 23-Nordeoxycholic Acid (NorDCA), Lithocholic Acid (LCA), Hyodeoxycholic acid (HDCA), Allocholic Acid (ACA), 12-Ketolithocholic Acid (12-ketoLCA), isolithocholic acid (isoLCA), 7,12-diketolithocholic acid (7,12-diketoLCA), 6,7-diketolithocholic acid (6,7-diketoLCA), Ursocholic Acid (UCA), Glycodeoxycholicacid (GDCA) were bought from Sigma Aldrich (USA). All other reagents were of the highest analytical purity.

### Animal experiment

C57BL/6 mice (aged 6 weeks) were purchased from Chengdu Dashuo Laboratory Animal Co., Ltd (Chengdu, China). During the experiment, the mice were housed in a controlled environment (23°C with 12/12 hr light-dark cycles) and provided with water and a normal chow diet on an as-needed basis. Animal experiments were approved by the Ethics Committee of West China Hospital of Sichuan University (No. 20220901001).

Five groups were formed randomly after the mice had acclimated for one week: control ($n$=8), STC ($n$=8), STC+ATL-III (10 mg/kg, $n$=8), STC+ATL-III (20 mg/kg, $n$=8), and STC+ATL-III (30 mg/kg, $n$=8). Mice were pretreated with 0.9% NaCl solution with or without compound diphenoxylate (2.5 mg/tablet, way of gavage, about 20.0 mg/kg/d) for 2 weeks. Then, the STC+ATL-III groups were gavaged with ATL-III (10.0 mg/kg; 20.0 mg/kg; 30.0 mg/kg. body weight), and administered daily from 7-21 d, the dosage of ATL was reported about previous studies.[13,22] In this study, both control group and STC group were gavaged with the same amount of saline. Meanwhile, all mice were fed with either distilled water or compound diphenoxylate. The mice were euthanized using ether anesthesia, and samples of their blood, intestinal contents, and gastrointestinal tissues were gathered for additional examination. The samples were kept at a temperature of -80°C.

### Assessment of Constipation Indices

Throughout the experiment, we conducted weekly monitoring of the body weight and 24-hr defecation volume of mice. Stool samples were collected separately after the animal experiment for further analysis. After weighing and drying at 60°C for 12 hr, desiccated fecal pellets were obtained. The wet/dry weight

ratio of collected fecal pellets was measured within 3 hr for each mouse. To evaluate intestinal transit capability, mice underwent an 18 hr fast followed by intragastric administration of 0.5 mL of a charcoal meal at the conclusion of the drug treatment. After 30 min, all mice were promptly sacrificed to retrieve the entire bowel from the pylorus to the end of the colon. The Gastrointestinal (GI) transit rate was determined as the percentage of the movement distance of activated charcoal relative to the full length of the intestine. The formula for calculating the intestinal transport rate is as follows:

Intestinal transport rate (%) = Distance from the pylorus to the front edge of the black semisolid paste/The full-length distance from the pylorus to the end of the colon×100%

## Enzyme-Linked Immunosorbent Assay (ELISA)

The serum levels of 5-HT, SP, NO, and VIP in mice were determined by strictly following the ELISA kit instructions and procedure. Briefly, standard, sample, and control wells were set up, standard and the sample were added, and incubated at 37°C for 60 min. Subsequently, add enzyme reagent and incubate for 30 min, add color developer to avoid light for 15 min, and terminate the reaction by adding termination solution. Absorbance was measured at 450 nm using a microplate reader to determine serum levels of 5-HT, SP, NO, and VIP.

## Immunohistochemistry

The proximal ileum and colon tissues were fixed with 4% paraformaldehyde, and the tissues were then embedded in paraffin wax before sectioning. After slicing the blocks into 4-μm-thick pieces, the fixed sections were hydrated with gradient ethanol, swilled with distilled water, and deparaffinized with xylene. The immunohistochemistry of colon tissues was analyzed using antibodies anti-Farnesoid X receptor (FXR, #A9033A, Invitrogen, USA), anti-Takeda G-protein-coupled receptor 5 (TGR5, #70-ab35833-050, MultiSciences, China) and anti-cyclic adenosine monophosphate (cAMP, #3567-30T, BioVision, USA). Following incubation with goat anti-mouse/anti-rabbit secondary antibodies (Zhongshan Goldbridge Biotechnology Co., Ltd, Beijing, China) at 37°C for 1 hr, all images were observed under a fluorescence microscope.

## Real-Time quantitative PCR (RT-qPCR)

Extraction of total RNA from colon tissues was performed using Trizol reagents (Invitrogen, USA), followed by reverse transcription into cDNA using a first-strand cDNA synthesis kit (Invitrogen, USA). For RT-qPCR analysis, the synthesized cDNA served as the template for amplification, conducted using SYBR qPCR mixture (TAKARA, Japan) on the CFX 96 Connect Real-time PCR System (BioRad, USA). Primer sequences utilized to assess the expression levels of various genes were provided in Table 1, encompassing FXR, TGR5, cAMP, and GAPDH. The amplification protocol consisted of a 10 min initial denaturation stage at 95°C, 40 cycles of denaturation at 95°C for 10 sec, and 30 sec of annealing/extension at 60°C. GAPDH served as the reference gene for calculating the relative expressions of target genes employing the $2^{-\Delta\Delta Ct}$ method.

## Western blot analysis

Mice intestinal homogenates were prepared for western blot analysis. Following centrifugation at 10,000 rpm for 10 min at 4°C, the supernatant was collected. Total protein concentration was determined using the BCA method, and equal protein amounts were loaded onto SDS-PAGE gels and transferred onto PVDF membranes (Millipore, USA). Membranes were then blocked with PBST containing 5% nonfat dry milk powder and incubated overnight at 4°C with primary antibodies against FXR (1:1000), cAMP (1:1000), TGR5 (1:1000), and β-actin (1:3000) (Signal way Antibody). Subsequently, membranes were probed with a secondary antibody, goat anti-mouse/rabbit IgG (HRP) (Signal way Antibody). Following removal of the secondary antibodies, the blots were washed and visualized using an ECL detection kit (Millipore, Billerica, MA, USA) and imaged on an automated gel imaging analysis system.

## Quantitative analysis of intestinal metabolites in fecal samples

In order to quantify BAs in feces, 50 mg of stool sample was dissolved in 1 mL of water-methanol-formic acid solution (25:74:1, V/V/V) containing 0.2 g/mL of d5-CA and d4-TCA as internal standards. Additionally, the mixtures were centrifuged at 12,000 g for 15 min at 4°C, and the supernatant was mixed with 600 μL of methanol and vortexed for 30 sec. Following filtration via a 0.22 μm water membrane, supernatants were collected for LC-MS analysis.

## 16S rDNA sequencing

The colon contents of the mice were collected under aseptic conditions and sent to a company for 16S rDNA sequencing. Before onboard sequencing, the libraries were quality checked on an Agilent Bio analyzer, after which the libraries were quantified on a Promega QuantiFluor Fluorescence Quantification System using the Quant-iT PicoGreen dsDNA Assay Kit. Subsequently, the data were analyzed using the QIIME toolkit developed by Quantitative Insights into Microbial Ecology, based in the United States. An Operational Taxonomic Unit (OTU) is considered a high-quality read if it exhibits a similarity of over 97%. Sequences shorter than 110 nucleotides and with an overlap of less than 10 base pairs were excluded from the analysis. Chimeric sequences were screened and removed using Usearch (version 8.1.1861, available at www.drive5.com/usearch/). The UCLUST algorithm was employed to match each 16S rDNA gene sequence against the Silva 16S rRNA database (Release 119, available at http://www.arb-silva.de) with a confidence threshold of 90%. Previously, LEfSe analysis was employed to identify microbial groups of

significant abundance.[23] Additionally, a study was conducted to explore the functional profile of bacterial communities using a database containing phylogenetically referenced genomes.[24]

### Statistical analysis

The obtained data were analyzed using the Statistical Product and Service Solutions (SPSS) software (version 20.0). One-way Analysis of Variance (ANOVA) was adopted for group comparisons, and *post hoc* analysis of the means were conducted with the Tukey *post hoc* test. A $p<0.05$ was considered statistically significant. All standard plots were created using GraphPad Prism version 7.0, headquartered in La Jolla, California, United States.

## RESULTS

### ATL-III alleviates constipation-related parameters in mice

We investigated the effects of ATL-III on constipation in mice pretreated with compound diphenoxylate for two weeks, then gavaged with ATL-III at 7 d-21 d (Figure 1A). Compared to the STC group, ATL-III treatment partially restored weight gain (Figure 1B) and accelerated gastrointestinal transit ratios of constipated mice (Figure 1C). Further, mice of the STC group had significantly less fecal pellet weight than those of the Control group, but these differences were reversed after treatment with ATL-III (Figure 1D). We also calculated the feces water content and found that it was low in STC group, and ATL-III have increased the feces water content of the mice in a dose-dependent manner (Figure 1E), suggesting that ATL-III could treat constipation in mice.

### ATL-III promotes the secretion of colonic neurotransmitters in constipated mice

Next, we explore the effect of ATL-III on enteric neurotransmitters in the serum of constipated mice by ELISA. As shown in Figure 2A, NO, VIP, 5-HT, and SP are all reduced in constipation conditions, while ATL-III restored their secretion. The FXR and TGR5 are the major receptors for bile acid action,[25] and cAMP contributes to body metabolism. ATL-III inhibited constipation-induced downregulation of FXR, cAMP, and TGR5 in intestinal tissue (Figures 2B and 2C). The low-expressed transcriptional levels of FXR, cAMP, and TGR5 were observed in constipated mice ($p<0.01$, vs Control group), all of which were up-regulated by ATL-III treatment ($p<0.05$, vs STC group) (Figure 2D). By western blot analysis, the decrease in the production of FXR, cAMP, and TGR5 in constipated mice were strikingly reversed after ATL-III treatment consistently (Figures 2E and 2F).

### ATL-III modulates the production of intestinal metabolites in mice with constipation

Bile acids have a strong connection to the movement of the intestines and inflammation in the gut, which is why we measured these compounds in mouse feces using LC-MS analysis. In Figure 3A, compound diphenoxylate-induced constipation down-regulated the levels of total BAs in the feces of mice ($p<0.01$, vs Control group). While ATL-III is still up-regulated the higher the dose, the more significant the difference. In terms of the abatement of individual BAs in constipated mice, NorDCA, LCA, 12-ketoLCA, and HDCA were all enhanced by ATL-III in a highly significant manner ($p<0.05$, vs STC group) (Figure 3B-D). Furthermore, CA, ACA, UCA, and TCA grew in number in constipated mice ($p<0.05$, vs Control group), and were significantly reduced by ATL-III ($p < 0.05$, vs STC group) (Figure 3C and 3D).

### Modifying the composition of gut bacteria in constipated mice using ATL-III

We used 16S rDNA sequencing to assess the variety, abundance, and structure of gut microbiota in five different experimental

**Table 1: Primers sequence.**

| Gene | Primer sequence | Amplification efficiency (%) |
|---|---|---|
| FXR | F: 5'-TCCGAAGAAGCATCACCAAA-3' R: 5'-CAGCCAACATTCCCATCTCTC-3' | 94.465 |
| cAMP | F: 5'-CCCGTGACGTTTACACCCGTA-3' R: 5'-TCTAGAAACTCGGTACCT-3' | 93.687 |
| TGR5 | F: 5'-CTGTTATCGCTCATCTCATTG-3' R: 5'-CTGGATTGTCCCTCTTG-3' | 109.469 |
| GAPDH | F: 5'-ACCCAGAAGACTGTGGATGG-3' R: 5'-CAGTGAGCTTCCCGTTCAG-3' | 99.889 |

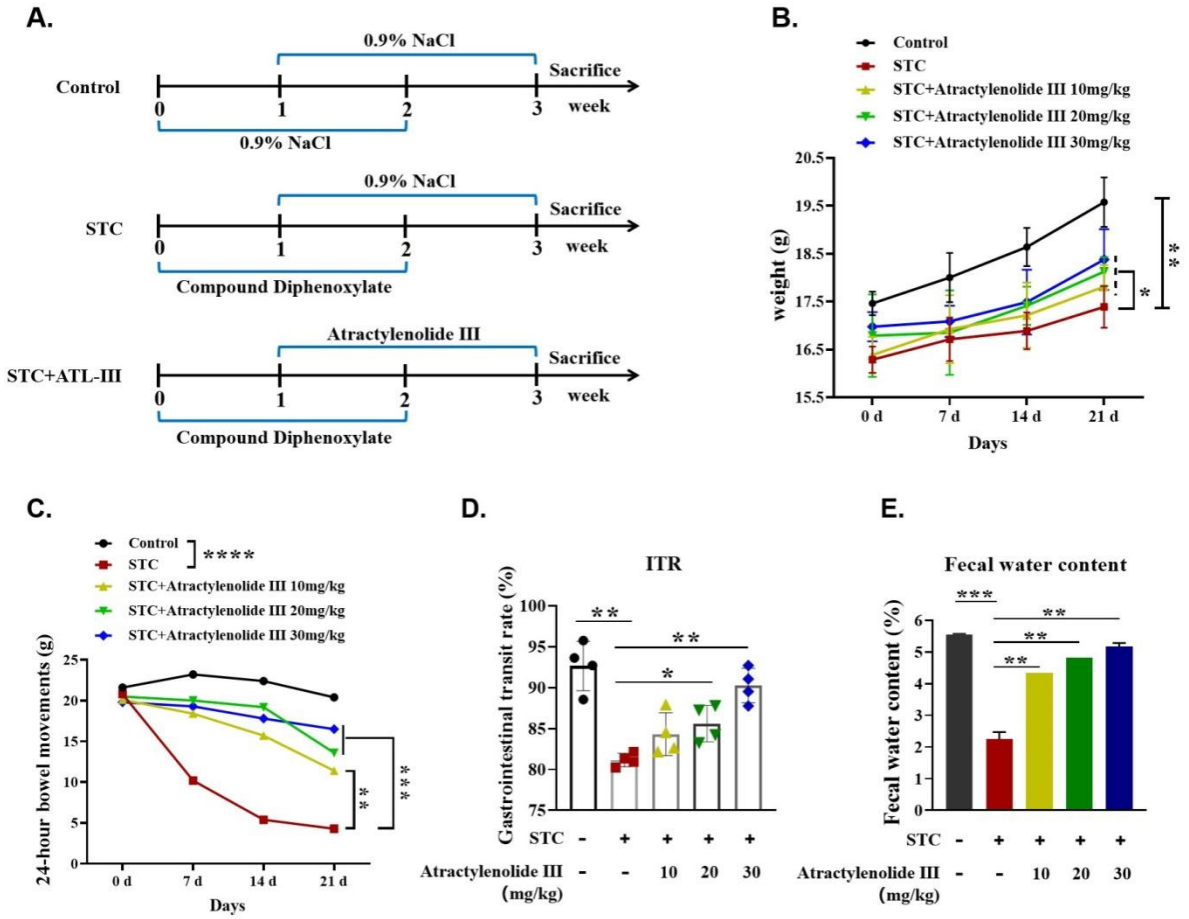


**Figure 1:** Constipated mice treated with ATL-III showed changes in physiological parameters. (A) Animal experiment schematic. (B) Changes in body weight of mice in each group (*n*=8). (C) Gastrointestinal transit rate (*n*=4). (D-E) Detection of fecal indices, including the changing trend of 24-hr defecation volume in mice (D, *n*=4), and fecal water content (E, *n*=4). Data were represented as mean±SEM. One-way analysis of variance was used to compare values between all groups. *p* values for Control or STC group: **p*<0.05, ***p*<0.01, ****p*<0.001, *****p*<0.0001.

groups, to investigate if imbalances in intestinal bacteria could be linked to constipation. Microbiome sample diversities, assessed through α and β diversity using the Chao index and Shannon index (Figure 4A), along with Non-Metric Dimensional Scaling (NMDS), revealed distinct separation into five clusters (Figure 4B), indicating significant differences in bacterial structures among groups.

At the phylum level, the STC group exhibited a decreased abundance of *Firmicutes* and elevated levels of *Bacteroidetes, Proteobacteria*, and *Deferribacteres*, which were significantly reversed by ATL-III treatment, except *Proteobacteria* (Figure 4C). At the family level, ATL-III treatment markedly suppressed the abundance of *S24-7, Lachnospiraceae*, and *Deferribacteraceae*, while increasing populations of *Lactobacillaceae, Desulfovibrionaceae*, and *Erysipelotrichaceae* in constipated mice (Figure 4D). Furthermore, heatmap analysis was conducted to investigate the modulatory effect of ATL-III on intestinal bacterial composition at the genus level. As depicted in Figure 4E, most of the alterations observed in the STC group were significantly attenuated following ATL-III treatment, including reductions in *Lactobacillus, Alistipes, Clostridium, Coprococcus, Dehalobacterium, Roseburia*, and *Butyricimonas*, among others.

We then performed LEfSe analysis on each experimental group to compare the microbial composition and specific bacterial taxa ('c', class; 'o', order; 'f', family). In Figure 5A, fourteen dominant families in the control group belonged to *deferribacteres, proteobacteria, campylobacterales, helicobacteraceae*, and *desulfovibrionales*; the higher taxonomies from five key families in the STC group were *flavobacteriales, flphaproteobacteria*, and *bacillales*. As shown in Figure 5C, compared with the STC group, after ALT_M intervention, *erysipelotrichi, alphaproteobacteria*, and *rhodobacterales* were the major family detected, while some significant reductions including *deferribacteres, clodtridia, lachnospiraceae*, and *ruminococcaceae*, etc., Subsequently, Linear Discriminant Analysis (LDA) score demonstrated that a rich abundance of p_*deferribacteres*, p_*proteobacteria* and o_*desulfovibrionales*, was captured in Control group, while c_*flavobacteriia*, c_*alphaproteobacteria*, and o_*bacillales* played major roles in STC group; *c_clodtridia, c_flavobacteriia, p_deferribacteres*, and *f_dehalobacteriaceae* were the most significant contributors in ALT_M group (Figures 5B and D).

## Impact of ATL-III on gut bacterial metabolic pathways

A PICRUSt analysis was performed on constipated mice to estimate the impact of ATL-III on metabolic pathways. Here we count the abundance of secondary functional pathways in the KEGG database (Figure 6A) and MetaCyc database (Figure 6B). The KEGG pathway enrichment analyses indicated that multiple pathways appeared to be affected, with all DEGs were mainly involved in the cell growth and death, cell motility, membrane transport, genetic information processing, and metabolism (Figure 6A). Among them, DEGs were significantly enriched in metabolic pathways, including carbohydrate metabolism, amino acid metabolism, metabolism of cofactors and vitamins, etc., (Figure 6A, blue pillars). GO analysis of the DEGs showed involvement in carbohydrate degradation, nucleoside and nucleotide degradation, fermentation, glycolysis, and mainly enriched in biosynthesis pathways, with nucleoside and

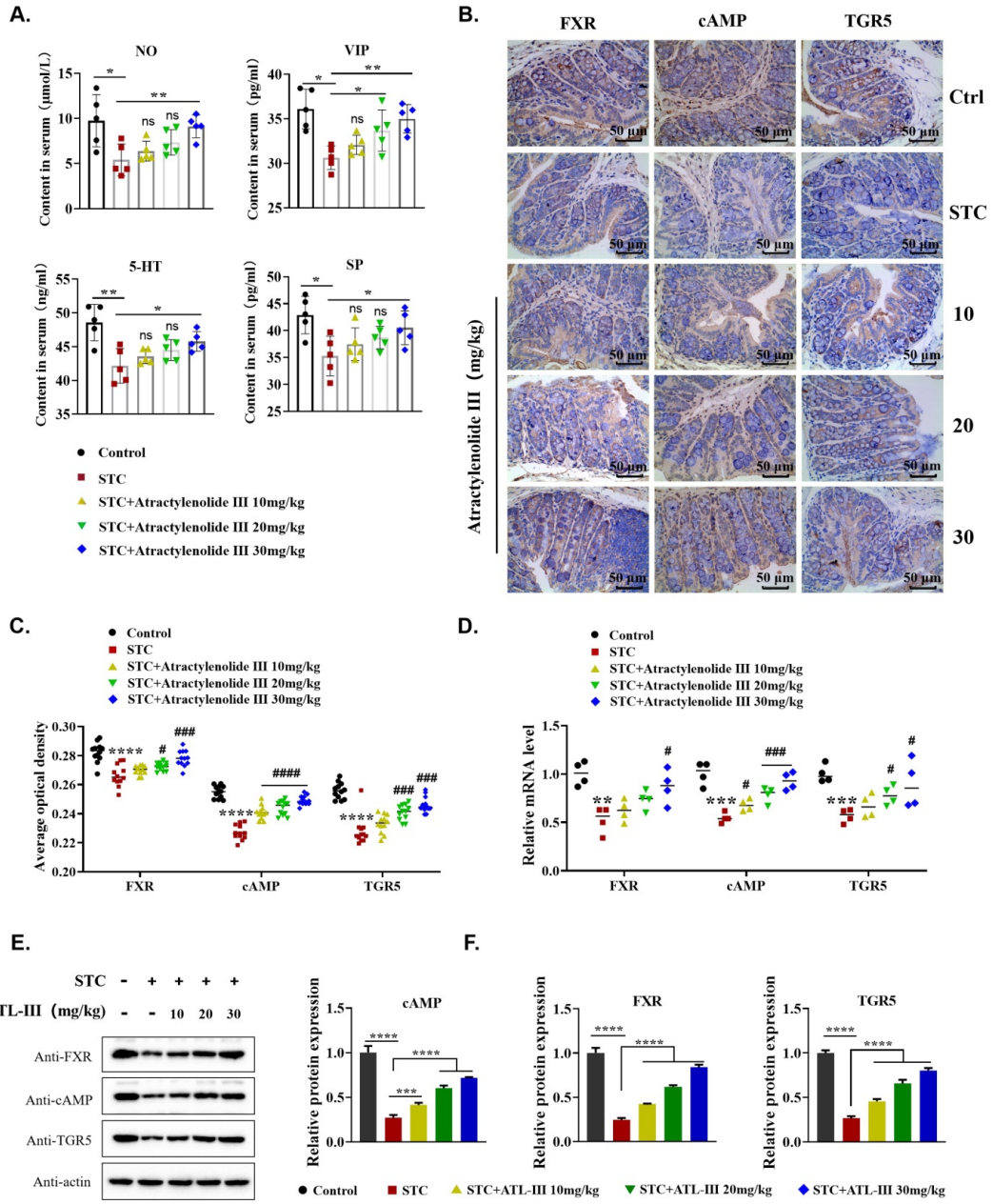


**Figure 2:** Improvement of ATL-III on colonic neurotransmitter secretion in constipated mice. (A) Detection of NO, VIP, 5-HT, and SP in serum of mice in each group by ELISA (*n*=5). (B) Immunohistochemical staining against FXR, cAMP and TGR5 in colon tissues (400×, scale bar, 50 μm). (C) Quantitative analysis of immunohistochemical results in (B) (*n*=12). (D) Transcriptional expressions of colonic mediators FXR, cAMP and TGR5 by RT-qPCR (*n*=4). (E) Expression levels of FXR, cAMP and TGR5 in colon detected by Western blot. (F) Quantitative analysis of WB results in (E) (*n*=4). Data were represented as mean±SEM. One-way analysis of variance was used to compare values between all groups. *p* values for Control or STC group: **p*<0.05, ***p*<0.01, ****p*<0.001, *****p*<0.0001.

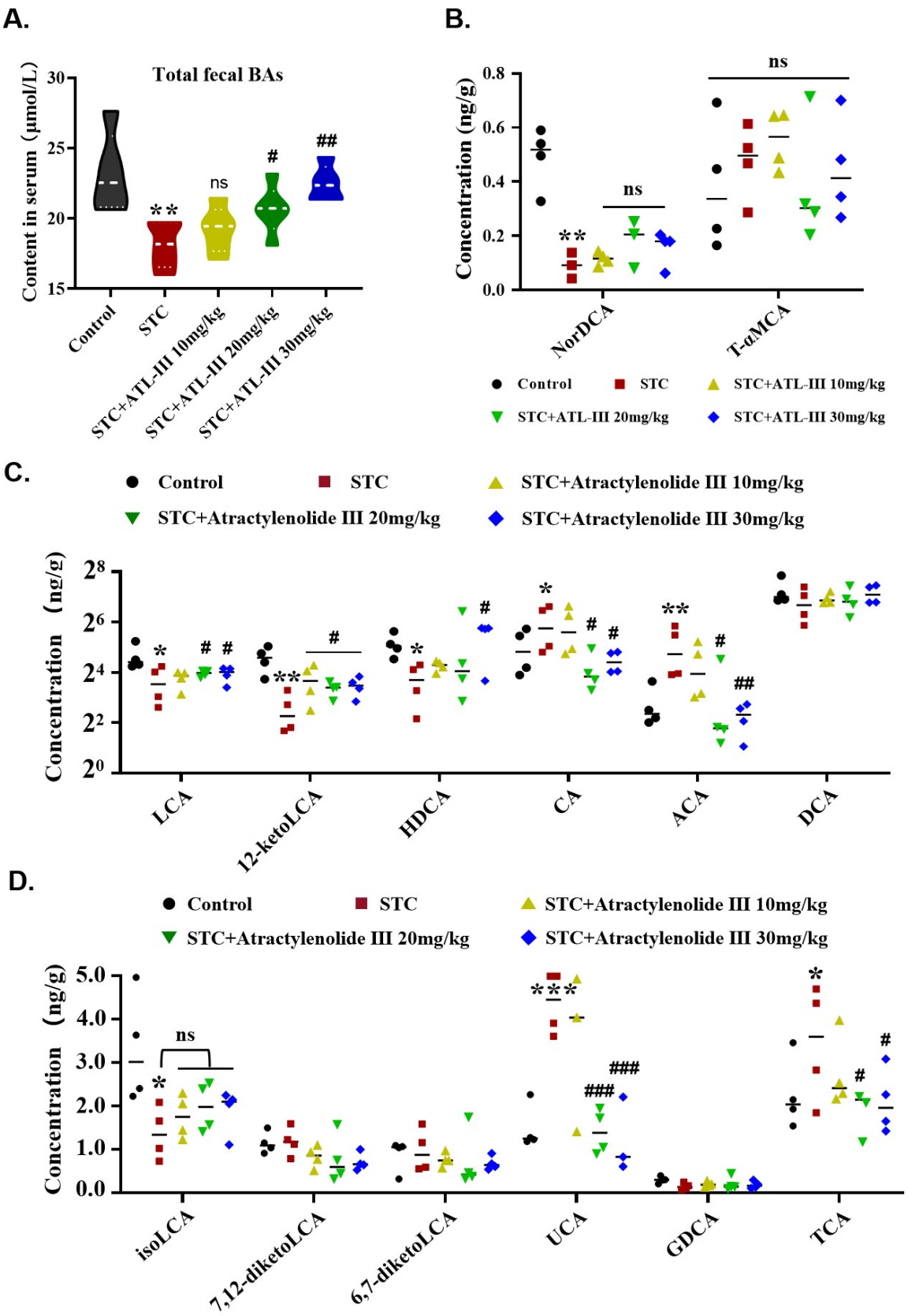


**Figure 3:** Regulatory effect of ATL-III on intestinal metabolites in feces of constipate mice. (A) Quantification of total fecal Bile Acids (BAs) by LC-MS ($n$=4). (B-D) Quantification of individual BAs in feces by LC-MS ($n$=4). Data were represented as mean±SEM. One-way analysis of variance was used to compare values between all groups. Compared with the control group, $^{*}p<0.05$, $^{**}p<0.01$, $^{***}p<0.001$; Compared with the STC group, $^{\#}p<0.05$, $^{\#\#}p<0.01$, $^{\#\#\#}p<0.001$, ns, no significant difference.

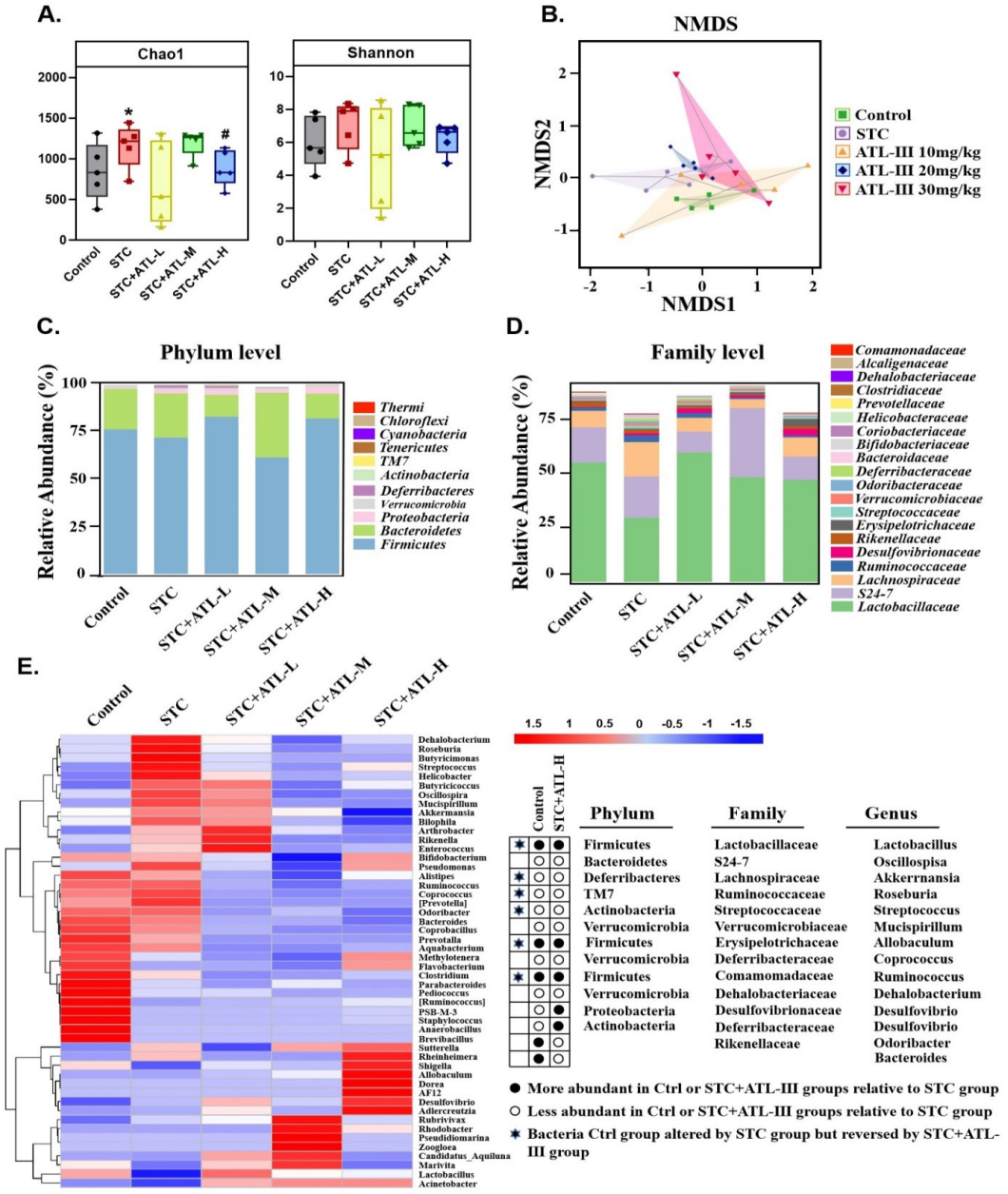


**Figure 4:** ATL-III restores gut microbiota dysbiosis in constipated mice. Following the conclusion of the animal experiment, all mice were euthanized, and their cecal contents were collected for 16S rRNA sequencing. (A) Assessment of alpha diversity using the Chao1 and Shannon index (*n*=4). (B) Evaluation of beta diversity using a Non-Metric Multidimensional Scaling (NMDS) plot (*n*=4). (C) Analysis of changes in gut microbiota at the phylum level (*n*=4). (D) Examination of changes in gut microbiota at the family level (*n*=4). (E) Heatmap comparison between experimental groups at the genus level (*n*=4). Data are presented as mean±SEM. Compared with the control group, $^{*}p<0.05$; Compared with the STC group, $^{\#}p<0.05$, ns: no significant difference.

nucleotide biosynthesis, amino acid biosynthesis, and fatty acid and lipid biosynthesis (Figure 6B).

## The relationship between bacterial abundance and metabolic biomarkers

To investigate the relationship between intestinal bacteria and constipation occurrence, Spearman's correlation coefficient was calculated (Figure 6C). *Sutterell*a was negatively correlated with bile acid metabolism and intestinal barrier and SCFA metabolism. *Shigella* was positively related to intestinal neurotransmitters and total BAs metabolism, but except for UCA, ACA, and TCA, etc., On the contrary, *Coprobacillus* was negatively correlated with intestinal neurotransmitters and total BAs metabolism, except for 7_12_diketoLCA, UCA, ACA, and TCA, etc., Other genera and physiochemical parameters of constipated mice, such as *Staphylococcus*_group, *Pseudidiomarina* _ group and *Ruminococcus*_group, etc., showed different correlations.

## DISCUSSION

Natural medicines are gaining increasing attention in the treatment of constipation due to their stimulating effect on the growth or activity of intestinal flora.[26,27] As an extract of *Atractylodes macrocephalala Koidz*, traditional Chinese medicine widely used to treat gastrointestinal diseases,[28] the biological function of ATL-III is similar to those of dietary supplements and prebiotics. Furthermore, ATL-III has the unique advantages of TCM treatment, such as good efficacy and low side effects. In this study, we demonstrate that ATL-III ameliorates compound diphenoxylate-induced constipation in mice by modulating gut bacteria and their metabolites.

Since constipation patients experience dryness and hardening of their feces.[29] Following ATL-III treatment, we observed a significant improvement in the dryness of feces as well as an increase in moisture content in these feces in all groups. Neurotransmitters play a key role in the regulation of physiological functions in the human body, among which NO, VIP, 5-HT, and SP are closely related to the functions of intestinal motility, secretion, and sensation. In the intestine, NO inhibits the contraction of intestinal smooth muscle and regulates intestinal propulsive movements, VIP promotes the propulsion of intestinal contents, 5-HT is present in large quantities in the gastrointestinal tract and regulates intestinal secretion, and SP promotes intestinal peristalsis.[30-32] In the present study, ATL-III

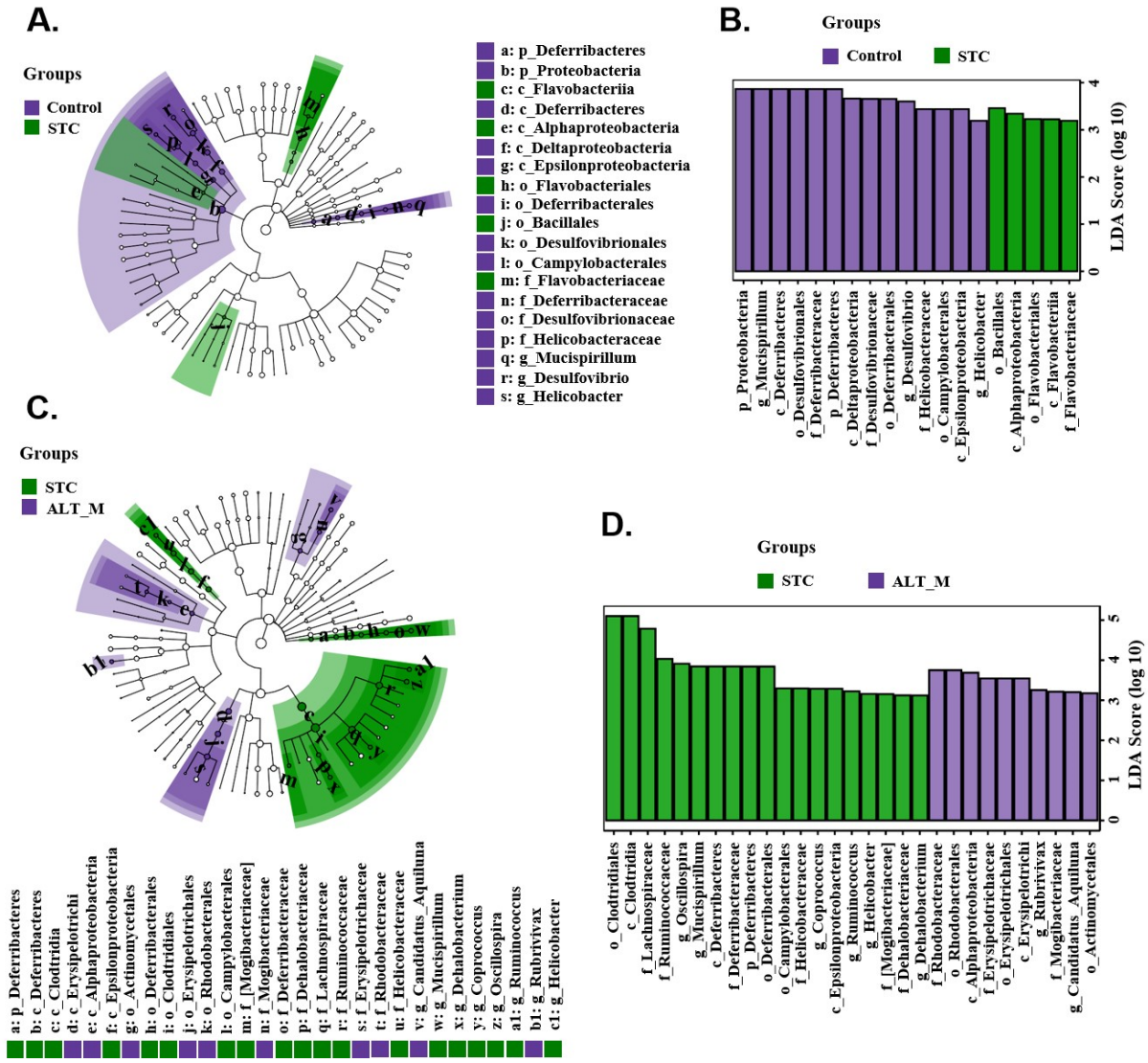


**Figure 5:** Analyzing the LDA Effect Size (LEfSe) to determine the most characteristic taxa between experimental groups. (A) Analysis of differentially enriched taxa between control and STC groups using LEfSe. Each circle represents its relative abundance (*n*=4). (B) Differences between Control group and STC group bacterial taxa after LDA using a threshold score higher than 3.0 (*n*=4). (C) The circle size is proportional to the relative abundance of each taxon in the STC and ALT_M groups (*n*=4). (D) The most substantial disparities in intestinal bacterial taxa between the STC and ALT_M groups were identified through LDA, employing a threshold score exceeding 3.0. The length of the bars in the LDA plot reflects the influence of significantly different species within each group (*n*=4).

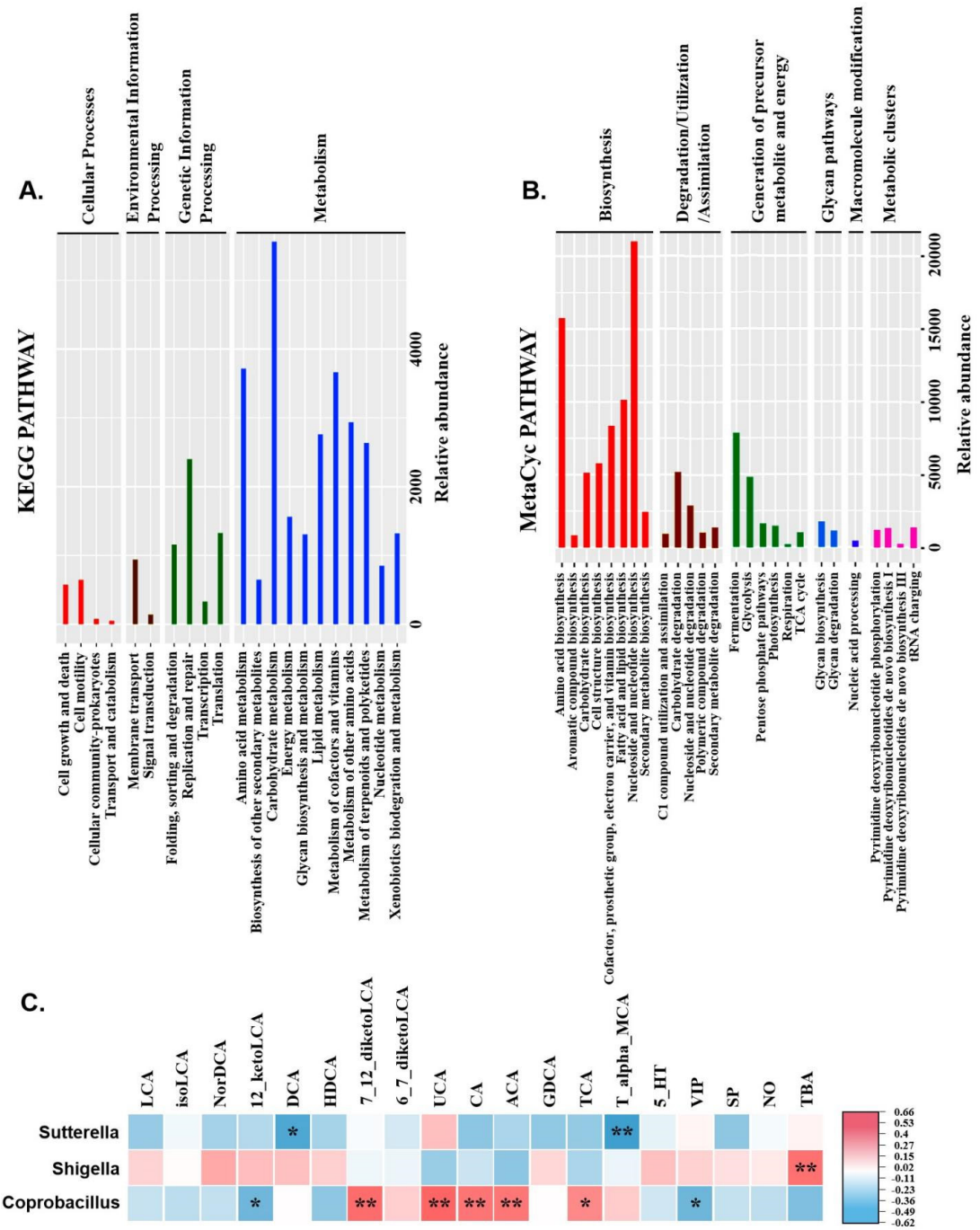


**Figure 6:** Analyzing Phylogenetic Relationships in communities by Reconstructing Unobserved States (PICRUSt). (A) Significantly enriched KEGG secondary functional pathway terms of the DEGs between groups were identified (*n*=4). (B) Predicted MetaCyc secondary functional pathway abundance map of the DEGs. The ordinate is the abundance of functional pathways, the abscissa is the functional pathway of the second classification/first-level pathway level of KEGG/MetaCyc (*n*=4). (C) A Spearman's correlation analysis was conducted between changed bacterial genera and constipation-related biomarkers (*n*=4). The colors ranged from blue (a negative correlation) to red (a positive correlation), and significant correlations were marked by *$p<0.05$, **$p<0.01$.

treatment increased the levels of NO, VIP, 5-HT, and SP in the intestinal tissues of constipated mice.

Studies have suggested that altered gut products (e.g., BAs) mediated by gut bacteria can cause constipation.[33] As an important signaling molecule, bile acid is involved in the regulation of glucose, lipid, and energy metabolism, and promotes the digestion, absorption and transport of lipid substances in the gut by binding to FXR and TGR5.[34] In addition, alterations in the mechanism of action of FXR directly affect the pool of BAs and lead to increased intestinal permeability as well as changes in the abundance and diversity of the gut microbiota, which can lead to intestinal motility disorders.[35]

Activation of TGR5 by BAs promotes the secretion of prokinetic intestinal peptides by enteroendocrine cells, such as Peptide YY (PYY) and Glucagon-Like Peptide-1 (GLP-1). These intestinal peptides can regulate intestinal motility and secretory function, promote intestinal peristalsis, and improve constipation.[36] When the production or action of cAMP is inhibited, it may lead to excessive contraction of the intestinal smooth muscle, which hinders the propulsion of intestinal contents, thus aggravating the symptoms of constipation.[37] In the present study, we detected decreased mRNA and protein levels of FXR, TGR5, and cAMP in the colon of constipated mice, and ATL-III was able to reverse this decrease in a dose-dependent manner. Drawing from the aforementioned information, we suggest that ATL-III could alleviate constipation by controlling the synthesis of intestinal metabolites. Therefore, we measured the amount of bile acids in the feces of constipated mice. The results showed that the amount of total and most individual bile acids was decreased, but that this effect was suppressed by ATL-III administration, suggesting that ATL-III modulates the metabolism of bile acids. Interestingly, in another study, most of the monomeric bile acids were elevated due to constipation, and chitosan oligosaccharides could regulate constipation in mice by inhibiting the excessive secretion of bile acids.[33] Our results appear to be contradictory, and we speculate that different metabolic dysregulation may be due to different inducing factors and the degree of disease. Taken together, in the present study, ATL-III may activate FXR and TGR5 signaling, thereby maintaining intestinal microecological balance and ensuring normal metabolism of BAs in the intestine and normal intestinal motility.

Intestinal microbes are responsible for the diversity of bile acids.[38] Gut microbiota plays a key role in bile acid synthesis, modification and signaling by converting host-derived primary bile acids to secondary bile acids and by decoupling bile salt hydrolases.[39] Inflammatory Bowel Disease (IBD), Colorectal Cancer (CRC), Hepatocellular Carcinoma (HCC), Type 2 Diabetes Mellitus (T2DM), and Polycystic Ovary Syndrome (PCOS) can all be caused by imbalances of bile acid and intestinal flora.[38] Several beneficial bacteria at genus level, such as *Faecalibacterium* and *Lactobacillus*, were enriched by ATL-III in this study. A higher concentration of *Faecalibacterium* promotes bowel transit and suppresses intestinal inflammation.[40] As a result of accumulating saturated long-chain fats, *Lactobacillus* and *Alistipes* stimulate colonic muscle contractions and increase defecation frequency.[41] In contrast, ATL-III repressed *Clostridium* and *Coprococcus*, which are both strongly associated with constipation in constipated mice.[42] From these results, it can be concluded that ATL-III can remodel the damaged intestinal flora of constipated mice.

PICRUSt analysis also found that ATL-III affects gut bacterial metabolism and has a modulating effect on gut microbial activity. ATL-III may be associated with the variation in fecal CA and MCA in constipated mice because Lactobacillus secretes bile salt hydrolase (BSH), which is required for the metabolism of both BAs.[43] Furthermore, *Bifidobacterium longum* changed the BAs metabolism by increasing the abundance of secondary BAs, such as -MCA, -MCA, LCA, CDCA, UDCA, HCA, isoLCA, isoalloLCA, regulated the gut microbiota composition.[44] The importance of gut microbiota homeostasis extends beyond the digestive system. In recent years, it has been reported that gut microbes can affect the connection of neurons and brain activity through metabolites.[45] Even gut microbes may affect the body's metabolic processes.[46] Based on this, we can't help looking forward to more functional regulation and clinical application of ATL-III.

## CONCLUSION

This study proves that ATL-III has beneficial effects on compound diphenoxylate-induced constipation in mice by promoting intestinal motility, regulating water and salt metabolism, and maintaining intestinal metabolic balance. In addition, ATL-III also remodeled the structure of the gut microbiota, which is critical for the production of gut metabolites. Our study points to the potential application of ATL-III in the treatment of constipation.

## CONFLICT OF INTEREST

The authors declare that they have no competing interests.

## ABBREVIATIONS

**ATL-III:** Atractylenolide; BAs:bile acids; **GF:** germ-free; GI:gastrointestinal.

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## ETHICAL APPROVAL AND CONSENT TO PARTICIPATE

The Experimental Animal Ethics Committee of West China Hospital of Sichuan University reviewed and approved the study (No. 20220901001).

## AUTHORS' CONTRIBUTIONS

Simin Chen and Xuegui Tang designed the experiment and write the draft, Simin Chen and Zhibin Zhang provided technical guidance in experiments and draft writing, Simin Chen, Jun Pang, Lina Guan, Qingfeng Wu, Qiuxiao Wang and Qian Li accomplished the main experiments, Simin Chen and Zhibin Zhang accomplished data collection and analysis. All authors

contributed to the design and interpretation of the study and further drafts.

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