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RESEARCH ARTICLE

Mechanism of Mulberry's Effect on Intestinal Flora and Motility

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Abstract: Studies have indicated that mulberry consumption promotes intestinal motility and alleviates constipation, but the specific mechanism remains unclear. This study adopted a constipation mouse model to empirically analyze the effects of mulberry on intestinal motility, gut microbiota composition, and neurotransmitter levels, aiming to elucidate its mechanism of action. Key findings are as follows: 1) Compared with the model group, the treatment with mulberry extract significantly increased the moisture content of feces (MD). Among them, the increase in the high-dose group compared to MD was 137.5% ($p< 0.05$). It also accelerated the small intestine transport speed by 3.4 times and shortened the latency period for the first occurrence of black feces by 73.7% ($p<0.05$). These parameters were comparable to those of the untreated control group. 2) In the constipation model caused by dehydration and restraint together, six intestinal neurotransmitters changed. Mulberry administration restored the levels of acetylcholine (ACh), γ-aminobutyric acid (GABA), serotonin (5-HT), and Norepinephrine (NE) relative to the model group, while Glutamate (Glu) was reduced. 3) The 16S rRNA gene sequencing of the intestinal microbiota revealed that there were 21 species of bacterial genera with varying abundances among different groups. Correlation analysis linked their relative abundances to the levels of intestinal neurotransmitters and motility parameters. Among them, *Dorea unclassified_f_Rikenellaceae* was identified as the constipation-associated genus. 4) Correlation analyses linked the altered genera with both neurotransmitter concentrations and motility indices. Notably, *Dorea* ($p < 0.05$) and *unclassified_f_Ruminococcaceae* ($p < 0.01$) showed the most pronounced changes after mulberry intervention and were strongly associated with restored 5-HT, Glu, NE, and ACh levels. Although *Citrobacter*, *Oscillospira*, and *unclassified_f_Rikenellaceae* remained unchanged, their baseline abundances correlated with constipation severity, suggesting potential biomarker roles. Collectively, these results demonstrate that mulberries markedly alleviate constipation in mice by restructuring the gut microbial ecosystem and normalizing intestinal neurotransmitter profiles, thereby enhancing peristalsis. Importantly, our integrated motility-microbiome-neurochemical data provide a novel mechanistic insight supporting the development of mulberry-based adjunct therapies for constipation.

Keywords: Mulberry, Gut Microbes, Intestinal Motility, Constipation

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Introduction

Functional constipation is a common functional bowel problem in clinical practice, which is mainly manifested by difficult defecation, less frequent bowel movements, and passing hard stools. Persistent constipation not only makes patients more likely to have proctological complications such as anal fissure, hemorrhoids and rectal prolapse, but also affects the mental health of patients. Issues like insomnia, depression, and anxiety are common, and may even raise the risk of cardiovascular

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and cerebrovascular events [1-3]. At present, ways to manage it include lifestyle changes, drug treatment and physical therapies [4], but such methods often don't reach the best treatment results; long-term use of laxatives can lead to dependence, may cause melanosis coli, and even make constipation worse instead [5]. The pathogenesis of functional constipation is multifactorial and closely linked to disturbances in intestinal motility. Recent evidence indicates that the integrity of the intestinal mucosal barrier is intimately associated with gut-microbiota composition and enteric neurotransmitter signaling, but the precise mechanisms are still not fully understood.

Consequently, systematic drug-screening studies targeting these pathways are scarce [6]. Mulberry (fruit), a dried fruit spike, belongs to Moraceae (Morus alba L.). It has a sweet and sour flavor, a cold property, functioning in channel tropism: heart, liver, and kidney. The mulberry, first written in *Xinxiu Bencao Tang*, affects nourishing the liver and kidney, enriching blood and promoting fluid production, nourishing fluids to calm wind, moistening the intestines, and relieving constipation [7]. Recognized by the National Health Commission as a "medicinal-and-edible homology" resource, mulberry is an attractive candidate for functional-health supplement development. The fruit contains more than 150 identified bioactive compounds and exhibits diverse physiological activities. Nevertheless, the mechanistic basis by which mulberry moistens the intestines, alleviates constipation, and enhances intestinal motility has not been elucidated. The present study investigates the therapeutic potential of mulberry fruit in a murine model of constipation. Specifically, we first characterize the phenotypic effects of mulberry supplementation on intestinal motility, including intestinal propulsion rate and first-defecation latency. We measured the concentrations of key enteric neurotransmitters, including Acetylcholine (ACh), γ aminobutyric acid (GABA), serotonin (5 HT), Norepinephrine (NE) and Glutamate (Glu). The aim is to explore how mulberry affects the neurochemical environment that controls gut motility. Then, we analyzed the gut microbiota composition by using 16S rRNA gene sequencing to identify bacterial taxa changed by mulberry treatment. Finally, we combined motility, neurochemical, and microbiome data to explore the relationship between specific bacterial changes and neurotransmitter levels. This gives mechanistic clues about mulberry's possible laxative effect. These findings will build a scientific basis. It supports developing mulberry-derived adjunct therapies for constipation. This depends on further translational validation in human studies.

Materials and Methods

Equipment and Reagents

Pilocarpine Nitrate 132-34-2, 98% (Weicai, China); Rhein 476-32-5, 98%; serotonin (5-HT) (091M5163V, American Sigma company); Norepinephrine (NE) (T10D6F7243, Shanghai Yuanye Biotechnology Co., Ltd); Dopamine (DA) (100070-201507, National Institute for Food and Drug Control); Glutamate (Glu) (S27M6G1, Shanghai Yuanye Biotechnology Co., Ltd); Acetylcholine (ACh)(R769IT59, NIFDC); Gamma-aminobutyric acid (GABA);(Z08A8H33553, Shanghai Yuanye Biotechnology Co., Ltd); Acetonitrile(Merck, Germany); Methanol(Merck, Germany); Chloral Hydrate(Shanghai Yuanye Biotechnology Co., Ltd). Mulberry, Botou Yuqing Biotechnology Co., Ltd., Figwort Root (Scrophularia ningpoensis), Dwarf Lilyturf Tuber (Ophiopogon japonicus), and Rehmannia Root (Rehmannia glutinosa) were purchased from TongRenTang Chinese Medicine.

Waters Acquity UPLC High Performance Liquid Chromatography (HPLC) System(Waters Corporation, Milford, MA, USA); Waters XEVO TQ-S Triple Quadrupole Mass Spectrometry (QQQ-MS) System(Waters Corporation, Manchester, UK); High-Speed Benchtop Refrigerated Centrifuge(Eppendorf 5424R, Eppendorf, Germany); Ultra-Micro Spectrophotometer(NanoDrop2000, Thermo Fisher Scientific, USA); PCR Sequencer(ABI GeneAmp® 9700, ABI, USA); MISEQ DNA Sequencer(Illumina Miseq, Illumina, USA); HISEQ Sequencer(Illumina Hiseq, Illumina, USA); Microplate Reader (BioTek ELx800, Biotek, USA); Covaris ultrasound DNA Tissue Disruptor(M220, Gene Company Limited, China); DNA Extraction Kit(D5625, Omega Bio-Tek, USA); FastPfu Polymerase(120428, TransGen, China); AxyPrep DNA Gel Extraction Kit(AP-GX-250, Axygen, USA)

Animals and Groups

Forty-two Specific-Pathogen-Free (SPF) female C57BL/6J mice, each weighing 180 ± 20 g, were acclimated for seven days in a controlled environment (temperature 20 ± 2 °C; relative humidity 60-70%). The sample size was determined based on a power analysis using GPower software (version 3.1), aiming for an effect size of f = 0.25 (medium effect), α = 0.05, and β = 0.20 (power = 0.80). The calculation shows that significant differences can be detected in at least six animals in each group. In response to the possible shedding situation, seven animals were actually placed in each group.

The mice took the lead in completing the adaptive training. It is randomly divided into six experimental groups through the random number table, with seven in each group. Among them, there is a blank control group (MB), and only physiological saline is given; another model control group (MD) is set up. The group did not receive any treatment after experiencing the process of induced constipation. There are also two groups of reference laxative groups: the positive high-dose group (GTH) and the negative low-dose group (GTL). Two groups of mulberry extract groups were included at the same time: high-dose group (XJH) and low-dose group (XJL). This study adopts a single-blind design. The staff who carried out the intervention do not know the affiliation of each group of mice. All experimental steps are approved by the Ethics Committee of Cangzhou Medical College (Approval No.: 2023-24) and follow the "Standards for the Management of Experimental Animals". Animal production license number: SCXK (Liao) 2020-000.

Animals Model Copy and Interruption

In order to determine the optimal dosage range, a preliminary dose-response study was done. It followed the equivalent efficacy conversion rule from clinical practice. The high-dose group (XJH) got 24 g· kg^{-1}· day^{-1}, which is about 90 times the clinical dosage for humans. The low-dose group (XJL) got 8 g· kg^{-1}· day^{-1}, which is 30 times the human clinical dosage. These doses were chosen based on past pharmacological studies, which show rodents need higher concentrations to reach the maximum biological activity.

Constipation was induced by the "compound diaphoresis-plus-purgative" method briefly. Mice were treated with pilocarpine (used to promote sweating) and a mild laxative for 14 consecutive days, which effectively produced typical symptoms such as gastrointestinal transit delay and reduced fecal moisture content. The model was successfully established by measuring the frequency of defecation, fecal weight, and serum levels of gastrointestinal hormones.

After verification of the model, the animals were treated as follows for seven consecutive days: the high-dose mulberry group (XJH) and the low-dose mulberry group (XJL) were given an oral gavage of mulberry fruit decoction at 24 $g \cdot kg^{-1} \cdot day^{-1}$ and 8 $g \cdot kg^{-1} \cdot day^{-1}$, respectively. The positive control groups mice received Zengye Decoction, which contains Radix Scrophulariae 38 g, Radix Ophiopogonis 31 g, and Rehmannia root 31 g. The low-dose subgroup was administered 8 $g \cdot kg^{-1} \cdot day^{-1}$, while the high-dose subgroup received 24 $g \cdot kg^{-1} \cdot day^{-1}$. The decoction was given once daily by oral gavage. The model control (MD) and the blank control (MB) groups were gavaged with an equal volume of normal saline to match the fluid load of the treatment groups.

Mulberry Intervention Constipation Model Correlated Efficacy Index Evaluation

Macroscopic Presentation Evaluation

General condition. We recorded the overall health of each mouse every day. We paid attention to mental state − how alert they were and their responsiveness, coat condition including hair shine, tail look, and fecal traits like shape, color and firmness. We also noted down any signs of distress. Lethargy and unusual grooming behaviors were documented too.

Defecation parameters. We measured four quantitative indices. They are for assessing bowel function:

1) Defecation quantity. The total number of fecal pellets expelled over a 24-h period was counted for each animal.
2) First black stool excretion time (FSE). After the final administration of the constipation-inducing regimen, the latency from the start of the observation period to the appearance of the first black-colored stool was recorded.
3) Fecal water content (FWC). Fresh feces were weighed (wet weight), then dried at 60 °C for 24 h and weighed again (dry weight). The water content was calculated as: $\mathrm{FWC}(\%) = \frac{\text{wet weight} - \text{dry weight}}{\text{wet weight}}$ x 100.
4) Intestinal propulsion rate. A 5 % charcoal suspension at a dose of 10 mL per kilogram of body weight was administered orally after a 12-hour fast. Ten minutes later the mice were euthanized, the small intestine was removed, and the distance traveled by the charcoal marker was measured. The propulsion rate was expressed as the ratio of the traveled distance to the total length of the small intestine, presented as a percentage.

All measurements were performed by investigators blinded to the group assignments, and the mean values for each group (n = 7) were used for subsequent statistical analysis.

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Microcosmic Presentation Evaluation

Enteric neurotransmitter detection. Approximately 100mg of frozen intestinal tissue was taken from each mouse, weighed, and transferred to pre-cooled tubes. Cold formic-acid/acetonitrile (1:9, v/v) solution was added, and the samples were subjected to ultrasonic disruption and homogenization in an ice bath. Then, an equal volume of cold formic-acid/acetonitrile (1:1, v/v) solution was added, and the samples were thoroughly vortexed and mixed. Finally, the suspension was left to stand at 4°C for 30 min. Centrifugation was done first. The supernatant was collected. It was used for quantitative analysis of six neurotransmitters. They are acetylcholine (ACh), 5-hydroxytryptamine (5-HT), Dopamine (DA), γ -Aminobutyric Acid (GABA), Norepinephrine (NE), and Glutamate (Glu).

Calibration curves for each neurotransmitter were 52.8d using standard solutions covering the expected concentration ranges in the tissue extracts. The Limits of Detection (LOD) and Limits of Quantification (LOQ) for all analytes met the requirements of the method validation (LOD < 0.05 ng·mL^{-1}, LOQ < 0.15 ng·mL^{-1}). All analyses were performed by an operator blinded to the group allocation, and each sample was measured in triplicate to ensure analytical reproducibility (Table 1).

Table 1: Specific conditions for quantitative analysis using an UPLC-TQ-MS system

Parameter	Setting
Column	Waters ACQUITY UPLC BEH C18 (2.1 mm × 100 mm, 1.7 μm)
Mobile phase	0.1 % formic acid (A) and acetonitrile (B) with a gradient elution program
Flow rate	1.2 mL min^{-1}
Ionization mode	Electrospray ionization (ESI$^+$)
Detection mode	Multiple-reaction monitoring (MRM)
Injection volume	5 μL
Column temperature	40 °C
MS parameters	Curtain gas = 35 psi; Ion source gas = 45 psi; Temperature = 500 °C; Capillary voltage = 3.5 kV

Intestinal Flora Diversity Study

Fecal pellets (≈200 mg) were collected from each mouse after a 12-h fast, stored at -80 °C, and DNA was extracted with the OMEGA-Soil DNA Kit. Full-length 16S rRNA genes were amplified using PacBio barcoded primers 27F (5′-AGRGTYYGATYMTGGCTCAG-3′) and 1492R (5′-RGYTACCTTGTTACGACTT-3′).

PCR (25 μL)

2× High-Fidelity PCR Master Mix 12.5 μL; Forward primer (10 μM) 0.5 μL.

Reverse primer (10 μM) 0.5 μL; Template DNA (10 ng/μL) 2 μL.

Nuclease-free water 9.5 μL.

Cycling (27 cycles)

95 °C 2 min; 95 °C 30 s; 55-57 °C 30 s; 72 °C 60 s; final 72 °C 5 min; hold 4 °C.

Products were checked on 1 % agarose gel (≈1.5 kb band) and verified with a Bioanalyzer. SMRTbell libraries were built and sequenced on the PacBio Sequel platform. CCS reads were filtered, denoised, and assigned taxonomy against SILVA 138 using QIIME 2.

All six groups (n = 7 per group: MB, MD, GTH, GTL, XJH, XJL) were processed identically, ensuring that observed microbiota differences reflect the respective treatments.

Statistical Analysis

All data were processed with SPSS 22.0 (IBM Corp., Armonk, NY, USA). Before conducting the parameter tests, the Shapiro-Wilk test was used to assess the normality of the data distribution and to test the homogeneity of variances.

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The Levene test was employed. For normally distributed data with equal variances, a one-way analysis of variance was used to evaluate the differences between groups. When the overall F-test result was significant, a post hoc Tukey test was conducted for pairwise comparisons. For non-normal distributed data or data that violated the assumption of homogeneity of variances, the Kruskal-Wallis H test was used. Then, the Mann-Whitney U test for paired comparisons was performed. The results were presented in the form of mean ± standard deviation (Mean ±SD), and a two-sided p-value < 0.05 was considered statistically significant.

Beta-Diversity of Fecal Microbiota

Principal Coordinates Analysis (PCoA) based on the unweighted UniFrac distance was performed to visualize differences in microbial community composition among the experimental groups. To identify the most discriminative bacterial taxa, a partial least squares discriminant analysis (PLS-DA) was subsequently applied, and the taxa with the highest Variable Importance in Projection (VIP) scores were selected as the key contributors distinguishing the groups.

Results

Affection Mulberry Solution Made on Feces

Fig. 1 clearly demonstrates the divergent fecal phenotypes among the experimental groups. The model control group (MD; Fig. 1b) excreted feces. Its feces are obviously long and slender, dark in color and hard in texture - these are the typical characteristics of the constipation model. The feces produced by the blank control group (MB; Fig. 1a) are different. Its feces are large, light in color and soft in granular form, indicating that the gastrointestinal function is normal. There is a difference between the low-dose mulberry treatment group (XJL; Fig. 1c) and the MB group. Its feces are slightly smaller, lighter in color and softer in texture, indicating that mulberry administration alleviates constipation-related changes to a certain extent.

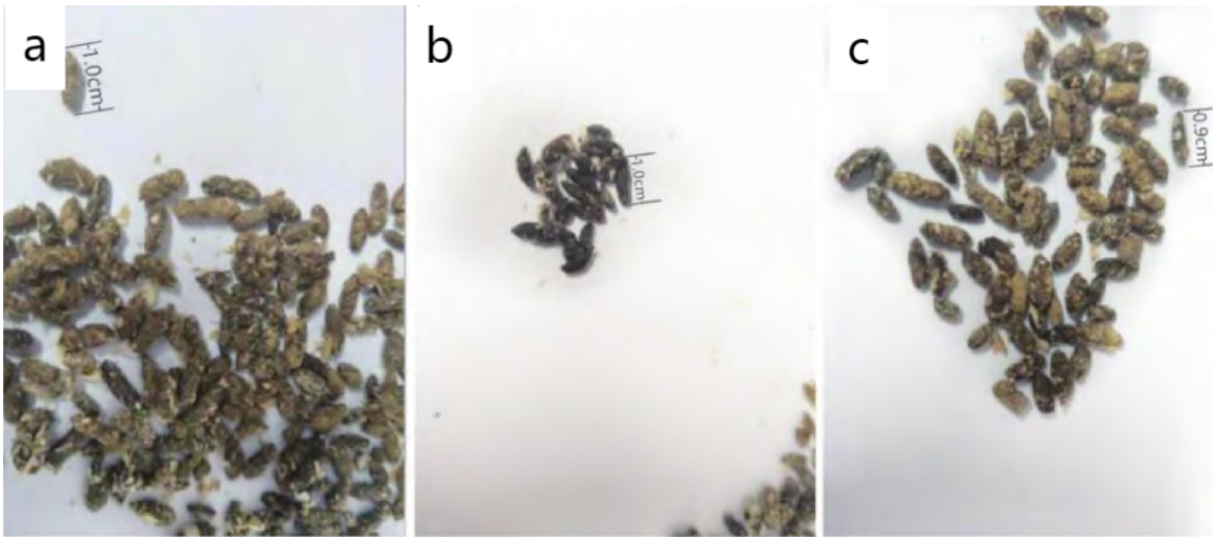

Fig. 1: Characteristics of feces in blank control group (MB), model group (MD), and low-dose mulberry group (XJL) mice

Affection Mulberry Solution Made on Feed and FWC

As shown in Fig. 2, there was a significant difference between the constipation model group (MD) and the blank control group (MB) after 14 days of induction. The fecal output of the MD group increased significantly (Fig. 2A), and the food intake also increased significantly (Fig. 2C), $p<0.05$. The water content (FWC) of feces decreased sharply (Fig. 2D), where $p<0.05$.

The two groups of mulberry treatment groups performed better in the above indicators. The feeding volume of the low-dose mulberry group (XJL) increased by 69.4% compared with that of the MD group. The high-dose mulberry group (XJH) received 102.9% higher food than that of the MD group, and both of them were $p<0.05$. There was no statistically significant difference between the mulberry group and the positive control group (GTH, GTL) and MB group. The results show that mulberry can effectively alleviate the clinical manifestations of constipation in the animal model.

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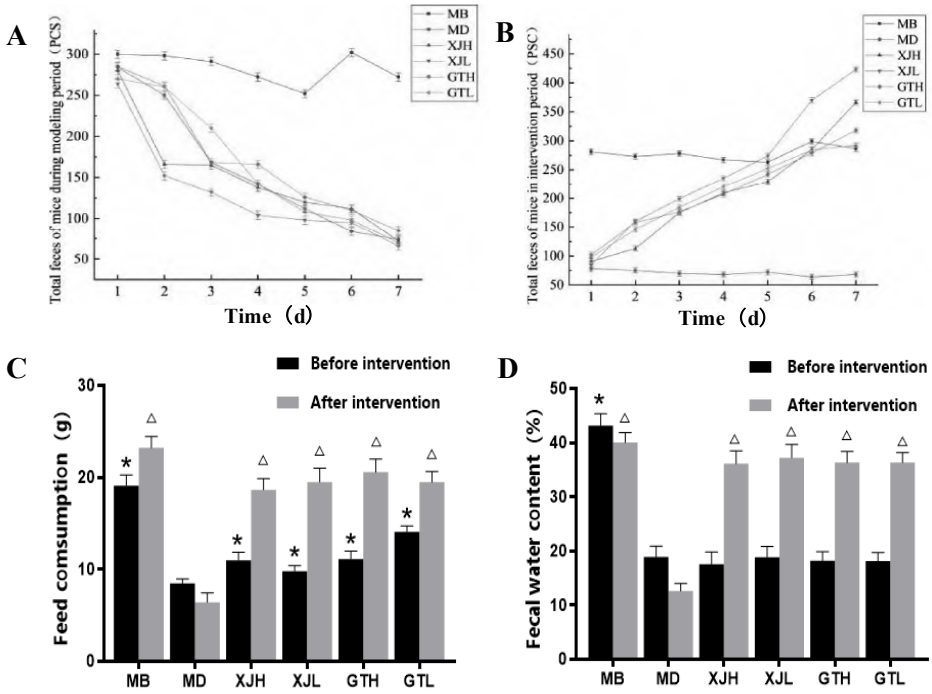

Fig. 2: Changes in food intake, total fecal matter, and water content indicators of mice in each group (Compared with the MD group

*p<0.05; △p<0.05)

Mulberry Stimulates Intestinal Motility

Fig. 3A illustrates the alterations in Intestinal Propulsion Rate (IPR) and First-Stool-Expulsion (FSE) latency following the 14-day modeling period. In the constipation model (MD) group, IPR decreased by 76.9 % relative to the blank-control (MB) group, while FSE time was prolonged by 206.9 % (both $p < 0.05$), confirming the successful establishment of the constipation model.

Both mulberry-treated groups (XJL and XJH) exhibited a significant increase in IPR and a reduction in FSE latency compared with the MD group ($p < 0.05$). The magnitude of these improvements was comparable to that observed in the positive-control groups (GTH and GTL), indicating that mulberry supplementation effectively normalised intestinal motility to a level similar to the established therapeutic reference.

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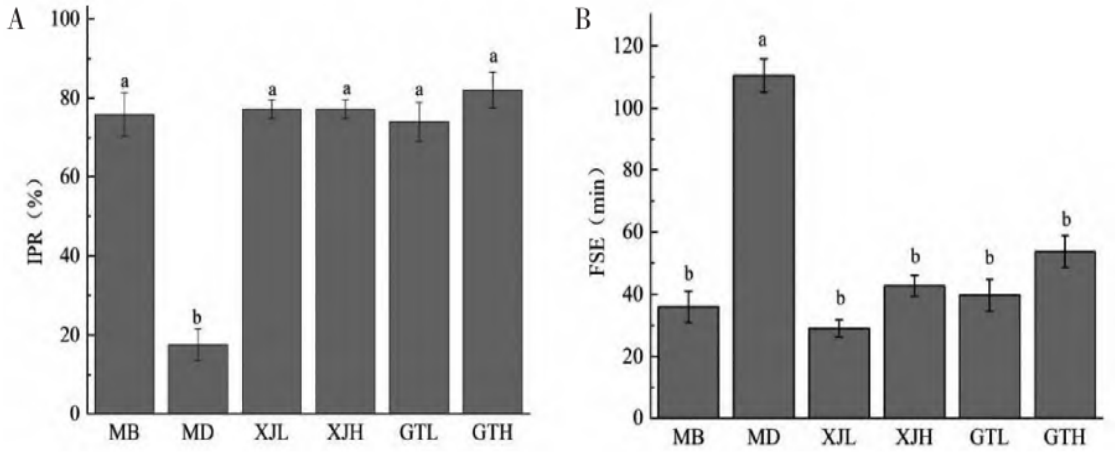

Fig. 3: Various functional indicators of IPR and FSE in each group of mice (Compared with the MD group, a p<0.05; Compared with the MB group, b p<0.05)

Mulberry Stimulates Intestinal Motility of Constipated Mice

The peak ion chromatograms and extracted-ion chromatograms of the reference solution for the six neurotransmitter ion pairs are presented in Fig. 4, demonstrating that each neurotransmitter is well separated and can be quantified with high accuracy. Table 2 shows the changes in the concentrations of the six enteric neurotransmitters relative to the blank-control group (MB). In the constipation model (MD) the following alterations were observed: acetylcholine (Ach) decreased by 53.8 %, γ-aminobutyric acid (GABA) decreased by 25.6 %, and Dopamine (DA), 5-hydroxytryptamine (5-HT) and Norepinephrine (NE) were all reduced to varying extents. GLUTAMATE (Glu) was increased. Notably, the DA level fell by 8.2 %, a change that did not reach statistical significance.

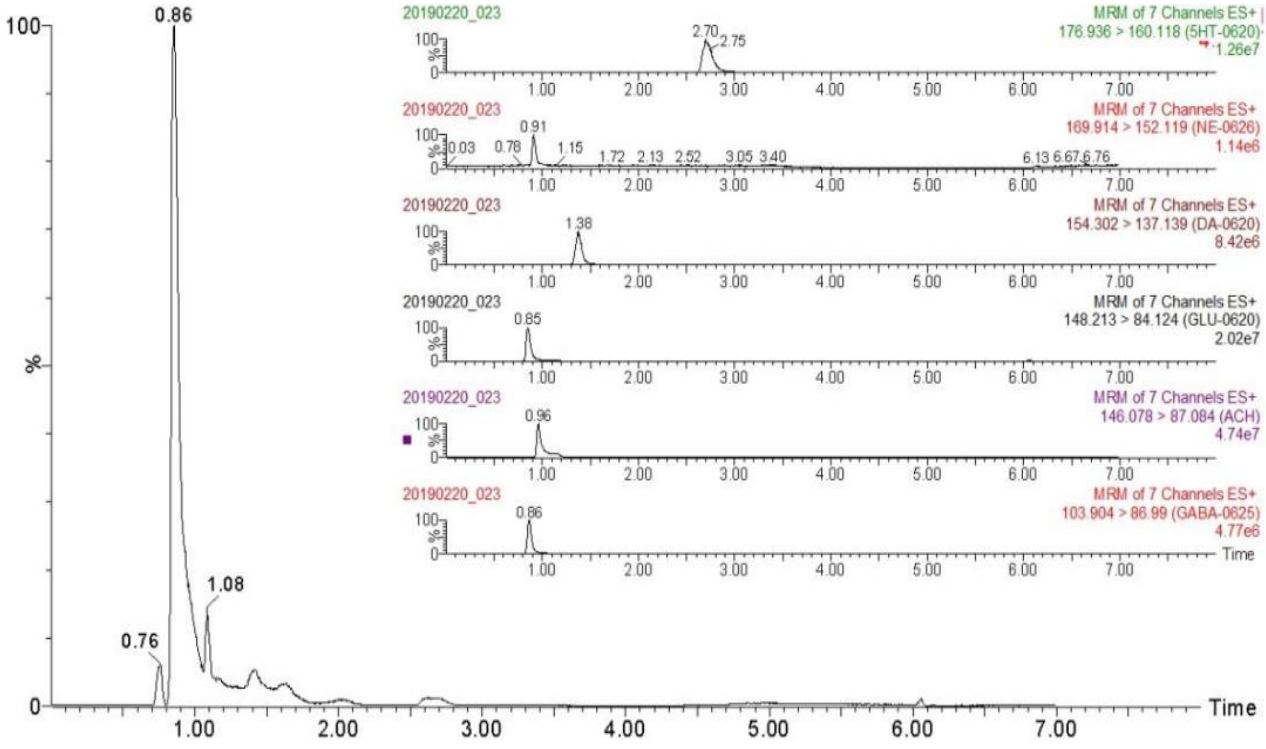

Fig. 4: Reference solution peak ion chromatogram and extraction ion chromatogram of 6 neurotransmitter ion pairs

Following mulberry intervention, the enteric neurotransmitter profile was markedly restored, approaching the values seen in the MB group. In the low-dose mulberry group (XJL) the increases were statistically significant ($p < 0.05$): GABA rose by

63.7 %, DA by 17.6 %, 5-HT by 11.7 %, and NE by 6.1 %. Conversely, the integrated peak area of 5-HT was reduced by 52.8 %, and that of Glu by 17.4 %, relative to the MD group.

These results indicate that mulberry treatment re-balances multiple neurotransmitters in the enteric nervous system, thereby contributing to the improvement of intestinal motility in constipated mice.

Table 2: Different treatment groups on neurotransmitters in the small intestine

Group	ACh	GABA	DA	5-HT	NE	Glu
MB	0.1977±0.02	0.7144±0.04	0.2044±0.01	0.3555±0.02	3.0155±0.06	84.78±3.78
MD	0.0907±0.02*	0.5316±0.02*	0.1884±0.01	0.3362±0.01	2.8371±0.03	82.27±5.67
XJL	0.1422±0.01	0.8666±0.03#	0.2218±0.01#	0.3757±0.06#	3.0224±0.04#	76.77±4.64
XJH	0.0621±0.02	0.4850±0.03	0.2051±0.01#	0.1581±0.06	2.7628±0.03	68.16±2.55#
GTL	0.1911±0.02&	0.9955±0.03&	0.2946±0.014	0.4933±0.07&	3.5829±0.04&	102.33±4.30
GTH	0.1068±0.03	0.4466±0.03	0.1357±0.01	0.2283±0.05	3.5388±0.08	39.44±2.67&

Note: contrast with MB, enteral neurotransmitter content of MD, * $p<0.05$; contrast with MD, after mulberry intervention, two groups enteral neurotransmitter, # $p<0.05$; contrast with MD, after positive drugs intervention, enteral neurotransmitter content, & $p<0.05$

Microbial Genera in Fecal Samples

To assess the compositional consistency of the fecal microbiota, we employed 16S rRNA sequencing. We analyzed the data. The relative content of different bacterial genera was significantly different between the blank control group (MB) and the constipation model group (MD). This change of microbial components confirmed that constipation induced significantly changed the fecal bacteria community structure (see Table 3).

Table 3: Differential bacterial genera of the MB and MD groups

No.	Genus	Trend	Comp
1	*Trabulsiella*	↓	-0.2848
2	*Ruminococcus*	↓**	-0.2578
3	*Parabacteroides*	↓*	-0.2538
4	*Oscillospira*	↑*	0.2314
5	*unclassified_f_Coriobacteriaceae*	↑*	0.2272
6	*f-S24-7*	↓*	-0.2217
7	*norank_f_Lachnospiraceae*	↓**	-0.2160
8	*unclassified_f_Ruminococcacea*	↓*	-0.2060
9	*unclassified_f_Rikenellaceae*	↑*	-0.2056
10	*Akkermansia*	↓**	-0.1799
11	*Enterobacteriaceae*	↑*	-0.1588
12	*Unclassified-f-Peptostreptococcaceae*	↑*	-0.1511
13	*Eubacterium*	↑*	-0.1462
14	*Erysipelotrichaceae*	↓**	-0.1361
15	*Enterococcus*	↑**	-0.1326
16	*Dorea*	↑**	0.1234
17	*Coprococcus*	↑*	0.1205
18	*Citrobacter*	↑*	0.1184
19	*Blautia*	↑*	0.1179
20	*Bacteroides*	↓***	0.1144
21	*Adlercreutzia*	↑*	0.1117

Note: Upward trend in the model group; ↓: Downward trend in the model group; * $p<0.05$; ** $p<0.01$; *** $p<0.001$

We used Pearson correlation analysis to examine how the relative abundance of differentially abundant bacterial taxa links to intestinal neurotransmitter concentrations. We assessed their connection with constipation.

The analysis revealed the following significant correlations ($|r| > 0.8$, $p < 0.05$): Norepinephrine (NE) was strongly positively correlated with *Enterococcus* ($r = 0.912$, $p<0.05$), *Ruminococcus* ($r = 0.898$, $p<0.05$), *Adlercreutzia* ($r = 0.828$, $p<0.05$) and *Trabulsiella* ($r = 0.907$, $p<0.05$). 5-Hydroxytryptamine (5-HT) showed a strong positive correlation with *Dorea* ($r = 0.926$, $p<0.05$) and a strong negative correlation with Citrobacter ($r = -0.845$, $p<0.05$). Glutamate (Glu) was positively correlated with *Oscillospira* ($r = 0.811$, $p<0.05$), *unclassified_f_Rikenellaceae* ($r = 0.771$, $p<0.05$) and, to a lesser extent, *unclassified_f_Ruminococcaceae* ($r = 0.678$, $p<0.05$), while Citrobacter displayed a strong negative correlation ($r = -0.845$, $p<0.05$). Acetylcholine (ACh) was positively associated with *Ruminococcus* ($r = 0.811$, $p<0.05$) and *Adlercreutzia* ($r = 0.828$, $p<0.05$). Subsequent correlation of neurotransmitter levels with intestinal motility parameters (Fig. 5B) demonstrated that the intestinal propulsion rate (IPR) was inversely related to both 5-HT ($r = -0.829$, $p < 0.05$) and Glu ($r = -0.829$, $p < 0.05$). Conversely, the first-defecation latency (FSE) was positively correlated with 5-HT ($r = 0.771$, $p < 0.05$). Collectively, these findings suggest that the bacterial genera *Dorea*, *Citrobacter*, *Oscillospira*, *unclassified_f_Ruminococcaceae* and *unclassified_f_Rikenellaceae* may modulate intestinal motility and consequently contribute to constipation by altering the local concentrations of key neurotransmitters.

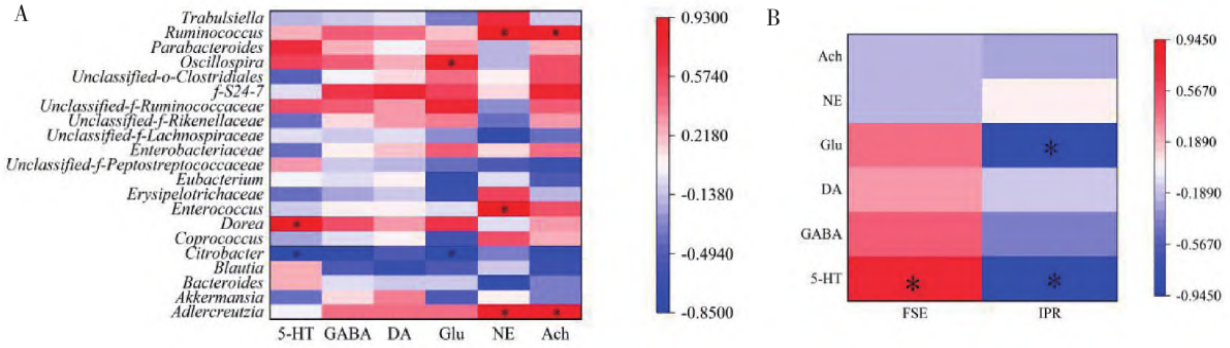

Fig. 5: Correlation analysis between constipation-related bacterial genera and intestinal neurotransmitter content (A), and correlation analysis between neurotransmitter content and intestinal motility indicators (B), *p < 0.05

Mulberry Modulates Constipation-Associated Gut Microbiota

High-throughput sequencing of the V3-V4 region of the bacterial 16S rRNA gene was performed on fecal samples from all experimental groups. After stringent quality filtering, a total of 1,237,622 high-quality reads were retained, corresponding to an average of 34,378 ± 9,394 reads per sample (range 23,112-56,721). The mean read length was 443 bp (range 423-486 bp). Clustering at a 97 % similarity threshold yielded 521 operational taxonomic units (OTUs). Comparative analysis revealed that the MD group exhibited a higher number of mapped reads and OTUs than the MB group, although the differences in overall OTU richness among all groups did not reach statistical significance ($p > 0.05$). These data indicate that mulberry fruit extract influences both the quantity and diversity of the intestinal microbiota in mice, suggesting a microbiota-mediated component to its laxative effect (see Table 4).

Table 4: Mapped Reads and OTU values of mouse gut microbiota

Group	Mapped Reads	OTU
MB	32297.8 ± 6314.79	220.8 ± 25.66
MD	28350.8 ± 2827.48	258.7 ± 36.10
XJL	38455.5 ± 4271.51	289.3 ± 35.11
XJH	40668.2 ± 20257.83	212.2 ± 22.10

Furthermore, to analyze the mouse feces sample and specifics in mulberry intervention group, see what Fig. 6 showed, the abundance in Dorea and unclassified f Ruminococcacea heavily varied. Meanwhile, *Citrobacter*, *Oscillospira* and *unclassified_f_Rikenellaceaes* stayed former. The result signified that mulberry might adjust the abundance of *Dorea* and *unclassified_f_Ruminococcacea*, having an effect on the intestinal neurotransmitter content, which would prevent the intestine from moving, moistening, and relieving constipation.

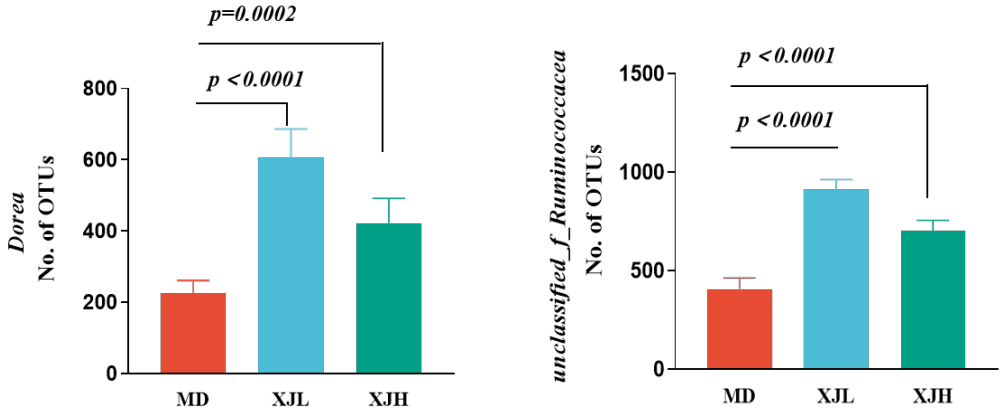

Fig. 6: Differential analysis of genus-level microbiota between the mulberry intervention group and the model group mice

Discussion

Compared with the MB control, the constipation model exhibited marked reductions in stool frequency, Fecal Water Content (FWC), and Intestinal Propulsion Rate (IPR). Compared with the model group, the two mulberry intervention groups (low dose XJL group and high dose XJH group) showed significant improvements in the above indicators. Its curative effect is beyond the traditional positive drug treatment, highlighting the excellent therapeutic potential of mulberry. Functional constipation is often accompanied by bowel motor dysfunction [8, 9]. This research data show that the fundamental pathological mechanism related to gut the specific biochemical changes of the nervous system. This difference reveals a more complex regulatory mechanism, and the negative correlation between 5-HT levels and the advancement rate (IPR) further confirms this view.

Gut-derived serotonin (5-HT) is recognized as a key neurotransmitter that promotes intestinal peristalsis. Our data show that 5-HT levels are negatively correlated with Intestinal Propulsion Rate (IPR), revealing a more complex regulatory mechanism. This difference may stem from the different activation patterns of receptor subtypes. Taking receptor types as an example: 5-HT4 receptors usually enhance peristalsis [10, 11]. Motility-promoting drugs target such receptors, including prucalopride [11] and mosapride [12] for the treatment of constipation. Excessive stimulation of 5-HT3 receptors may lead to temporary inhibition or spasm, which is common in specific pathological conditions [13, 14]. In addition, we found that the mechanism of restoring intestinal peristalsis by morberry extract was not only to enhance excitatory signals, but also to rebalance the entire intestinal neurotransmitter network. Specifically, decreased levels of inhibitory Glutamate (Glu), which may be achieved by impinging metabolic receptor activation, or preventing the desensitization of excitotoxic neurons; at the same time, the excitatory acetylcholine (ACh) level increases. These two effects work together to promote peristalsis, linking the observed biochemical changes to improved bowel motor function.

We observed dose-dependent differences in neurotransmitter profiles between the low- and high-dose groups. These differences may be affected by interactions with the gut microbiota, and the hypothesis needs to be further studied. Through 16S rRNA sequencing, we found 21 bacterial genera with differences between the experimental groups. We analyzed the correlation between these differential genera and intestinal neurotransmitter levels, and focused on ten genera with significant correlations. Among them, key taxa such as *Dorea*, *Citrobacter*, *Oscillospira*, *unclassified_f_Ruminococcaceae*, and *unclassified_f_Rikenellaceae* may affect neurotransmitter dynamics and intestinal peristalsis function at the same time. Dorea belongs to the *Lachnospiraceae* family, which is part of Firmicutes, which is related to the pro-inflammatory process and is associated with obesity, Irritable Bowel Syndrome (IBS) and colorectal cancer [12, 15, 16]. Its relative abundance increased after mulberry treatment. This indicates a modulatory role in the intestinal inflammatory milieu. *Unclassified f Ruminococcaceae* is often more abundant in constipated patients [13, 17]. It participates in Bile-Acid (BA) metabolism via the enterohepatic circulation. BAs can activate certain receptors. These include the Farnesoid X Receptor (FXR) and various G-Protein-Coupled Receptors (GPCRs) [8, 14, 18]. This process can activate the peristaltic reflex and impact systemic metabolism. These findings support the dual mechanism for mulberry’ s pro-kinetic effects. One is direct modulation of

enteric neurotransmitters. The other is indirect regulation by reshaping specific microbial communities. These communities in turn affect neurotransmitter synthesis and signaling.

Our research has revealed a significant correlation between the changes in specific microbial communities and the restoration of neurotransmitter balance. However, we realize that this correlation itself does not prove a causal relationship. Future research will be through the tube feeding specific strains or targeted microorganisms interventions such as broad-spectrum antibiotics to fill this gap, these methods will help to clarify the exact mechanism of the interaction.

Gut microbiota overall index of steady state is to assess the intestinal health. The relative abundance ratio of *Bacteroidetes* to *Firmicutes* (B/F ratio) is a marker to assess gut microbiota homeostasis [19]. The B/F ratio of the moderate constipation model group increased to 350.83% of the normal control group, indicating that the model animals had obvious intestinal flora imbalance. The intervention with mulberry significantly reversed this abnormal trend, restoring the B/F ratio to the normal physiological range, achieving the overall reconstruction of the intestinal ecosystem. Further cross-sectional analysis showed that after mulberry intervention, the abundance of intestinal Trabulsiella increased by 6 times in the low-dose group, 7 times in the high-dose group, and only 3 times in the positive control group. This significant difference clearly verified the core value of Astragalus as a specific biomarker for mulberry regulation of intestinal function, and reflected that mulberry had a broad spectrum repair effect compared with the positive control group, and the treatment specificity was more accurate. This study empirically confirmed that mulberries can promote intestinal peristalsis and restore intestinal function.

In this study, association analysis was mainly based on 16S rRNA.To establish causality, functional validation of the identified taxa is required (e.g. by quantitative real-time PCR, metagenomics, or targeted metabolomics).In addition, the dose dependent change of neurotransmitters reaction is worth further exploration, to clarify the potential microbial - host signal path.In this study, Zengye Decoction was selected as the traditional Chinese medicine control group, ensuring the clinical relevance of the research.We recognize that there are limitations in using only TCM criteria, which may limit the universality of research conclusions to biomedical researchers.In order to overcome this defect, future research should introduce Linaclotide and other pharmacological standards to construct a double control system. This system can provide a more comprehensive reference benchmark. The results reveal the effectiveness of the source of mulberry plant chemicals, future research should further to explore the influence of these compounds of bile acid pool, and focus on FXR/GPCR activation mechanism.This will more directly connect microbial metabolism with host motor function, filling the cognitive gap between traditional efficacy and modern biomedical interpretation.

Conclusion

We established a mouse model of functional constipation to detect changes in intestinal motility, intestinal flora and neurotransmitter content. Mulberries supplements by regulating intestinal neurotransmitter release, significantly lower levels of Glutamate (Glu) and serotonin signaling, which both can promote smooth muscle relaxation and enhance peristalsis function. Mulberry reshapes intestinal microbial community, significantly increases the abundance of *Dorea* and *Trabulsiella*, and affects bile acid metabolism through enterohepatic circulation. Bile acids further stimulate the gut peristaltic reflex by activating FXR receptors and GPCR receptors. These mechanisms together form the basis of the anti-constipation effect of mulberries and provide a mechanism framework for the development of mulber-based therapeutic strategies. Subsequent studies should focus on validating these microbial-neurochemical interactions and translating the findings to clinical applications.

Future work should focus on validating these microbial-neurochemical interactions and translating the findings into clinical applications.

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Author's Contributions

All the authors have made significant contributions to this work and have agreed to submit this paper.

Huizhen Zhao and Yuanyuan Zhang: Were responsible for most of the experimental work.

Shijing Xie and Yan Jiao: Were responsible for the conception and design of the experiments.

Yuwen Li: Supervised the work and revised the final version of the manuscript.

All authors have read and approved the final version of the manuscript.

Ethics

All experimental procedures were approved by the Ethics Committee of Cangzhou Medical College (Approval No. 2023-24) and complied with the national Guidelines for the Care and Use of Laboratory Animals. The animal production license number is SCXK (Liao) 2020-000.

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