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Targeting intestinal flora and its metabolism to explore the laxative effects of rhubarb

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Abstract

Rhubarb, a traditional herb, has been used in clinical practice for hundreds of years to cure constipation, but its mechanism is still not clear enough. Currently, growing evidence suggests that intestinal flora might be a potential target for the treatment of constipation. Thus, the aim of this study was to clarify the laxative effect of rhubarb via systematically analyzing the metagenome and metabolome of the gut microbiota. In this study, the laxative effects of rhubarb were investigated by loperamide-induced constipation in rats. The gut microbiota was determined by high-throughput sequencing of 16S rRNA gene. Ultra-high performance liquid chromatography-quadrupole time-of-flight mass spectrometry was used for fecal metabolomics analysis. The data showed that rhubarb could significantly shorten gastrointestinal transit time, increase fecal water content and defecation frequency, improve gastrointestinal hormone disruption, and protect the colon mucus layer. Analysis of 16S rRNA gene sequencing indicated that rhubarb could improve the disorder of intestinal microbiota in constipated rats. For example, beneficial bacteria such as *Ligilactobacillus*, *Limosilactobacillus*, and *Prevotellaceae UCG-001* were remarkably increased, and pathogens such as *Escherichia-Shigella* were significantly decreased after rhubarb treatment. Additionally, the fecal metabolic profiles of constipated rats were improved by rhubarb. After rhubarb treatment, metabolites such as chenodeoxycholic acid, cholic acid, prostaglandin F2α, and α-linolenic acid were markedly increased in constipation rats; in contrast, the metabolites such as lithocholic acid, calcidiol, and 10-hydroxystearic acid were notably reduced in constipation rats. Moreover, correlation analysis indicated a close relationship between intestinal flora, fecal metabolites, and biochemical indices associated with constipation. In conclusion, the amelioration of rhubarb in constipation might modulate the intestinal microflora and its metabolism. Moreover, the application of fecal metabolomics could provide a new strategy to uncover the mechanism of herbal medicines.

Key points

- *Rhubarb could significantly improve gut microbiota disorder in constipation rats.*
- *Rhubarb could markedly modulate the fecal metabolite profile of constipated rats.*

Keywords Rhubarb · Constipation · Gut microbiota · Metabolites

Introduction

Constipation, which is characterized by tense defecation, dry or hard stools, painful bowel movement, and the sensation of anorectal obstruction (Chatoor and Emmnauel 2009; Bharucha et al. 2013; Mearin et al. 2016), is the most common chronic gastrointestinal disorder. Currently, its global prevalence ranges from 2 to 35% (Andromanakos et al. 2006; Mugie et al. 2011). Complications of constipation include hemorrhoids, anal fissures, diverticulitis, and impaction of stool (Bharucha and Lacy 2020). Constipation is associated

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with many factors, including intestinal nervous system dysfunction, visceral hypersensitivity, aberrant distribution of interstitial Cajal cells, and low gastrointestinal motility (Bharucha and Wald 2019). Emerging evidence suggests that gut microbiota dysbiosis is another risk factor for the development of constipation (Dimidi et al. 2017).

The gut microbiota includes 3.9×10^{13} bacteria that belong to more than 1000 species in the gastrointestinal tract (Sender et al. 2016). They regulate the host's metabolism, immunity, digestion, and development (Tremaroli and Bäckhed 2012; Obata and Pachnis 2016). Recently, research indicated that disruption of the gut microbiota was closely related to constipation (Ohkusa et al. 2019). In vivo studies showed that the colon in normal mice was more contractile and the intestinal time was shorter than in germ-free mice (Kashyap et al. 2013). Similarly, clinical studies have shown a close correlation between gut microbiota and constipation in patients (Mancabelli et al. 2017). Furthermore, intestinal bacteria such as *Bacteroides*, *Faecalibacterium*, *Actinobacteria*, and *Prevotella* could notably affect intestinal motility, colonic mucin secretion, or transport time in the colon (Parthasarathy et al. 2016). Some studies suggested that the metabolites from gut flora could ameliorate the symptoms of constipation. For example, butyrate from gut flora could regulate intestinal motility and strengthen the integrity of the intestinal barrier by upregulating the expression of tight junction proteins or mucins (Morrison and Preston 2016). Tryptophan derivatives produced by the intestinal microbiota were tightly associated with the development of constipation (Bhattarai et al. 2018). Additionally, *Bacteroides* could stimulate colonic motility by altering the composition of the bile acid pool (Wahlström et al. 2016).

Recently, new therapies for constipation by altering the structure of the gut flora have been clinically proved (Jayasimhan et al. 2013; Waitzberg et al. 2013). Some probiotics and commensal bacteria could improve constipation by regulating gut motility and mucus secretion (Dimidi et al. 2017; Chandrasekharan et al. 2019). Similar results have been found in some studies on prebiotics. For instance, inulin and isomalto-oligosaccharide could increase the abundance of *Lactobacillus* to accelerate the frequency of defecation (Lan et al. 2020). To date, fecal microbial transplantation (FMT) therapy for constipation has gradually moved into clinical practices (Kelly et al. 2021). In addition, some studies indicated that herbs used to treat constipation could also improve the structure and function of the intestinal flora (He et al. 2020; Yang et al. 2021).

Rhubarb, an herbal stimulant laxative, has been used in Europe and Asia with its dried roots. The major laxative components in rhubarb are anthraquinone glycosides, of which sennoside A has the most potent effect (Takayama et al. 2012). Clinically, rhubarb is used to treat constipation caused by various reasons (Hou et al. 2015; Shimizu et al. 2018; Wei et al. 2021). Modern pharmacological studies have shown that rhubarb exerts its laxative effect by regulating intestinal water and salt balance, intestinal motility, and mucosal barrier (Xiang et al. 2020). For example, sennoside A could inhibit the expression of AQP3 in the colon, thereby reducing the transport of water from blood vessels into the intestinal lumen to produce a laxative effect (Kon et al. 2014). Rhubarb also promoted the proliferation of goblet cells of intestinal mucosa, which could secrete large amounts of mucus and then strengthen the intestinal mucosal barrier (Neyrinck et al. 2017). Additionally, emodin could activate calcium-activated chloride channels to enhance the contraction of colonic smooth muscle (Xu et al. 2009). Emodin could also improve the disorder of intestinal flora in chronic kidney disease (Lu et al. 2020). Recently, some researches also showed that rhein could regulate the gut microbiota to improve colitis in mice (Wu et al. 2020). These results implied that rhubarb had the potential to regulate intestinal flora. However, there was insufficient evidence that rhubarb improved constipation by modulating gut flora and its metabolism.

In this study, the pharmacodynamic effects of rhubarb on loperamide-induced constipation in rats were systematically and comprehensively evaluated. Moreover, the modulation of rhubarb on the intestinal microbiota and its metabolism was investigated in detail by 16S rRNA gene sequencing and fecal metabolomics. Thus, the laxative mechanism of rhubarb was clarified based on a multi-omics exploration, which was helpful for its clinical application.

Materials and methods

Chemicals and reagents

Acetonitrile (UPLC grade) was obtained from Merck (Darmstadt, Germany). Formic acid and methanol (HPLC grade) were purchased from Merck (Darmstadt, Germany). Loperamide was purchased from Shanghai Yuanye Bio-Technology Co., Ltd. (Shanghai, China). Bisacodyl was bought from Hebei Kangtai Pharmaceutical Co., Ltd. (Hebei, China). India ink was purchased from Nanjing Duly Biotech Co., Ltd. (Jiangsu, China). Ultra-pure water was produced by EPED purification system (Nanjing, China). The dried roots of rhubarb (*Rheum tanguticum* Maxim. ex Balf) were bought from Hunan Songlingtang Chinese Medicine Decoction Pipe Co., Ltd. (Hunan, China).

Preparation of rhubarb extract

According to the Chinese Pharmacopoeia, rhubarb was pulverized, and its powder was fully infiltrated in cold eightfold distilled water (DW). Then, it was heated to reflux for 10 min

under boiling condition. The extract was immediately filtered with gauze, and then the residue was immersed in six-fold DW to be boiled for 5 min. Filtrates were combined and concentrated with a rotary vacuum evaporator (50 °C) to prepare rhubarb solution.

Animals

Male 5-week-old Sprague Dawley rats (SPF, weighting 170 ± 20 g) were purchased from Qinglong Mountain Animal Breeding Farm, Jiangning District, Nanjing, China (license number: SCXK (Zhejiang) 2019–0002). Animal welfare and experimental procedures were strictly carried out according to the guidelines of the Animal Ethics Committee of Nanjing University of Chinese Medicine, and this study was based on the Guide for the Care and Use of Laboratory Animals. Before the experiment, rats were fed adaptively for one week in an environment-controlled rearing chamber (temperature at 20–26 °C, humidity at 60 ± 5%, and under artificial 12-h light/12-h dark cycles).

Induction of constipated rats, treatment, and oral-anal transmission test

After 1 week of the adaptation period, all rats (220 ± 10 g) were randomly and equally divided into the following six groups: the normal group (*N*), the model group (*M*), the positive control group with 2 mg/kg bisacodyl (Bis), rhubarb-treated low-dose group (1.0 g/kg, RL), rhubarb-treated medium-dose group (3.0 g/kg, RM), and rhubarb-treated high-dose group (9.0 g/kg, RH). The constipated rats were induced by oral administration of 5 mg/kg loperamide (freshly dissolved in saline solution), once a day for ten continuous days. The normal group was given saline. Constipated rats were treated with bisacodyl and rhubarb for 3 days, respectively. Subsequently, each rat was given 2 ml of Indian ink, recording the first black feces discharge time. Finally, all the rats were fasted overnight for at least 12 h and were killed the next day.

Sample collection and preservation

Fresh feces of rats were collected with sterile Eppendorf tubes. All rats were anesthetized with pre-prepared 10% chloral hydrate, and blood was taken from the abdominal aorta. The colonic tissue of rats was taken and stored in 10% formalin solution with 0.5 cm at 4 °C, and the rest was put into the frozen tube. All biological samples were stored at − 80 °C. Rat motilin (MTL), gastrin (GAS), vasoactive intestinal peptide (VIP), and somatostatin (SS) ELISA kits (Shanghai FanKe Industrial Co., Ltd., Shanghai, China) were respectively used to detect the concentrations of MTL, GAS, VIP, and SS in rat serum.

Metabolic profiling by UPLC-Q-TOF/MS

Metabolites in pretreated fecal samples were separated and detected by ultra-high performance liquid chromatography-quadrupole time-of-flight mass spectrometry (UPLC-Q-TOF/MS), and then the data from MassLynx™ v4.1 (Waters Corp, Milford, MA, USA) were analyzed by SIMCA-P 14.0 software (https://umetrics.com/products/simca). The differential metabolites were refined, analyzed, predicted, and identified by XCMS Online (http://xcmsonline.scripps.edu), METLIN (http://metlin.scripps.edu), HMDB (http://www.hmdb.ca/), MassBank (https://massbank.eu/), and Metaboanalyst 5.0 (http://www.MetaboAnalyst.ca/) (Pang et al. 2021; Guijas et al. 2018; Wishart et al. 2018; Tautenhahn et al. 2012; Horai et al. 2010). The details were listed in Supplemental Text S1.

Sequencing analysis of gut microbes

Intestinal microbiological DNA was extracted from fresh fecal samples by the E.Z.N.A.® Soil DNA Kit (Omega Biotek, Norcross, GA, USA). The extracted genomic DNA was detected by 1% agarose gel electrophoresis. The V4–V5 regions of the bacteria 16S ribosomal RNA gene were amplified by PCR. PCR products were detected and quantified by QuantiFluor™-ST blue fluorescence quantitative system (Promega Company, Madison, Wisconsin, USA). The extracted genes were detected, and the data were analyzed on the IlluminaPE250 platform. The details were listed in the Supplementary material (Text S2).

Histological analysis

The rat colon was fixed with 10% formalin for 48 h, embedded in paraffin, cut into Sects. 4.0 μM thick, then dewaxed with xylene and rehydrated. Sections were rinsed with distilled water and stained with the AB-PAS staining kit (Wuhan Servicebio Technology Co., Ltd., Wuhan, Hebei, China). Finally, the morphological characteristics of stained colon sections were observed by upright optical microscopes (Nikon, Minato, Tokyo, Japan).

Statistical analysis

Graphpad prism 9.0 (GraphPad Software, San Diego, CA, USA, www.graphpad.com) was used for statistical analysis. After the homogeneity of variance test, *t*-test was used to analyze the differences between the two groups, and one-way ANOVA was used to compare the differences between more than two groups. The results of all indexes were expressed

in mean ± standard error of mean (SEM), $P < 0.05$ indicated that the difference of the measured data was statistically significant, $P < 0.01$ showed that there was a very significant statistical significance. Pearson correlation analysis was used in correlation analysis.

Nucleotide sequence accession number

The 16S rRNA sequences obtained in this study were deposited into the NCBI Sequence Read Archive (SRA) database with accession number PRJNA767447.

Results

Amelioration of rhubarb on pathological symptoms of constipation

In this study, the constipation model was successfully induced by loperamide in rats. After oral administration of rhubarb, the fundamental defecation indexes (defecation frequency, oral-anal transit time, and fecal water content) and body weight growth rate of constipated rats were significantly improved, and the constipation symptoms of rats were alleviated (Supplemental Fig. S1). ELISA kits were used to detect the constipation-related biochemical indicators in serum, and the histopathological changes of the colon were observed by AB-PAS staining in each group (Fig. 1). Compared with the normal group, the levels of gastrointestinal hormones including MTL, GAS, and VIP in serum were remarkably decreased in the model group; in contrast, the levels of SS were markedly increased (Fig. 1D–G). However, treatment with rhubarb could notably reverse these gastrointestinal hormones in constipated rats. Additionally, the results of colon histology showed that the colonic mucus layer of model rats was seriously damaged (Fig. 1A), the mucin content in the mucosa was markedly decreased, and the muscle layer became thinner (Fig. 1B, C). After treatment with rhubarb, the mucin content was significantly increased and the muscle layer in the colon was markedly thickened.

Effects of rhubarb on the composition of gut microbiota in constipated rats

To explore the effect of rhubarb on the gut microbiota, the fecal microflora of 36 samples from six groups were analyzed by 16S rRNA sequencing. A total of 1,450,829 high-quality reads were generated, which were subsequently clustered. The 5669 operational taxonomic units (OTUs) were eventually obtained according to 97% similarity level. According to the trend of individual rarefaction curves, Shannon–Wiener curves, rank-abundance distribution curves, and species accumulation curves, the sequencing data were adequate and could reflect the microbial information in all samples (Supplemental Fig. S2). Chao1 and Shannon indices were used to evaluate the microbial α diversity in fecal samples (Fig. 2A, B). Compared with the normal group, Chao1 index was significantly decreased, and Shannon index was markedly increased in model rats. Rhubarb treatment could increase the Chao1 index, especially in the RH group ($P < 0.05$, vs. *M* group), and decreased Shannon index, especially in the RL and the RM group ($P < 0.05$, vs. *M* group). Regarding the β diversity, nonmetric multidimensional scaling (NMDS) analysis was used to evaluate the differences of gut microbiota among the six groups (Fig. 2C). It showed that the normal and model groups were separated and the different rhubarb-treated groups were close to the normal group. These results indicated the disorder of gut microbiota in constipated rats, while rhubarb could improve the dysbiosis of gut microbiota.

Next, the gut flora was evaluated at the taxonomic levels of phylum, family, and genus. At the phylum level (Fig. 2D), there were no obvious differences between groups. At the family level (Fig. 2E), the abundances of *Muribaculaceae*, *Oscillospiraceae*, *Christensenellaceae*, and [*Eubacterium*] *coprostanogenes group* were significantly increased in the model group compared with the normal group and were markedly reduced after rhubarb treatment. Besides, the abundances of *Bacteroidaceae*, *Lactobacillaceae*, and *Butyricicoccaceae* were notably decreased in the model group, while these trends were reversed by rhubarb. Heat map analysis was used to observe the changes in gut microbiota between six groups at the genus level (Fig. 2F). Twenty-nine kinds of bacteria were different between the model and normal groups in the genus level, of which ten were significantly affected by rhubarb (Supplemental Table S1). Compared with the normal group, the abundance of *Ligilactobacillus*, *Limosilactobacillus*, *Lachnospiraceae_uncultured*, and *Prevotellaceae UCG-001* were markedly reduced in model group, and *Blautia*, *Oscillospiraceae UCG-005*, *Christensenellaceae R-7 group*, *[Eubacterium] coprostanoligenes group_norank*, *Erysipelotrichaceae_uncultured*, and *Escherichia-Shigella* were notably increased. In comparison, the abundance of these bacteria was remarkably reversed by rhubarb.

Linear discriminant analysis effect size (LEfSe) method was used to identify the specific bacterial taxa, and a total of 56 specific bacteria (LDA scores > 3) were identified in six groups (Fig. 3A, B). There were 11 specific bacteria in normal group, 12 specific bacteria in model group, 4 specific bacteria in the Bis group, 8 specific bacteria in the RL group, 10 specific bacteria in the RM group, and 11 specific bacteria in the RH group. These phylotypes played a key role in distinguishing the composition of gut microbiota in six groups.

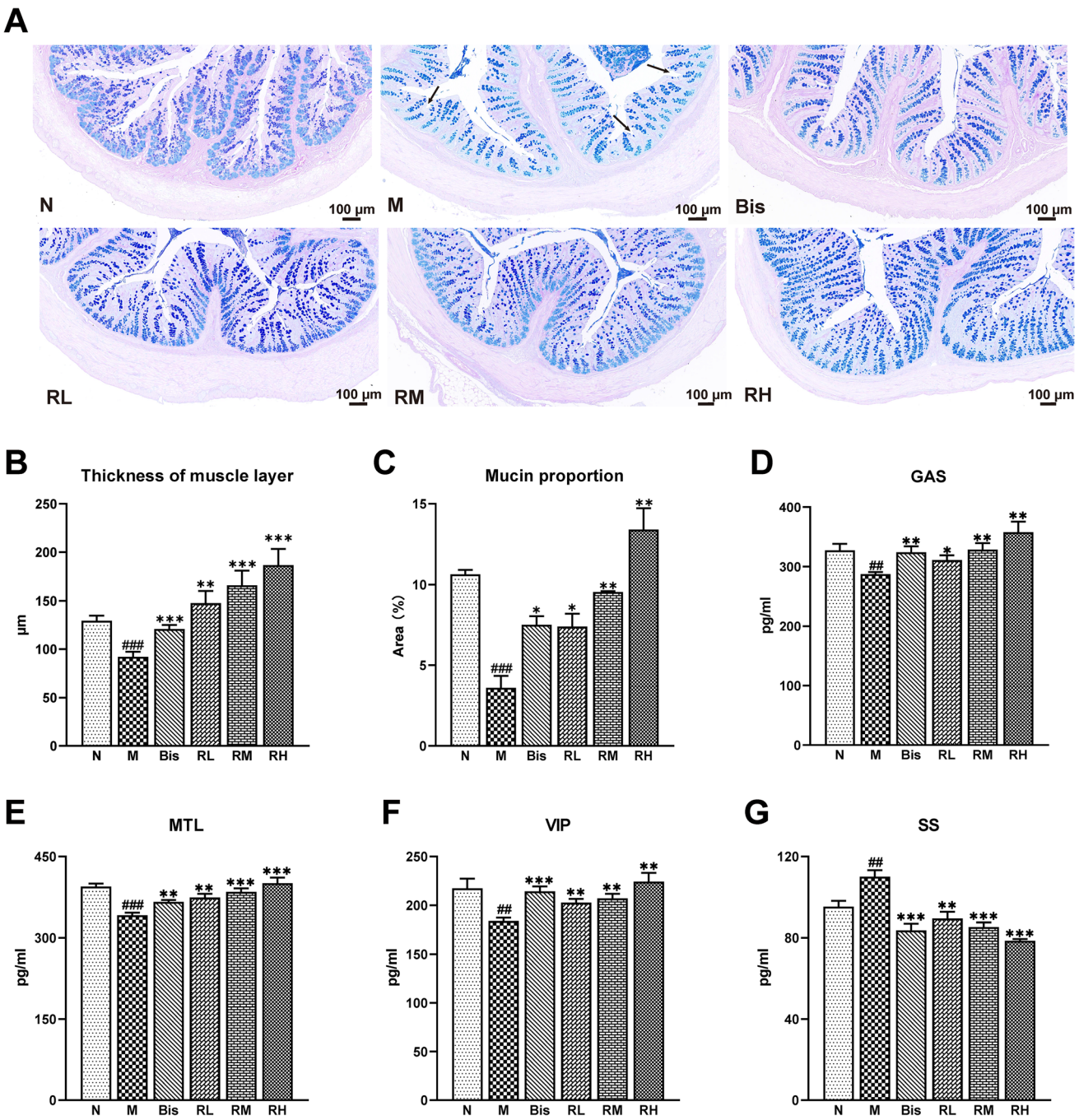
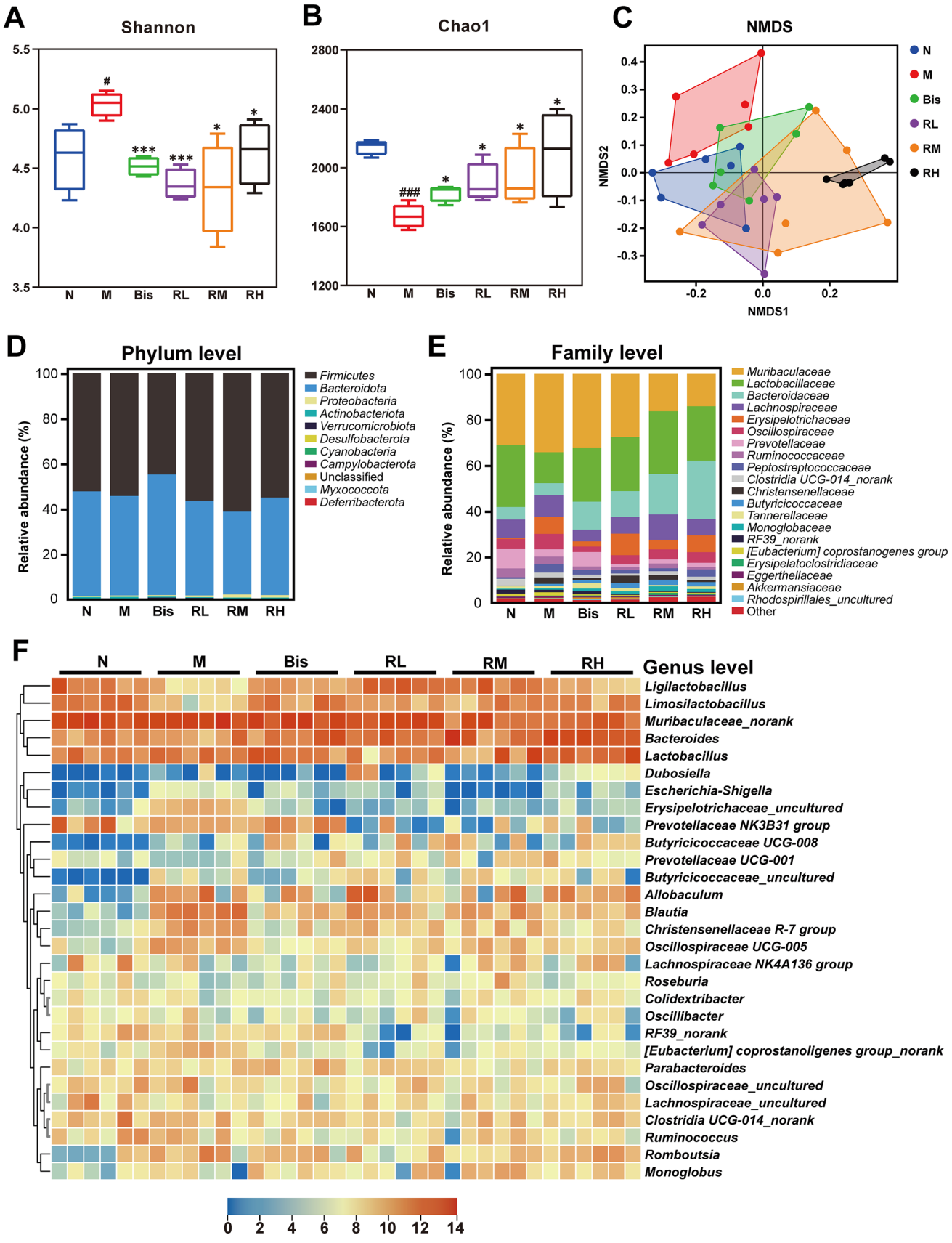


Fig. 1 Evaluation of rhubarb on the laxative effect. Histopathological observation of colonic tissue (**A**), intestinal wall muscle thickness (**B**) and mucin content (**C**) in different group. Biochemical index level of GAS (**D**), MTL (**E**), VIP (**F**) and SS (**G**) in each group. The results of data statistics were expressed by means ± SEM. $^{*}P < 0.01$, $^{**}P < 0.05$, $^{***}P < 0.001$ vs. control; $^{\#}P < 0.05$, $^{\#\#}P < 0.01$, $^{\#\#\#}P < 0.001$ vs. model

Modulation of rhubarb on the fecal metabolic profiles of constipated rats

To explore the actual situation of intestinal flora metabolism in each group, the fecal samples were analyzed by an UHPLC-Q-TOF/MS system in positive and negative ion models, and the corresponding metabolic fingerprints in each group were obtained (Supplemental Fig. S3). Next, the dataset generated by the MassLynx™ v4.1 workstation was imported into SIMCA-P for multivariate statistical analysis. The discrepancy in fecal metabolic profile between different treatment groups was assessed via partial least squares discriminant analysis (PLS-DA). As shown in Fig. 4C, D, the metabolic profiles of rhubarb-treated groups were markedly


A
Shannon
B
Chao1
C
NMDS
N
M
Bis
RL
RM
RH
NMDS1
NMDS2
D
Phylum level
Relative abundance (%)
Firmicutes
Bacteroidota
Proteobacteria
Actinobacteriota
Verrucomicrobiota
Desulfobacterota
Cyanobacteria
Campylobacterota
Unclassified
Myxococcota
Deferribacterota
E
Family level
Muribaculaceae
Lactobacillaceae
Bacteroidaceae
Lachnospiraceae
Erysipelotrichaceae
Oscillospiraceae
Prevotellaceae
Ruminococcaceae
Peptostreptococcaceae
Clostridia UCG-014_norank
Christensenellaceae
Butyricicoccaceae
Tannerellaceae
Monoglobaceae
RF39_norank
[Eubacterium] coprostanogenes group
Erysipelatoclostridiaceae
Eggerthellaceae
Akkermansiaceae
Rhodospirillales_uncultured
Other
F
Genus level
Ligilactobacillus
Limosilactobacillus
Muribaculaceae_norank
Bacteroides
Lactobacillus
Dubosiella
Escherichia-Shigella
Erysipelotrichaceae_uncultured
Prevotellaceae NK3B31 group
Butyricicoccaceae UCG-008
Prevotellaceae UCG-001
Butyricicoccaceae_uncultured
Allobaculum
Blautia
Christensenellaceae R-7 group
Oscillospiraceae UCG-005
Lachnospiraceae NK4A136 group
Roseburia
Colidextribacter
Oscillibacter
RF39_norank
[Eubacterium] coprostanoligenes group_norank
Parabacteroides
Oscillospiraceae_uncultured
Lachnospiraceae_uncultured
Clostridia UCG-014_norank
Ruminococcus
Romboutsia
Monoglobus

◂**Fig. 2** Analysis of intestinal microbial diversity and species composition. The alpha diversity of intestinal microorganisms was evaluated through Chao1 (**A**) and Shannon (**B**) index. The beta diversity of intestinal microorganisms was evaluated through NMDS (**C**). Relative abundances of the main phyla (**D**), families (**E**) and genera (**F**) of intestinal microbiota in different groups. The results of data statistics were expressed by means ± SEM. $^{*}P < 0.01$, $^{**}P < 0.05$, $^{***}P < 0.001$ vs. control; $^{\#}P < 0.05$, $^{\#\#}P < 0.01$, $^{\#\#\#}P < 0.001$ vs. model

separated from the model group and closed to the normal group, which suggested that rhubarb could ameliorate the disorder of fecal metabolic profiles in model rats.

Next, potential biomarkers were screened based on orthogonal partial least square discriminant analysis (OPLS-DA) between the normal and model groups. The score scatter plot of OPLS-DA showed that the metabolic profiles of the normal and model groups were completely separated (Fig. 4A). A combination plot of loading S-plot and VIP-values was used to identify differential metabolites (Fig. 4B). According to the restrictions (VIP > 1, $P < 0.05$), 308 (ESI+) and 143 (ESI-) features were obtained as potential differential metabolites related to constipation.

Based on MS spectrum information, these potential differential metabolites were identified by the HMDB and METLIN database. A total of 17 potential biomarkers were finally identified. The detailed information of these biomarkers was listed in Table 1. Compared with the normal group, the significantly increased metabolites in the model group included calcidiol, 3α,7α-dihydroxy-5β-cholestane, 27-deoxy-5β-cyprinol, 10-hydroxystearic acid, and lithocholic acid, while the levels of 12 biomarkers (17α,21-dihydroxypregnenolone, cholic acid, chenodeoxycholic acid docosahexaenoic acid, arachidonic acid, and others) were markedly decreased in constipation rats. After rhubarb treatment, the level of these differential metabolites were notably reversed. The MetPA databases (https://www.metaboanalyst.ca) and the KEGG databases (https://www.genome.jp/kegg/) were used for metabolic pathways analysis. Based on 17 identified biomarkers, 8 metabolic pathways were obtained, in which 3 metabolic pathways were selected as the most important metabolic pathways ($P < 0.05$, impact > 0.1) that were related to metabolic disturbances (Fig. 5). These metabolic pathways included arachidonic acid metabolism, primary bile acid biosynthesis, and α-linolenic acid metabolism.

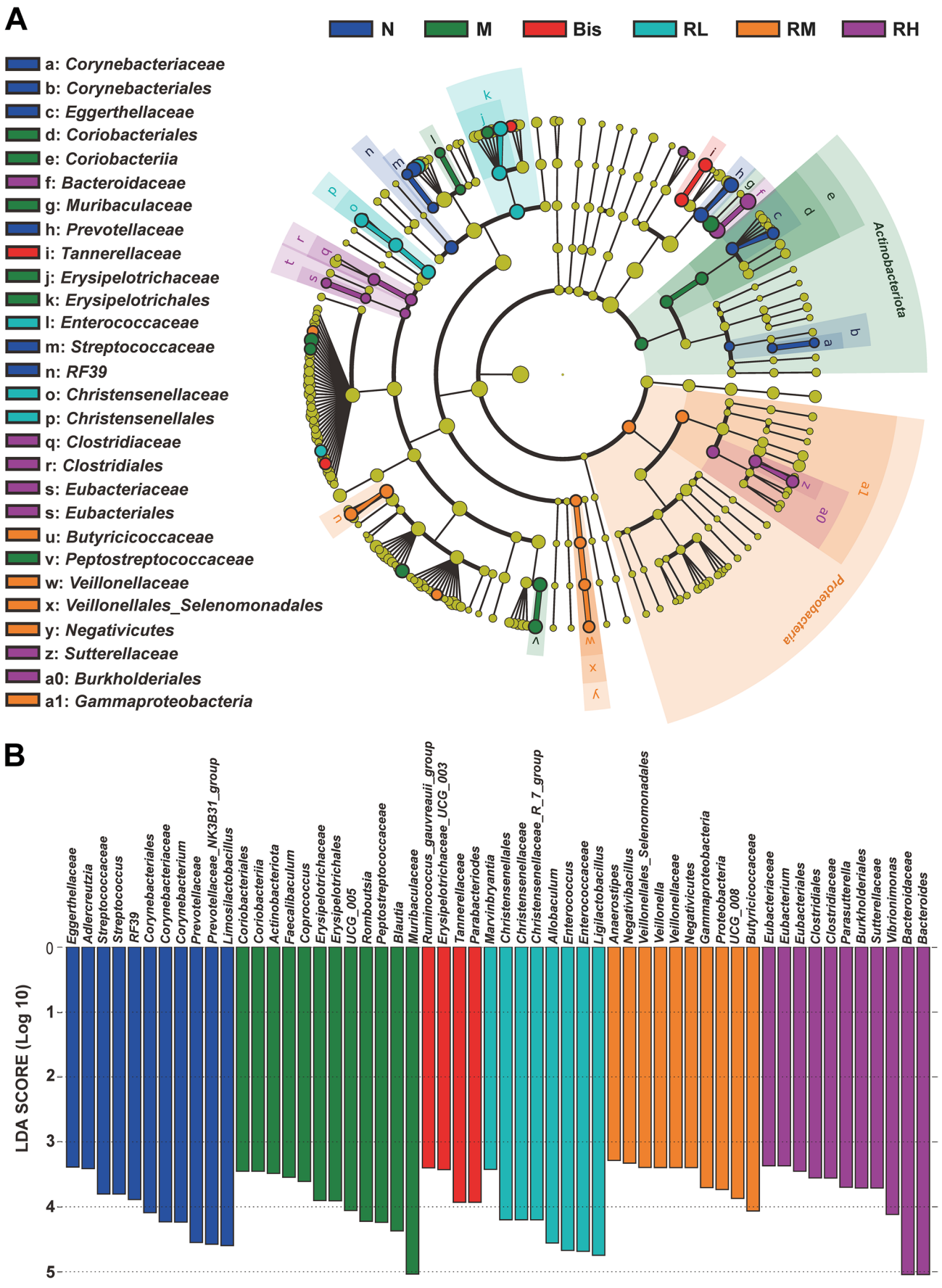
Correlation among intestinal microflora, fecal metabolites, and constipation-related biochemical indexes

To explore the relationship among the gut microbiota, fecal metabolites, and constipation-related biochemical indexes, Pearson correlation analysis was carried out and the correlation coefficient heatmap was obtained (Fig. 6). Results showed that MTL, GAS, and VIP were positively correlated with *Ligilactobacillus*, *Limosilactobacillus*, *Lachnospiraceae_uncultured*, and *Prevotellacaae UCG-001*, while SS positively correlated with *Christensenellaceae R-7 group*, *Erysipelotrichaceae_uncultured*, *Escherichia-Shigella*, *Oscillospiraceae UCG-005*, *Blautia*, and *[Eubacterium] coprostanoligenes group_norank*. Additionally, bacteria such as *Ligilactobacillus*, *Limosilactobacillus*, *Lachnospiraceae_uncultured*, and *Prevotellacaae UCG-001* were positively correlated with 12 metabolites including cholic acid, *α*-linolenic acid, chenodeoxycholic acid, prostaglandin F2α, and 7α,27-dihydroxycholesterol, and others (Fig. 6A). *Oscillospiraceae UCG-005*, *[Eubacterium] coprostanoligenes group_norank*, *Christensenellaceae R-7 group*, *Erysipelotrichaceae_uncultured*, *Escherichia-Shigella*, and *Blautia* were positively related to calcidiol, lithocholic acid, 10 − hydroxystearic acid, 3α,7α-dihydroxy-5β-cholestane, and 27-deoxy-5β-cyprinol. Furthermore, the relationship between 4 biochemical indexes and 17 metabolites were analyzed (Fig. 6B). The 12 metabolites such as cholic acid, *α*-linolenic acid, chenodeoxycholic acid, prostaglandin F2α, 7α,27-dihydroxycholesterol, and others were positively correlated with GAS, MTL, and VIP. SS was positively related to calcidiol, lithocholic acid, 10 − hydroxystearic acid, 3α,7α-dihydroxy-5β-cholestane, and 27-deoxy-5β-cyprinol.

Discussion

Rhubarb, a traditional herb, has been used to treat constipation and other gastrointestinal diseases for more than 2000 years (Wang et al. 2013; Cirillo and Capasso 2015). The rhubarb is rich in polyphenols, particularly in anthraquinones, which can be metabolized by specific colonic bacteria and impact directly on gut microbiota composition. Some studies indicated that some rhubarb components, such as rhein and emodin, could regulate the gut microbiota (Lu et al. 2020; Wu et al. 2020). Currently, increasing evidence has shown a close correlation between the gut microbiota dysbiosis and constipation (Mancabelli et al. 2017). Additionally, some metabolites of intestinal flora play an important role in health, such as bile acid, short-chain fatty acid, and indoles (Fan and Pedersen 2021). Thus, this study attempted to explore the laxative effect of rhubarb through the combination of the intestinal microbiome and fecal metabolome.

Constipation seriously affects people's quality of life and is a vast medical burden on society. In daily life, the inducing factors of constipation are multifactorial, including physical inactivity, inadequate fiber intake, excessive stress, and administration of some opioids, calcium channel blockers, and anticholinergics, but the exact pathogenesis of constipation is still unclear (Vriesman et al. 2020). In experimental


A
N
M
Bis
RL
RM
RH
a: Corynebacteriaceae
b: Corynebacteriales
c: Eggerthellaceae
d: Coriobacteriales
e: Coriobacteriia
f: Bacteroidaceae
g: Muribaculaceae
h: Prevotellaceae
i: Tannerellaceae
j: Erysipelotrichaceae
k: Erysipelotrichales
l: Enterococcaceae
m: Streptococcaceae
n: RF39
o: Christensenellaceae
p: Christensenellales
q: Clostridiaceae
r: Clostridiales
s: Eubacteriaceae
s: Eubacteriales
u: Butyricicoccaceae
v: Peptostreptococcaceae
w: Veillonellaceae
x: Veillonellales_Selenomonadales
y: Negativicutes
z: Sutterellaceae
a0: Burkholderiales
a1: Gammaproteobacteria
Actinobacteriota
Proteobacteria
B
LDA SCORE (Log 10)
0
1
2
3
4
5
Eggerthellaceae
Adlercreutzia
Streptococcaceae
Streptococcus
RF39
Corynebacteriales
Corynebacteriaceae
Corynebacterium
Prevotellaceae
Prevotellaceae_NK3B31_group
Limosilactobacillus
Coriobacteriales
Coriobacteriia
Actinobacteriota
Faecalibaculum
Coprococcus
Erysipelotrichaceae
Erysipelotrichales
UCG_005
Romboutsia
Peptostreptococcaceae
Blautia
Muribaculaceae
Ruminococcus_gauvreauii_group
Erysipelotrichaceae_UCG_003
Tannerellaceae
Parabacteriodes
Marvinbryantia
Christensenellales
Christensenellaceae
Christensenellaceae_R_7_group
Allobaculum
Enterococcus
Enterococcaceae
Ligilactobacillus
Anaerostipes
Negativibacillus
Veillonellales_Selenomonadales
Veillonella
Veillonellaceae
Negativicutes
Gammaproteobacteria
Proteobacteria
UCG_008
Butyricicoccaceae
Eubacteriaceae
Eubacterium
Eubacteriales
Clostridiales
Clostridiaceae
Parasutterella
Burkholderiales
Sutterellaceae
Vibrionimonas
Bacteroidaceae
Bacteroides

◂**Fig. 3** Linear discriminant analysis effect size. A cladogram showed specific bacteria among six groups (**A**). Linear discriminant analysis (LDA) showed scores of these specific bacteria (**B**)

studies, the constipation model is commonly used to investigate the efficacy and mechanism of laxatives. Although some studies also showed that rhubarb had a laxative effect on the common model, rhubarb is only used to establish a diarrhea model (Sun et al. 2020; Peng et al. 2014). Additionally, the constipated model of rats is consistent with the pathophysiology of clinical constipation patients with intestinal dysfunction, which is helpful for us to investigate the mechanism of rhubarb in the treatment of constipation. Therefore, we used a rat model of constipation to investigate the laxative effect of rhubarb. Our data showed that gastrointestinal hormones (GAS, MTL, VIP, and SS) were disordered in constipation. These gastrointestinal hormones play an essential role in regulating the physiological function of the gut. For example, GAS could promote the growth of the mucosa of digestive tract, the contraction of gastrointestinal smooth muscle, and the relaxation of the pyloric sphincter, thereby relieving constipation (Suo et al. 2014). MTL could stimulate the secretion of gastric juice and pepsin and affect the water and electrolyte transport of the intestinal lumen (Feighner et al. 1999). SS could inhibit the secretion of gastrointestinal hormones that promoted defecation (Qian et al. 2018). VIP could significantly stimulate the secretion of water and electrolytes in the intestinal tract and increase gastrointestinal motility (Iwasaki et al. 2019). In this study, rhubarb significantly improved constipation by reducing gastrointestinal transit time, increasing the stool water content, regulating gastrointestinal hormones disruption, and protecting the intestinal barrier (Fig. 1 and Supplemental Fig. S1). Compared with the normal group, the levels of GAS, MTL, and VIP decreased significantly, while the level of SS increased remarkably in the model group. However, rhubarb could notably reverse this trend.

With the increasing understanding of the gut microbiota, it has been proved to be involved in the development of various diseases such as diabetes, inflammatory bowel disease, and obesity. In recent years, the dysbiosis of gut microbiota in constipation has been confirmed, and regulation of the gut microbiota is gradually becoming a new treatment for constipation (Ohkusa et al. 2019). The disorder of gut microbiota in constipated rats was also observed in this study, and treatment with rhubarb could markedly improve the disorder of gut microbiota (Fig. 2). The abundance of *Lachnospiraceae_uncultured*, *Prevotellaceae UCG-001*, *Limosilactobacillus*, and *Ligilactobacillus* in constipated rats was remarkably increased by rhubarb. Previous studies have shown that *Prevodellaceae UCG-001* could degrade fiber to produce short-chain fatty acids (Bekele et al. 2010). As a source of energy for the intestinal epithelium, short-chain fatty acids play an important role in intestinal homeostasis. Additionally, short-chain fatty acids could promote intestinal mucus secretion and protect the intestinal barrier (Mangian and Tappenden 2009; Wrzosek et al. 2013). As two members of *Lactobacillusaceae*, the levels of *Limosilactobacillus* and *Ligilactobacillus* as intestinal probiotics were raised by rhubarb. Clinical studies indicated that some species of *Limosilactobacillus* were able to improve constipation (Riezzo et al. 2019; Trivić et al. 2021). A recent clinical study demonstrated that the abundance of *Erysipelotrichaceae* was increased in constipated patients (Tian et al. 2020). *Erysipelotrichaceae* was found to be closely associated with inflammation-related gastrointestinal diseases and metabolic disorders (Kaakoush 2015). Our results also showed that *Erysipelotrichaceae* was significantly enriched in constipated rats (Fig. 3), and *Erysipelotrichaceae_uncultured* was markedly reduced by rhubarb (Supplemental Table S1). Additionally, the conditional pathogen *Escherichia-Shigella* in the model group was notably reduced after the treatment with rhubarb. Therefore, it was reasonable to assume that rhubarb could restore the microecological homeostasis of intestine by promoting the proliferation of beneficial bacteria and inhibiting harmful bacteria, further ameliorating constipation.

Metabolomics based on UPLC-Q-TOF–MS has been widely used to screen and identify the biomarkers associated with various diseases. To establish the metabolic profiles of gut microbiota, non-targeted metabolomics analysis method was applied to screen differential metabolites in feces. Finally, 17 metabolites were identified in fecal samples and these metabolites are involved in 8 metabolic pathways (Table 1 and Fig. 5). Furthermore, metabolic pathways including primary bile acid biosynthesis, arachidonic acid metabolism, and α-linolenic acid metabolism were disturbed in constipated rats and were markedly improved by rhubarb. The levels of chenodeoxycholic acid and cholic acid in the model rats were significantly increased by rhubarb. Previous studies also confirmed that intestinal chenodeoxycholic acid and cholic acid levels were lower in constipated patients than in the healthy population (Shin et al. 2013; Vijayvargiya et al. 2018). Chenodeoxycholic acid and cholic acid could relieve constipation by accelerating intestinal transit and promoting colonic motility and secretion (Mekjian et al. 1971; Vijayvargiya et al. 2018). Colonic administration of chenodeoxycholic acid showed clinical efficacy in treatment of irritable bowel syndrome with constipation (Steiger et al. 2020). Thus, the alteration of bile acid metabolism might be related to the pathophysiology of constipation. In addition, the arachidonic acid metabolic pathway was affected in constipated rats. Arachidonic acid metabolism could play an important role in intestinal motility and secretion of intestinal fluid (Lai and Manley 1984). Prostaglandin F2α could stimulate chloride secretion in colon epithelial

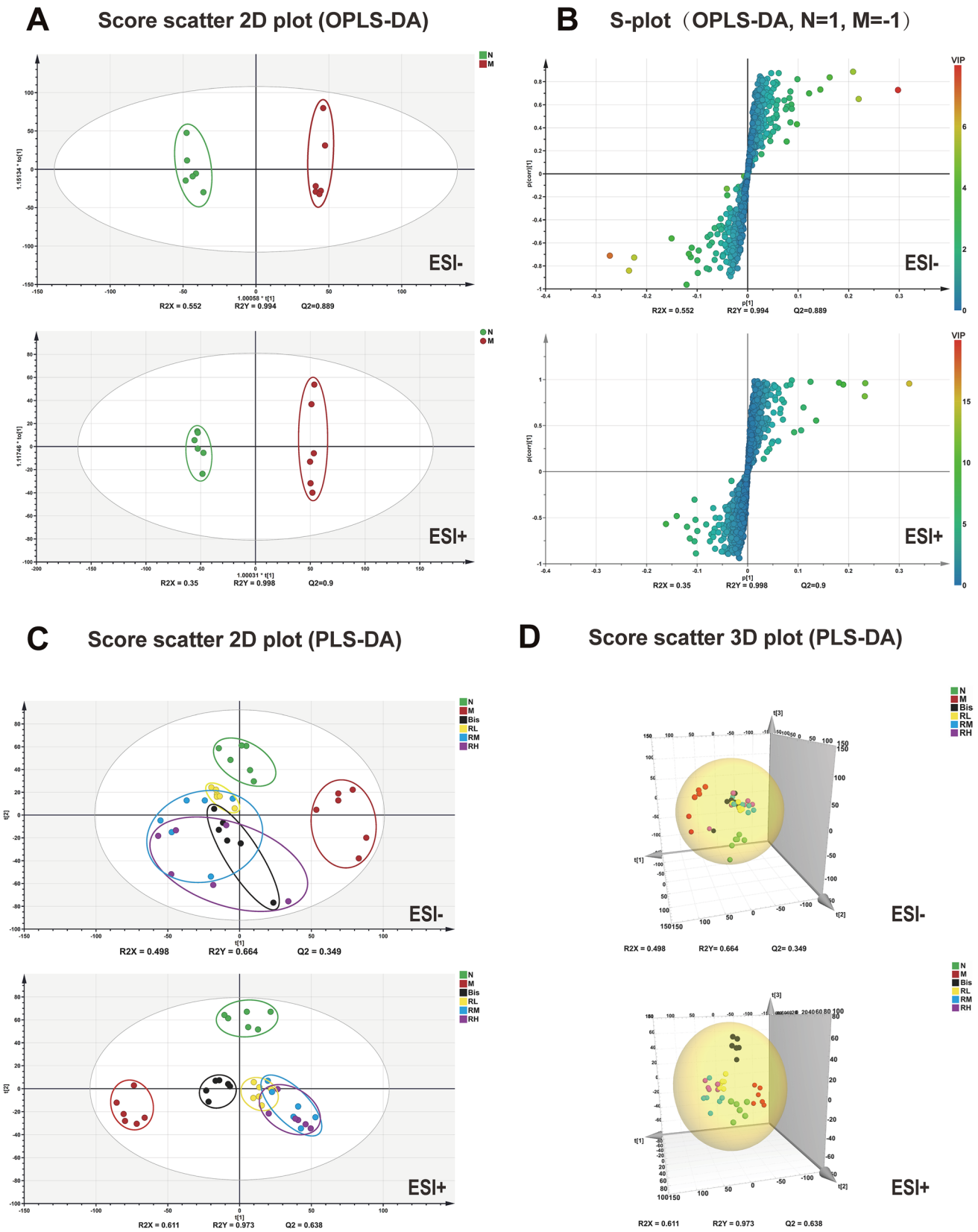


Fig. 4 Multivariate statistical analysis and differential metabolic screening. Score scatter plot (**A**) and S-plot (**B**) were obtained by OPLS-DA analysis between normal and model groups in positive and negative ion mode. In positive and negative ion mode, score scatter 2D (**C**) and 3D (**D**) plot of each group were obtained by PLS-DA analysis

Table 1 The identified and change trend of the potential biomarkers in constipation rats intervened by rhubarb

Metabolites	Formula	HMDB	Mass (m/z)	RT (min)	Molecular ions	VIP	Model	Effects of test drugs				Pathway	Ion mode
							M/N	**Bis**	**RL**	**RM**	**RH**		
α-Linolenic acid	$C_{18}H_{30}O_2$	0,001,388	279.2304	12.29	[M+H]+	2.3234	$\downarrow^{\#\#}$	↑**	↑**	↑**	↑*	α-Linolenic acid metabolism	ESI+
5-L-glutamyl-taurine	$C_7H_{14}N_2O_6S$	0,004,195	255.0598	7.34	[M+H]+	2.2500	$\downarrow^{\#\#\#}$	↑*	↑***	↑*	↑**	Taurine and hypotaurine metabolism	ESI+
3-Dehydrosphinganine	$C_{18}H_{37}NO_2$	0,001,480	300.2873	13.13	[M+H]+	1.8460	$\downarrow^{\#\#\#}$	↑***	↑***	↑**	↑*	Sphingolipid metabolism	ESI+
Prostaglandin F2α	$C_{20}H_{34}O_5$	0,001,139	355.2509	10.79	[M+H]+	2.4159	$\downarrow^{\#\#\#}$	↑*	↑***	↑***	↑***	Arachidonic acid metabolism	ESI+
17α,21-dihydroxypregnenolone	$C_{21}H_{32}O_4$	0,006,762	371.2475	10.89	[M+Na]+	2.1759	$\downarrow^{\#\#\#}$	↑***	↑***	↑***	↑***	Steroid hormone biosynthesis	ESI+
7α,27-dihydroxycholesterol	$C_{27}H_{46}O_3$	0,006,281	421.3390	12.81	[M+H]+	2.0291	$\downarrow^{\#\#\#}$	↑***	↑***	↑***	↑***	Primary bile acid biosynthesis	ESI+
Calcidiol	$C_{27}H_{44}O_2$	0,003,550	423.3153	10.92	[M+Na]+	1.1970	$\uparrow^{\#\#}$	↓***	↓**	↓***	↓***	Steroid biosynthesis	ESI+
Campesterol	$C_{28}H_{48}O$	0,002,869	423.3535	12.47	[M+Na]+	3.8756	$\downarrow^{\#\#\#}$	↑***	↑***	↑*	↑***	Steroid biosynthesis	ESI+
3α,7α-dihydroxy-5β-cholestane	$C_{27}H_{48}O_2$	0,006,893	427.3517	13.66	[M+Na]+	1.9446	$\uparrow^{\#\#\#}$	↓***	↓***	↓***	↓***	Primary bile acid biosynthesis	ESI+
27-deoxy-5β-cyprinol	$C_{27}H_{48}O_4$	0,001,231	459.3422	13.45	[M+Na]+	2.9113	$\uparrow^{\#}$	↓***	↓***	↓***	↓***	Primary bile acid biosynthesis	ESI+
Arachidonic acid	$C_{20}H_{32}O_2$	0,001,043	303.2311	13.45	[M-H]-	3.0244	$\downarrow^{\#\#\#}$	↑**	↑***	↑***	↑**	Arachidonic acid metabolism	ESI-
Docosahexaenoic acid	$C_{22}H_{32}O_2$	0,002,183	327.2328	13.27	[M-H]-	1.7754	$\downarrow^{\#\#\#}$	↑**	↑**	↑	↑	Biosynthesis of unsaturated fatty acids	ESI-
Chenodeoxycholic acid	$C_{24}H_{40}O_4$	0,000,518	391.2846	11.27	[M-H]-	5.1159	$\downarrow^{\#}$	↑*	↑	↑*	↑**	Primary bile acid biosynthesis	ESI-
Cholic acid	$C_{24}H_{40}O_5$	0,000,619	407.2787	9.86	[M-H]-	5.2408	$\downarrow^{\#\#}$	↑**	↑**	↑**	↑**	Primary bile acid biosynthesis	ESI-
Lithocholic acid	$C_{24}H_{40}O_3$	0,000,761	375.2896	11.94	[M-H]-	1.9844	$\uparrow^{\#\#\#}$	↓***	↓*	↓***	↓***	Bile acid metabolism	ESI-
10-Hydroxystearic acid	$C_{18}H_{36}O_3$	0,037,396	299.2581	12.45	[M-H]-	3.6774	$\uparrow^{\#}$	↓**	↓**	↓*	↓**	Fatty acid metabolism	ESI-
7-Ketodeoxycholic acid	$C_{24}H_{38}O_5$	0,000,391	405.2630	10.19	[M-H]-	3.6458	$\downarrow^{\#}$	↑*	↑***	↑***	↑***	Fatty acid metabolism	ESI-

The levels of potential biomarkers are labeled with upregulation (↑) and downregulation (↓). RT, retention time

"#" indicates significant change of *M* vs. N ($^{\#\#\#}P<0.001$; $^{\#\#}P<0.01$; $^{\#}P<0.05$); "*" indicates significant change of different treatment groups vs. *M* ($^{***}P<0.001$; $^{**}P<0.01$; $^{*}P<0.05$)

N, normal group; *M*, model group; *Bis*, bisacodyl (2 mg/kg); *RL*, low dose of rhubarb (1 g/kg); *RM*, medium dose of rhubarb (3 g/kg); *RH*, high dose of rhubarb (9 g/kg)

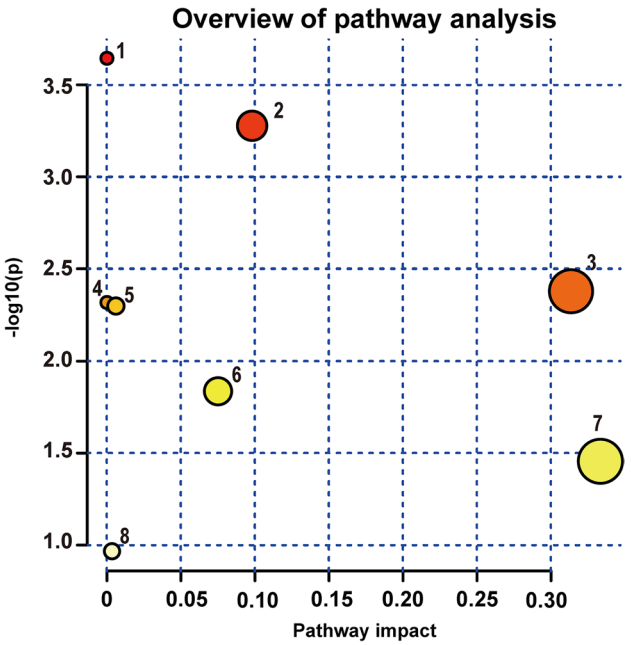


Fig. 5 Metabolic pathways of potential biomarkers. 1. Taurine and hypotaurine metabolism; 2. primary bile acid biosynthesis; 3. arachidonic acid metabolism; 4. biosynthesis of unsaturated fatty acids; 5. steroid hormone biosynthesis; 6. sphingolipid metabolism; 7. α-Linolenic acid metabolism; 8. steroid biosynthesis

cells (Collins et al. 2009). In the results, prostaglandin F2α was notably increased by rhubarb in constipated rats (Table 1). α-Linolenic acid, an ω-3 fatty acid, was significantly increased by rhubarb, and it could promote gastrointestinal motility in cecum resected rats (Zhang et al. 2011), reduce inflammation of the colonic mucosa, and increase the abundance of bacteria associated with the intestinal mucosa (Pearl et al. 2014; Plissonneau et al. 2021). Moreover, lithocholic acid, a toxic secondary bile acid produced by the metabolism of colonic microorganisms, was significantly increased in the model group, which was probably related to the prolonged intestinal transit time during constipation. Lithocholic acid could exert a cancer-promoting effect in the intestinal mucosa (Kozoni et al. 2000). A clinical study showed that the level of lithocholic acid in the feces of patients with constipation was significantly higher than that of the healthy population (Vijayvargiya et al. 2019), which was consistent with our findings. However, rhubarb could significantly reduce the level of lithocholic acid in the feces of rats with constipation. Thus, rhubarb might alleviate constipation by modulating metabolic pathways such as primary bile acid biosynthesis, arachidonic acid metabolism, and α-linolenic acid metabolism and reducing toxic metabolites of gut flora.

In this study, a correlation between intestinal flora and its metabolites was comprehensively evaluated (Fig. 6). Two free bile acids (chenodeoxycholic acid and cholic acid) were positively correlated with *Ligilactobacillus* and *Limosilactobacillus*. Bile salts in the large intestine were mainly hydrolyzed by bile salt hydrolases from dominant bacteria such as *Bifidobacterium* and *Lactobacillus* to free bile acids (Ridlon et al. 2016), and the latter were subsequently degraded to secondary bile acids (Wahlström et al. 2016). Increasing evidence indicated that bile acids could promote the movement and secretion of the colon. In this study, a positive relation between secondary bile acids like lithocholic acid and pathogenic bacteria such as *Escherichia-Shigella* and *Erysipelotrichaceae_uncultured* was observed. However, lithocholic acid was one of the most toxic bile acids (Halvorsen et al. 2000), which suggested that the disorder of intestinal bacteria might be one of the causes of intestinal toxin accumulation in the progress of constipation. Furthermore, the lever of α-linolenic acid in constipation rats was notably upregulated by rhubarb, while α-linolenic acid was also found to be positively correlated with *Ligilactobacillus* and *Limosilalactobacillus*. α-Linolenic acid was reported to promote the proliferation and adhesion of beneficial bacteria to intestinal epithelial cells (Liu et al. 2021). In addition, docosahexaenoic acid, as the downstream product of α-linolenic acid in the biosynthesis of polyunsaturated fatty acids (PUFAs), was also positively correlated with *Ligilactobacillus* and *Limosilalactobacillus*. Similarly, arachidonic acid, as a lipid in the biosynthesis of PUFAs, was also positively related to the beneficial bacteria. The homeostasis of intestinal PUFAs played an important role in maintaining intestinal physiology, including intestinal barrier and inflammation (Durkin et al. 2021; Warner et al. 2019). Evidence suggested that ω-3 PUFA could change gut microbial composition in human (Costantini et al. 2017). Therefore, it was intimated that rhubarb might improve the disorder of intestinal flora in constipated rats through maintaining the homeostasis of intestinal PUFAs.

Furthermore, the prediction of gut microbiota function could provide a complement to researches on fecal metabolism. The results of function prediction and analysis of gut flora showed that some metabolic pathways were changed by rhubarb, especially retinol metabolism, steroid hormone biosynthesis, primary and secondary bile acid biosynthesis, butanoate metabolism, methan metabolism, and propionic acid metabolism (Supplemental Fig. S4). Anaerobic methanogens in the gut could use methane biosynthesis for energy production (Triantafyllou et al. 2014). Recently, some studies have shown that methane could slow down intestinal peristalsis (Pimentel et al. 2006; Park et al. 2017). Moreover, *Methanobrevibacter smithii* was considered to be the primary methane producer (Kim et al. 2012). Interestingly, rhubarb could significantly reduce methane metabolism in this research (Supplemental Fig. S4B), which hinted that rhubarb might improve constipation by inhibiting the growth of methane-producing bacteria. Function prediction of the gut

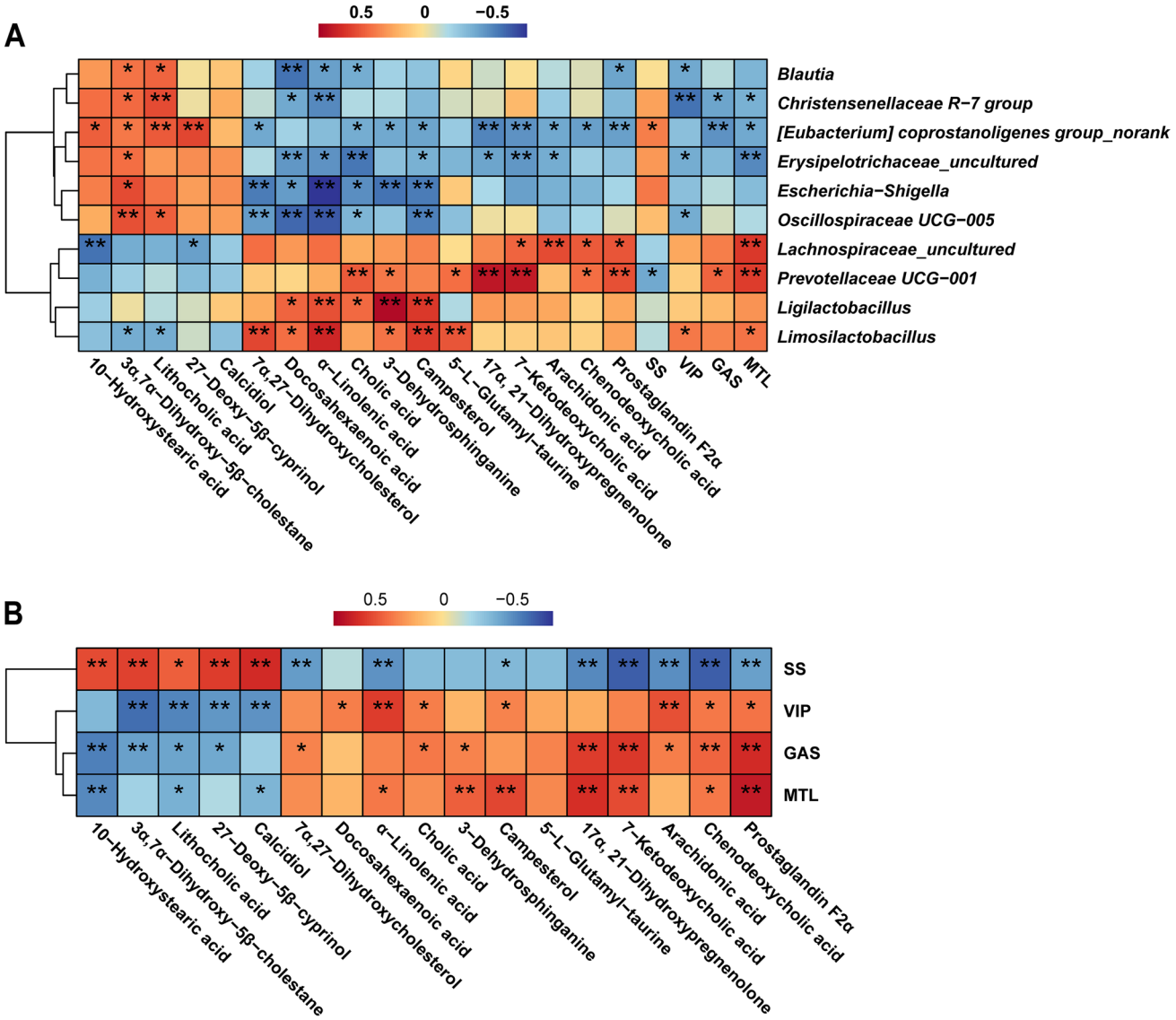


Fig. 6 Correlation analysis among constipation-related biochemical indexes, intestinal flora and fecal metabolites. **A** Pearson's correlation analysis was performed between intestinal bacteria abundance, fecal metabolites and constipation-related biochemical index. **B** Pearson's correlation analysis was carried out between constipation-related biochemical index and fecal metabolites

microbiota also indicated that primary bile acid biosynthesis was increased by rhubarb in constipated rats, consistent with the results of pathway analysis in the fecal metabolome. Bile acids are natural laxatives that affect the colon secretion and promote the intestinal motility as prokinetic agents (Rao et al. 2010). Bile acids are synthesized in the liver and form conjugates with some amino acids. When they arrive in the colon, they can be uncoupled and converted into secondary bile acids by the intestinal flora. Therefore, we speculated that rhubarb might influence the levels of intestinal bile acids by regulating the intestinal flora to achieve a laxative effect. Retinol, known as vitamin A, is essential for maintaining the integrity of the epithelium and mucous membranes, but it can easily become deficient (Pattanakitsakul et al. 2020). Other studies showed that retinol derivatives produced by symbiotic bacteria had protective effects on the intestinal epithelial barrier (Yamada and Kanda 2019; Takahashi et al. 2020). In this study, retinol metabolism was downregulated in the model group, while rhubarb could reverse this trend in constipated rats. In summary, predicting of the metabolic function of gut flora might be helpful to better understand the co-metabolism between gut flora and host and further clarify the mechanism of rhubarb.

In conclusion, loperamide-induced constipation rat was successfully established and applied to investigate the therapeutic effect of rhubarb. Results showed that different dosages of rhubarb had a good laxative effect.

Furthermore, 16S rRNA gene sequencing and fecal metabolomic analysis showed that rhubarb could significantly ameliorate the dysbiosis of gut microbiota and the disorder of fecal metabolite profiles in constipation rats. Beneficial bacteria such as *Ligilactobacillus*, *Limosilalactobacillus*, *Lachnospiraceae_uncultured*, and *Prevotellaceae UCG-001* decreased in constipation rats were remarkably enriched after the treatment with rhubarb, while opportunistic pathogens such as *Escherichia-Shigella* were notably decreased by rhubarb. Additionally, fecal metabolomics analysis indicated that fecal metabolic profiles of rats with constipation were improved after rhubarb treatment. Rhubarb significantly increased pro-defecation metabolites such as chenodeoxycholic acid, cholic acid, prostaglandin F2α, and α-linolenic acid and markedly decreased toxic metabolites such as lithocholic acid in constipated rats. Additionally, the correlation analysis showed that chenodeoxycholic acid, cholic acid, prostaglandin F2α, and α-linolenic acid were positively correlated with *Ligilactobacillus*, *Limosilalactobacillus*, *Lachnospiraceae_uncultured*, and *Prevotellaceae UCG-001*, while lithocholic acid was proportional to *Escherichia-Shigella*. These results suggested that an interplay among gut microbiota, microbiota-derived metabolites, and the host might play an important role in the constipation process. Rhubarb might treat constipation by improving the disorder of intestinal flora and its mechanism, and restoring intestinal flora homeostasis. Additionally, this study also showed that the combined application of multi-omics was a potential strategy to reveal the mechanism of herbal medicines.

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Author contributions YL performed experiments, analyzed the data, and wrote the manuscript. YW, WWL, CL, HFL, ZLD, KZ, EXS, and DWQ helped with performing experiments and analyzed data. SJ and JAD designed the study and critically reviewed the manuscript. All authors read and approved the manuscript.

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Data availability All data generated or analyzed during this study are included in this published article (and its supplementary information files).

Declarations

Ethical approval All applicable international, national, and/or institutional guidelines for the care and use of animals were followed.

Conflict of interest The authors declare no competing interests.

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