Original Article

Limonitum Ameliorates Castor Oil-Induced Diarrhoea in Mice by Modulating Gut Microbiota

(traditional Chinese medicine / mineral Chinese medicine / mineral drug / mineral medicine / diarrhoea / Limonitum / gut microbiota / short-chain fatty acids)

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Abstract. Diarrhoea is a common clinical condition; its pathogenesis is strongly associated with gut microbiota dysbiosis. Limonitum is a well-known traditional Chinese medicine that exerts appreciable benefits regarding the amelioration of diarrhoea. However, the mechanism through which Limonitum ameliorates diarrhoea remains unclear. Here, the efficacy and underlying mechanism of Limonitum decoction (LD) regarding diarrhoea were explored from the aspect of gut microbiota. Castor oil (CO) was used to induce diarrhoea in mice, which were then used to evaluate the effects of LD regarding the timing of the first defecation, diarrhoea stool rate, degree of diarrhoea, diarrhoea score, intestinal propulsive rate, and weight of intestinal contents. The concentrations of short-chain fatty acids (SCFAs), including acetic, propionic, isobutyric, butyric and valeric acids, were analysed by gas chromatography-mass spectrometry (GC-MS). The 16S rRNA high-throughput sequencing technology was applied to evaluate changes in the gut microbiota under exposure to LD. LD was found to effectively ameliorate the symptoms of diarrhoea, and the diversity and relative abundance of gut microbiota were restored to normal levels following LD treatment. Additionally, LD significantly restored the observed reductions in SCFAs. These results provide strong evidence that LD can sufficiently ameliorate diarrhoea in mice by regulating their gut microbiota. The findings presented here highlight that Limonitum may constitute a prospective remedy for diarrhoea.

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Abbreviations: CO – castor oil, GC-MS – gas chromatography-mass spectrometry, HCA – hierarchical clustering analysis, LD – Limonitum decoction, LH – loperamide hydrochloride, NMDS – non-metric multidimensional scaling analysis, OUT – operational taxonomic units, PCA – principal component analysis, PCR – polymerase chain reaction, SCFAs – short-chain fatty acids, TCM(s) – traditional Chinese medicine(s), UV-vis – ultraviolet-visible.

Introduction

Diarrhoea is a high-incidence, multi-factor and multi-pathogen intestinal disease. As a common clinical affliction, diarrhoea is not only an independent disease, but also a common symptom in the pathological processes of many other diseases (Huang et al., 2021). The pathological basis of diarrhoea comprises intestinal oedema, mucosal inflammation, hyperactivity regarding intestinal secretions and motor function, and loose and frequent stools, which can occur under the stimulation of either infectious or non-infectious factors (Aziz et al.,

2020). Unfortunately, improper handling of diarrhoea can easily lead to dehydration and severe sequelae. Long-term studies have shown that gut microbiota play an important role in many processes, such as regulating their host's immune and endocrine functions, and transmitting neural signals (Ling et al., 2014). Moreover, they are closely related to problems associated with intestinal diseases (Ling et al., 2014). Thus, gut microbiota may constitute a potential therapeutic target for diarrhoea.

Traditional Chinese medicines (TCMs) have made great contributions to the treatment of many diseases in China for centuries, including diarrhoea. For instance, TCMs such as *Cortex Phellodendri* (Xu et al., 2020), *Eriosema chinense* Vogel (Prasad et al., 2017) and *Atractylodes lancea* (Thunb.) DC. (Xie et al., 2021) have been shown to act through multiple pathways and against multiple targets to relieve diarrhoea. Limonitum, which is a frequently used TCM (named Yuyuliang in Chinese), is a hydroxide mineral (limonite) that is officially listed in the Chinese Pharmacopoeia. Notably, it was identified in Shen Nong's herbal classic (200–300 AD, Han Dynasty) as having an impressive ability to stop diarrhoea when combined with astringents; astringents constitute an important base for Limonitum-containing TCMs that have historically been used to treat diarrhoea (Liu et al., 2015).

Castor oil (CO) can promote the release of various mediators, including histamine and tachykinins, by binding to receptors on intestinal smooth muscle cells. This ultimately stimulates intestinal motility and elimination of electrolytes from the intestine (Jahan et al., 2022). In addition, CO can produce ricinoleic acid, which has a definite laxative effect due to the action of small intestinal lipase (Adela et al., 2022). CO has therefore been commonly used to induce acute diarrhoea in experimental models. Our previous research has demonstrated that Limonitum can protect against CO-induced diarrhoea in mice through analysis of the diarrhoea score, degree of diarrhoea and intestinal motility (Ma et al., 2019). Accordingly, Limonitum can play a significant role in treating diarrhoea. However, the mechanism by which Limonitum acts against diarrhoea remains unclear.

In this paper, the efficacy of LD was evaluated using a murine model of CO-induced diarrhoea. For the first time, the potential mechanism of Limonitum was investigated from the aspect of the gut microbiota, based on the 16S rRNA high-throughput sequencing technology. The findings of this study can provide further evidence for the prevention and treatment of diarrhoea with Limonitum.

Material and Methods

Materials and reagents

Limonitum (batch No. 20160510) was provided by Yinquan Medicinal Materials Station (Henan, China) and authenticated by Professor Dekang Wu of Nanjing University of Chinese Medicine. A voucher specimen was deposited in the Teaching and Research Section of Authentication of Chinese Materia Medica, School of Pharmacy, Nanjing University of Chinese Medicine, China. CO was purchased from Lingfeng Chemical Reagent Co., Ltd. (Shanghai, China). Loperamide hydrochloride (LH) was purchased from Xi'an Janssen Pharmaceutical Co., Ltd. (Shanxi, China). Charcoal powder was purchased from Zhuxi Activated Carbon Co., Ltd. (Jiangsu, China). Acetic acid, propionic acid, isobutyric acid, butyric acid and valeric acid (purity ≥ 99 %) were obtained from Aladdin Biochemical Technology Co., Ltd. (Shanghai, China).

Preparation of Limonitum decoction

LD was prepared in accordance with a previously published method (Ma et al., 2019). Briefly, Limonitum fragments (250 g) were boiled at 100 °C in 10 times the volume of distilled water (2500 ml) for 1 hour. The filter residue was then boiled with 8 times the volume of distilled water (2000 ml) for 1 hour after filtration. Then, the LD was combined in a container, concentrated under reduced pressure, and precisely adjusted to a concentration of 0.125 g/ml for subsequent experiments.

Animals

Male six-week-old ICR mice weighing 20 ± 2 g were obtained from Qinglong Mountain Animal Breeding Farm, Jiangning District, Nanjing (Jiangsu, China). The above animals were given free access to a basal diet and kept in a chamber at 23 ± 2 °C, under 55 ± 5 % humidity and a 12-h light/dark cycle. All experimental procedures were approved by and conducted in accordance with the Animal Ethics Committee of Nanjing University of Chinese Medicine (Permission No. 201808A005).

Experimental design

All mice were allowed to adapt for 7 days before the experiment. Subsequently, the mice were randomly divided into the following five groups (10 mice per group): normal, CO, LH (8 mg/kg) and LD (1 and 2.5 g/kg, respectively). Mice in the normal group were consistently given sterile water, while mice in the other groups were administered with 20 ml/kg CO for 6 days to induce diarrhoea. Then, mice in the LH group were given LH, a clinical drug for diarrhoea, by oral gavage once per day, for an additional 14 days. Similarly, mice in the LD group were given a corresponding dose of LD by oral gavage once per day for an additional 14 days.

Assessment of diarrhoea

After the last gavage, mice were placed in observation cages, and the filter paper was changed every hour (for 8 hours) to observe their diarrhoea. The diarrhoea of experimental mice was evaluated in terms of the first defecation time, diarrhoea stool rate, degree of diarrhoea and diarrhoea score, as previously described (Liu et al., 2021). In brief, the diarrhoea stool rate was calculated as follows: diarrhoea stool rate = number of loose stools per mouse / number of defecations per mouse

(stains on the filter paper were considered as loose stool, while each particle or pile of faeces on the filter paper was regarded as a defecation). The degree of diarrhoea was calculated as follows: degree of diarrhoea = sum of loose stools' grades per mouse / number of loose stools per mouse. The loose stools were divided into four grades according to the size of the stain on the filter paper: Grade I (< 1 cm), Grade II (1–1.9 cm), Grade III (2–3 cm) and Grade IV (> 3 cm). The diarrhoea score was calculated as follows: diarrhoea score = diarrhoea stool rate × degree of diarrhoea.

At the end of the treatment, 0.5 % charcoal powder was given by gavage after fasting for 24 hours. Then, the mice were euthanized by cervical dislocation under anaesthesia with 0.3 % sodium pentobarbital, after 30 minutes. Subsequently, the abdomen of each mouse was cut open, and the intestine was removed and placed in a clean tray, where it was laid flat on a meter ruler. The distance from the pylorus to the end of the charcoal powder was taken as the advancing length, and the distance from the pylorus to the ileocecal valve was taken as the full length. The intestinal propulsive rate was calculated as follows: intestinal propulsive rate = advancing length / full length. The total weight of the intestine was determined. Furthermore, the contents of the intestine were thoroughly extruded and weighed to obtain the net weight. In detail, the weight of the intestinal contents was calculated as follows: weight of intestinal contents = total weight – net weight.

Determination of short-chain fatty acids (SCFAs)

At the end of the treatment, mice were euthanized in the same way as described above. Mouse faeces in the colon were collected in plastic tubes, placed in liquid nitrogen for snap-freezing, and then stored at –80 °C for further analysis. The concentrations of SCFAs in mouse faeces (including acetic, propionic, isobutyric, butyric and valeric acids) were determined using previously described expression profiles, with slight modifications (Jiang et al., 2020). Analysis was carried out in an Agilent 7000C gas chromatography-mass spectrometer (GC-MS; Agilent, CA) with a HP-5MS capillary column (30 m × 0.25 mm, 0.25 μm).

16S rRNA sequencing

Microbial DNA was extracted from mouse faeces using an E.Z.N.A.® soil DNA Kit (Omega Bio-Tek, Norcross, GA) following the manufacturer's protocols. A NanoDrop 2000 ultraviolet-visible (UV-vis) spectrophotometer (Thermo Fisher Scientific, Waltham, MA) was used to determine the concentration and purification of the DNA. Primers containing 338F (5'-ACTCCTACGGGAGGCAGCAG-3') and 806R (5'-GGACTACHVGGGTWTCTAAT-3'), determined with a GeneAmp 9700 polymerase chain reaction (PCR) system (Applied Biosystems, Waltham, MA), were used to amplify the V3-V4 regions of the bacterial 16S rDNA genes. The PCR reactions were performed as previously described (Cui et al., 2021). An Illumina MiSeq platform (Illumina, San Diego, CA) was used to pool and sequence the purified amplicons following the standard protocols (Majorbio Bio-Pharm Technology, Shanghai, China). The sequencing data were processed according to previous reports (Cui et al., 2021).

Statistical analysis

Statistical analysis and results visualization were performed using GraphPad Prism 8 software (GraphPad Software Inc., San Diego, CA). Results are presented as means ± standard deviation (SD), $N \geq 6$. To determine statistically significant differences, the Student's *t*-test was applied for statistical analysis between pairs of groups, while one-way analysis of variance (ANOVA) was used for statistical analysis among multiple groups. Assessment of statistical correlation was carried out using Pearson's correlation analysis. $P < 0.01$ and $P < 0.05$ were regarded to represent statistically significant differences.

Results

LD alleviates CO-induced diarrhoea

As demonstrated in Fig. 1A–D, compared with the normal group, CO remarkably shortened the first defecation time while increasing the diarrhoea stool rate, degree of diarrhoea and diarrhoea score. Treatment with LD alleviated these symptoms in a dose-dependent manner. Diarrhoea is often accompanied by disturbances in intestinal motility, as evaluated by the intestinal propulsive rate and weight of intestinal contents (Ma et al., 2019). As illustrated in Fig. 1E, the intestinal propulsive rate increased significantly following the administration of CO, which was relieved after the treatment with LD. In addition, the weight of intestinal contents was decreased by CO, but was observably alleviated by the LD treatment (Fig. 1F). Noticeably, high dosages of LD presented comparable therapeutic effects to those of LH. Collectively, these results indicate that LD effectively ameliorates CO-induced diarrhoea.

LD improves production of SCFAs in mice with CO-induced diarrhoea

A growing body of evidence suggests that SCFAs are the main metabolites of gut microbiota and that they participate in the maintenance of intestinal function (Cui et al., 2021). A proven GC-MS method was used to determine the contents of SCFAs (Fig. 2A–E). In comparison to the normal group, the acetic, propionic, isobutyric, butyric and valeric acid contents all decreased obviously after CO induction, while LD observably redressed this reduction (Fig. 2H–J).

LD restores the general composition of gut microbiota in mice with CO-induced diarrhoea

Alterations in the gut microbiota represent a vital driver for the progression of several intestinal diseases

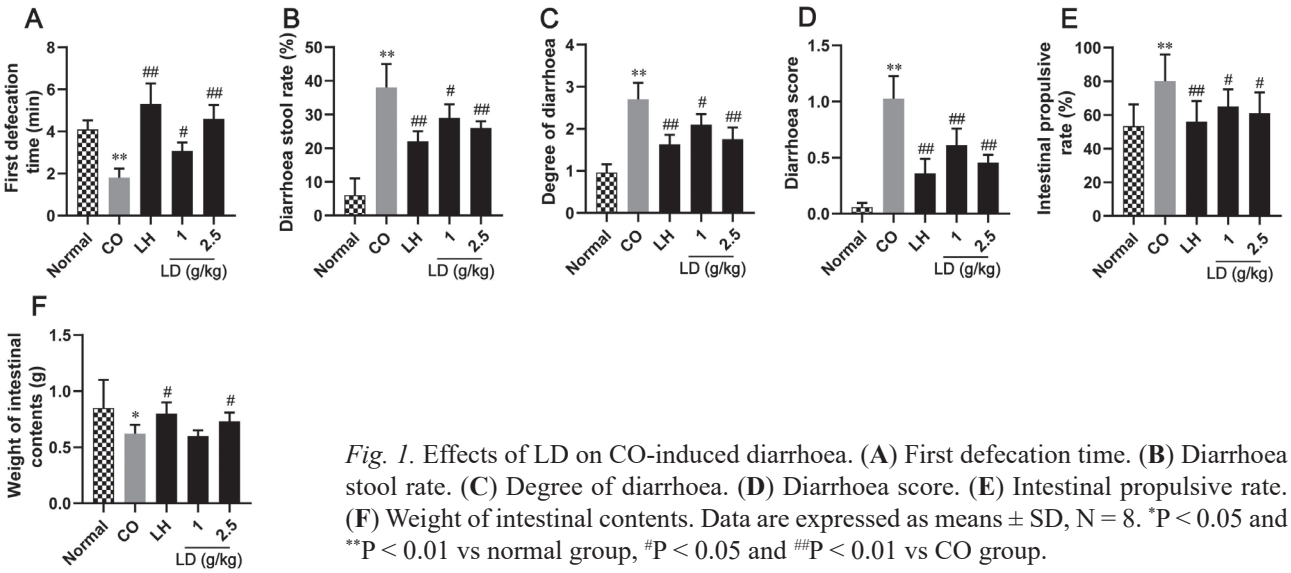

Fig. 1. Effects of LD on CO-induced diarrhoea. (**A**) First defecation time. (**B**) Diarrhoea stool rate. (**C**) Degree of diarrhoea. (**D**) Diarrhoea score. (**E**) Intestinal propulsive rate. (**F**) Weight of intestinal contents. Data are expressed as means ± SD, N = 8. $^{*}P < 0.05$ and $^{**}P < 0.01$ vs normal group, $^{\#}P < 0.05$ and $^{\#\#}P < 0.01$ vs CO group.

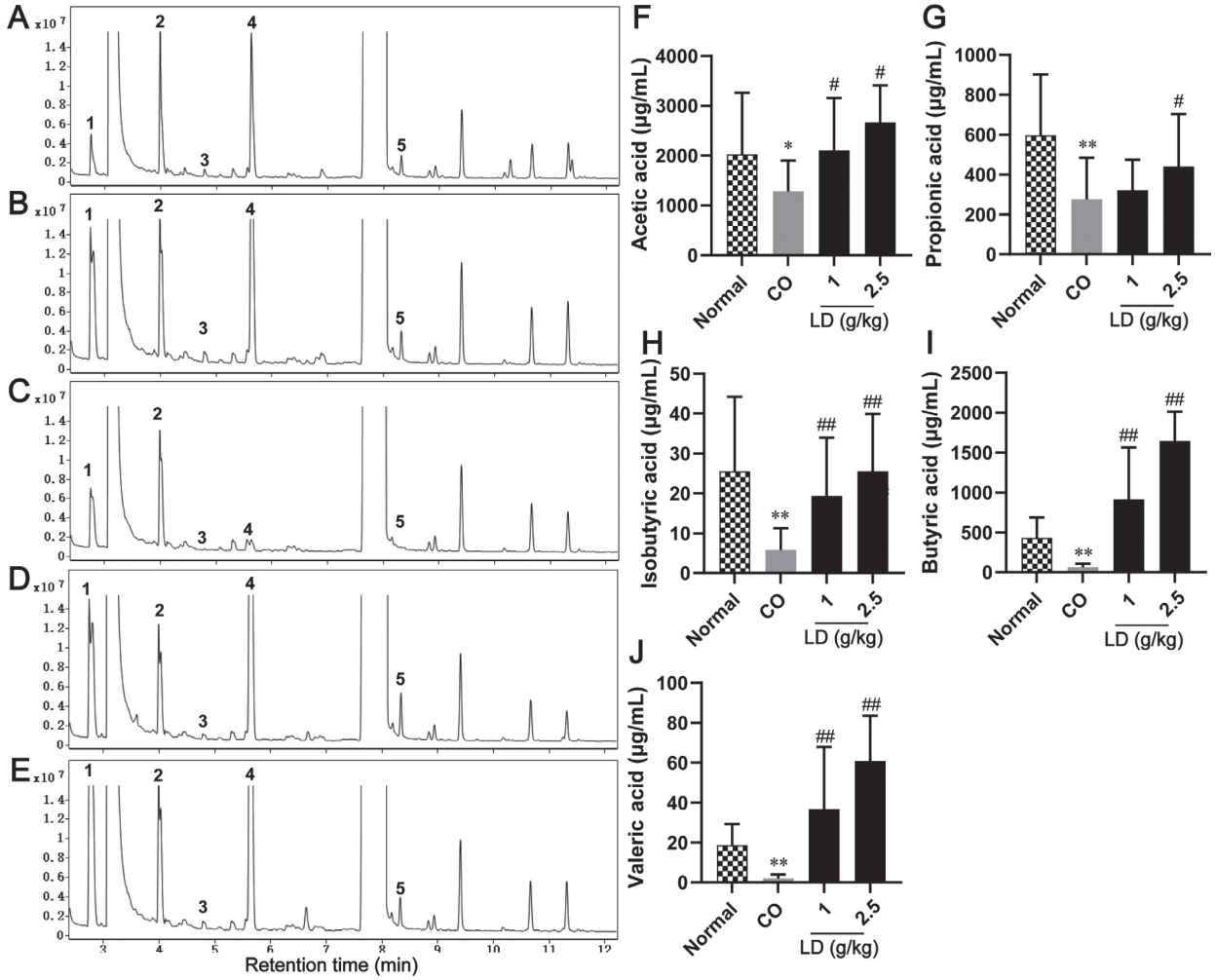

Fig. 2. Effects of LD on the production of SCFAs in mice with CO-induced diarrhoea. GC-MS chromatography profiles of standard mixture solutions (**A**) and different treatments: normal group (**B**), CO group (**C**), LD (1 g/kg) group (**D**), LD (2.5 g/kg) group (**E**). Peak identification: (1) acetic, (2) propionic, (3) isobutyric, (4) butyric and (5) valeric acids. Contents of (**F**) acetic, (**G**) propionic, (**H**) isobutyric, (**I**) butyric and (**J**) valeric acids in mouse faeces. Data are expressed as means ± SD, N = 8. $^{*}P < 0.05$ and $^{**}P < 0.01$ vs normal group, $^{\#}P < 0.05$ and $^{\#\#}P < 0.01$ vs CO group.

(Shen et al., 2018). Here, the plateaued Smith-Wilson rarefaction curves on the operational taxonomic units (OTU) level showed that all bacterial species in the analysed samples were covered by the depth of sequencing (Fig. 3A). Alpha diversity is an ecological indicator that measures how many taxa exist in each sample and whether said taxa are evenly distributed (Cui et al., 2021). Bacterial richness and diversity were estimated by the Chao and Shannon indices, respectively. After CO treatment, the alpha diversity (including the Chao and Shannon indices) was distinctly disturbed (Fig. 3B and C). The LD groups showed corrected alpha diversity, however.

Beta diversity refers to comparing the composition of microbial communities and assessing the differences among them (Cui et al., 2021). Hierarchical clustering analysis (HCA), principal component analysis (PCA) and non-metric multidimensional scaling analysis (NMDS) have all been used to describe beta diversity, so they were applied in the current study. These analytical methods enable us to visualize the similarities between individual samples in a highly intuitive way. For example, if several samples are clustered together, it indicates that the similarity between these samples is extremely close; on the contrary, if several samples are dispersed from each other, it indicates that the similarity between these samples is relatively low (Yan et al., 2017). As shown in Fig. 3D, HCA divided the CO groups from the other three groups into two clearly delineated categories, a and b. Moreover, PCA and NMDS revealed that the gut microbiota were markedly altered in that the model had a distinct cluster significantly far away from that of the other three groups (Fig. 3E and F). These results indicate that the gut microbiota of CO groups were significantly different from those of the normal group. Additionally, LD treatment significantly reduced this difference, making LD-treated groups similar to the normal group in terms of the gut microbiota. In summary,

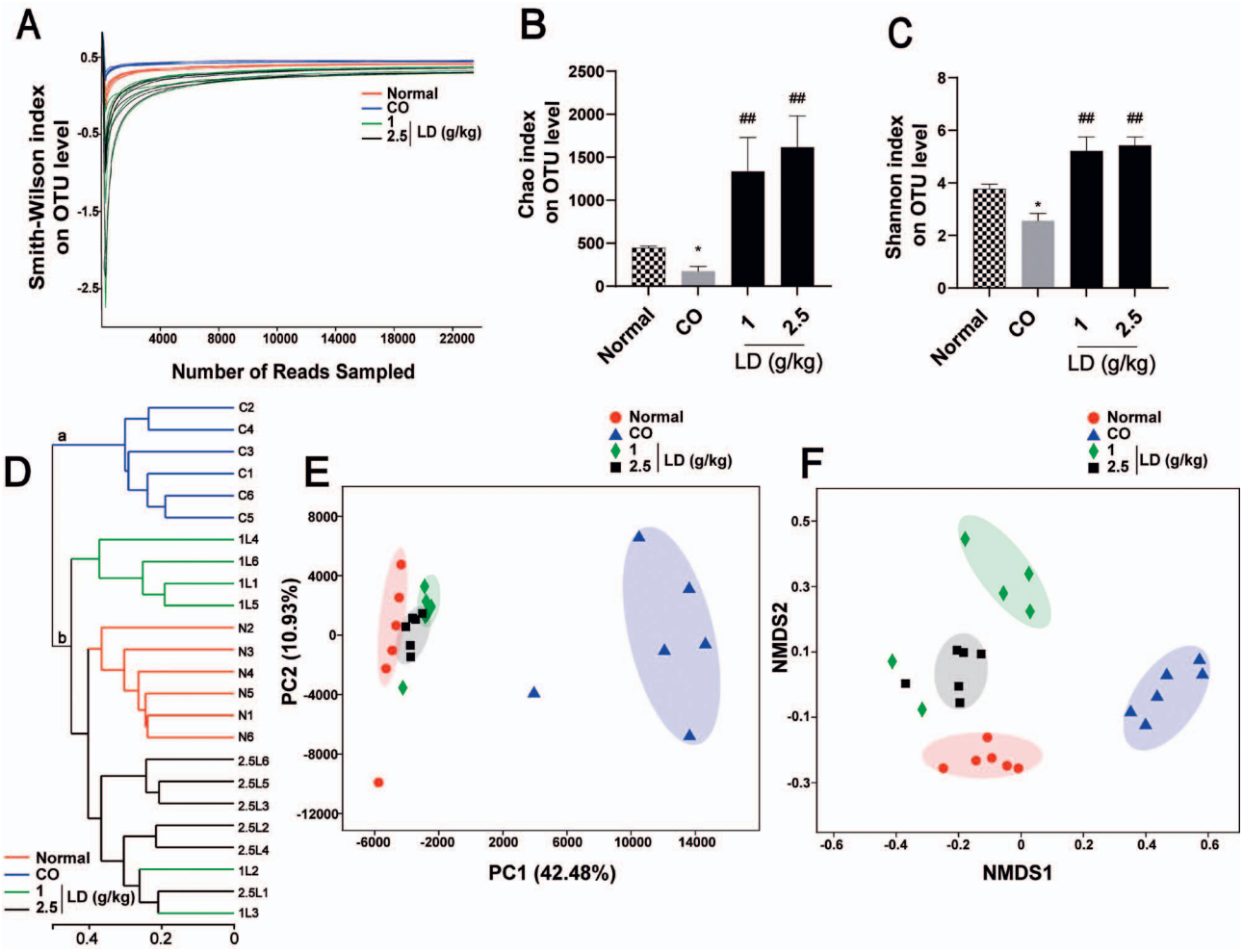

Fig. 3. Effects of LD on the general composition of gut microbiota in mice with CO-induced diarrhoea. (**A**) Smith Wilson rarefaction curves on the OTU level of gut microbiota in mice with diarrhoea. Alpha diversity of gut microbiota in mice with diarrhoea is shown via (**B**) Chao and (**C**) Shannon indices on the OTU level. Beta diversity of gut microbiota in mice with diarrhoea is shown via (**D**) HCA, (**E**) PCA and (**F**) NMDS. Data are expressed as means ± SD, N = 6. $^{*}P < 0.05$ vs normal group, $^{\#\#}P < 0.01$ vs CO group.

these results indicate that the therapeutic effect of LD on CO-induced diarrhoea may be closely related to remodelling of the gut microbiota.

LD regulates gut microbiota abundance in mice with CO-induced diarrhoea

Remarkable shifts in the gut microbial composition in response to both diarrhoea and LD treatment were revealed at the level of phylum (Fig. 4A–E) and genus (Fig. 4F–O).

There was no significant difference in the relative abundance of phyla Bacteroidetes, Actinobacteria, Acidobacteria and Chloroflexi between CO and LD groups. Analysis at the phylum level revealed that the gut microbiota of the CO group were characterized by a significant decrease in the abundances of Firmicutes and Deferribacteres ($P < 0.01$) and an increase in the abundances of Proteobacteria and Verrucomicrobia ($P < 0.01$); the abundances of these phyla were restored towards their normal status in the LD treatment groups (Fig. 4A–E).

Further analysis at the genus level revealed that CO markedly decreased the abundances of the *norank_f_Bacteroidales_S24-7_group*, *Lactobacillus*, *Lachnospiraceae_NK4A136_group*, and *norank_f_Lachnospiraceae* ($P < 0.01$) and increased the abundances of *Bacteroides*, *Klebsiella*, *Lachnoclostridium*, *Akkermansia* and *Parabacteroides* ($P < 0.01$), which confirmed that LD reshaped the abundances of these genera (Fig. 4F–O). Notably, the high dosage of LD (2.5 g/kg) specifically enriched the genera *norank_f_Bacteroidales_S24-7_group*, *Lactobacillus*, *Lachnospiraceae_NK4A136_group*, and *norank_f_Lachnospiraceae* ($P < 0.01$). Overall, the above results suggest that LD modulates the abundances of gut microbiota in mice with CO-induced diarrhoea to maintain intestinal homeostasis.

As shown in Fig. 5A and B, Pearson's correlation analysis at the phylum and genus levels showed that Firmicutes, *Lachnospiraceae_NK4A136_group* and *norank_f_Lachnospiraceae* were negatively associated with the intestinal propulsive rate, while Proteobacteria, *Klebsiella* and *Parabacteroides* were positively associated with the intestinal propulsive rate. *Lactobacillus* had a positive correlation with the weight of intestinal contents as well as a negative correlation with the degree of diarrhoea and diarrhoea score. *Bacteroides* showed a significant negative correlation with the first defecation time. In addition, Proteobacteria exhibited a significant positive relation with the degree of diarrhoea, diarrhoea stool rate and diarrhoea score, whereas the *norank_f_Bacteroidales_S24-7_group* was negatively correlated with these diarrhoea symptoms.

Discussion

Diarrhoea has been identified as the leading cause of malnutrition and death in children under five years of age, and more than half a million children die of diarrhoea-related causes worldwide every year (Huang et al., 2021). Antibiotics are commonly used to treat diarrhoea, but they have demonstrated limited efficacy and frequent side effects in clinical treatments (Ma et al., 2019). Accordingly, efficient alternative drugs for the prevention or treatment of diarrhoea are urgently required. Here, LD significantly improved the symptoms of diarrhoea, indicating that it may constitute a promising agent for the treatment of diarrhoea.

Dysbiosis of intestinal microecology, which is caused by the disturbance of water and electrolyte metabolism in the intestinal tract, represents one of the main causes of diarrhoea (Sun et al., 2021). The gut microbiota and their metabolites form the core part of intestinal microecology. Here, the oral administration of LD was found to reverse CO-induced gut microbiota dysbiosis, as evidenced by an increase in the microbial diversity and the recovery of the microbial community composition to normal levels. Variations were also observed in the relative abundances of certain bacteria in LD-treated mice, as evidenced by higher levels of Firmicutes, Deferribacteres, *norank_f_Bacteroidales_S24-7_group*, *Lactobacillus*, *Lachnospiraceae_NK4A136_group* and *norank_f_Lachnospiraceae*, and lower levels of Proteobacteria, Verrucomicrobia, *Bacteroides*, *Klebsiella*, *Lachnoclostridium*, *Akkermansia* and *Parabacteroides* (compared with the CO-treated mice). It is worth noting that these bacteria are intimately associated with the pathogenesis of diarrhoea. Earlier studies have shown that Firmicutes are usually highly represented in the gut microbiota of healthy individuals and are seen to decrease with the disease progression. Conversely, a significant increase in the abundance of Proteobacteria may lead to chronic abdominal pain/diarrhoea and a range of gastrointestinal inflammatory conditions (Bi et al., 2017; Kang et al., 2019; Yang et al., 2022). Moreover, Proteobacteria include a variety of pathogenic bacteria, and the massive proliferation of these bacteria has been identified as a microbial marker for the progression of diarrhoea (Shin et al., 2015). The *norank_f_Bacteroidales_S24-7_group* belongs to *Bacteroidales*, which play an important role in breaking down complex molecules in the intestine and help the body's immune system fight potentially harmful pathogens (Wexler, 2007). *Lactobacillus* is a type of beneficial bacteria that exert intestinal digestion and correct imbalances in the gut microbiota. Notably, *Lactobacillus* has the ability to alleviate the severity of diarrhoea (Fan et al., 2020). The *Lachnospiraceae_NK4A136_group* and *norank_f_Lachnospiraceae* can both be filed under *Lachnospiraceae*, which may be a potentially beneficial bacterium involved in the metabolism of many carbohydrates (Altomare et al., 2021). *Bacteroides* are crucial pathogenic bacteria associated with diarrhoea, and their abundance is positively correlated with the degree of this disorder (Villa et al., 2018). *Klebsiella*, meanwhile, is an opportunistic pathogen that causes acute and chronic diarrhoea after infection (Liu et al., 2022). The literature on the association between Deferribacteres, Verrucomicrobia, *Lachnoclostridium*,

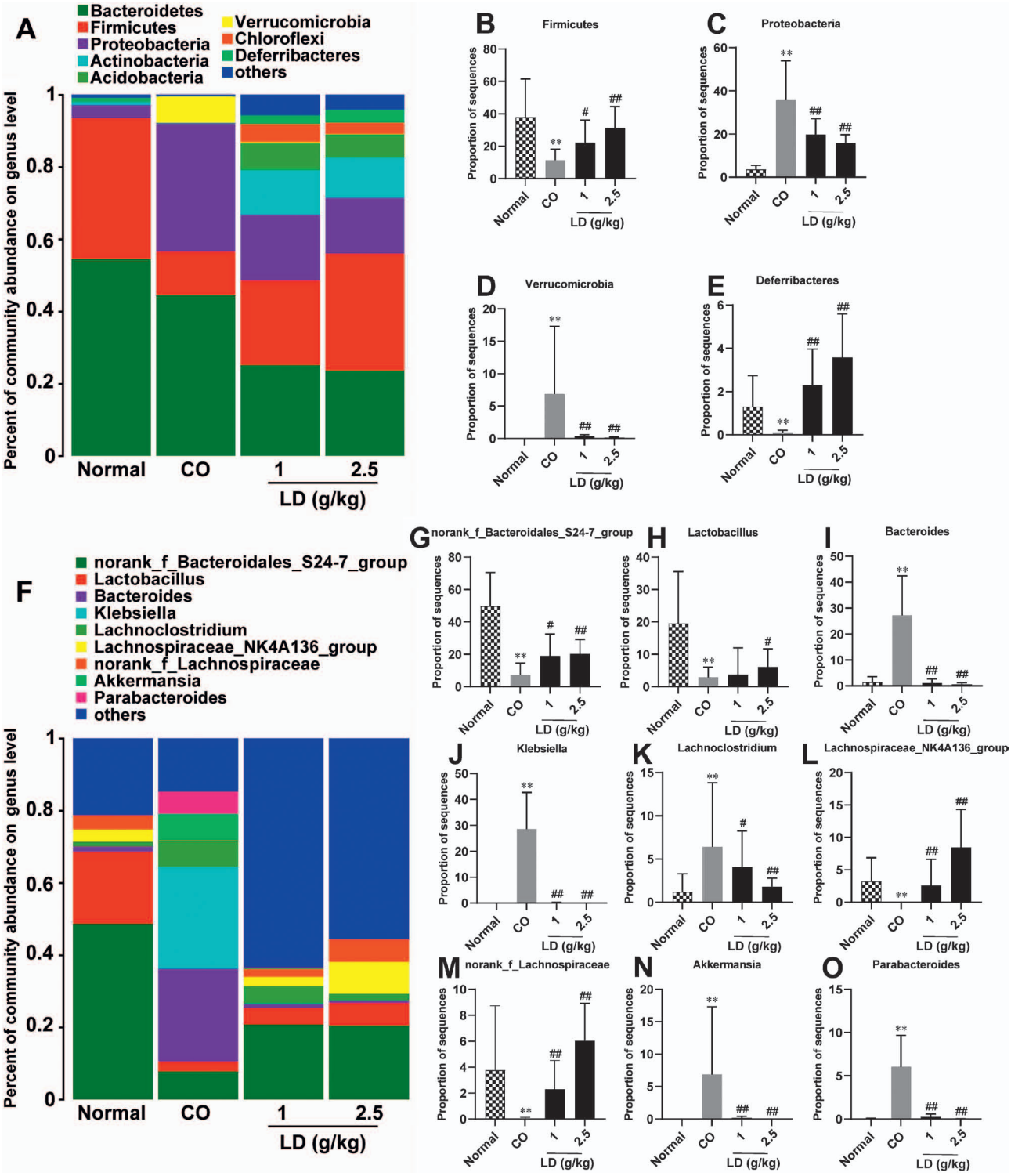

Fig. 4. Effects of LD on the abundance of gut microbiota in mice with CO-induced diarrhoea. (**A**) Relative abundances of species at the phylum level, including (**B**) Firmicutes, (**C**) Proteobacteria, (**D**) Verrucomicrobia and (**E**) Deferribacteres. (**F**) Relative abundances of species at the genus level, including (**G**) *norank_f_Bacteroidales_S24-7_group*, (**H**) *Lactobacillus*, (**I**) *Bacteroides*, (**J**) *Klebsiella*, (**K**) *Lachnoclostridium*, (**L**) *Lachnospiraceae_NK4A136_group*, (**M**) *norank_f_Lachnospiraceae*, (**N**) *Akkermansia* and (**O**) *Parabacteroides*. Data are expressed as means ± SD, N = 6. $^{**}P < 0.01$ vs normal group, $^{\#}P < 0.05$ and $^{\#\#}P < 0.01$ vs CO group.

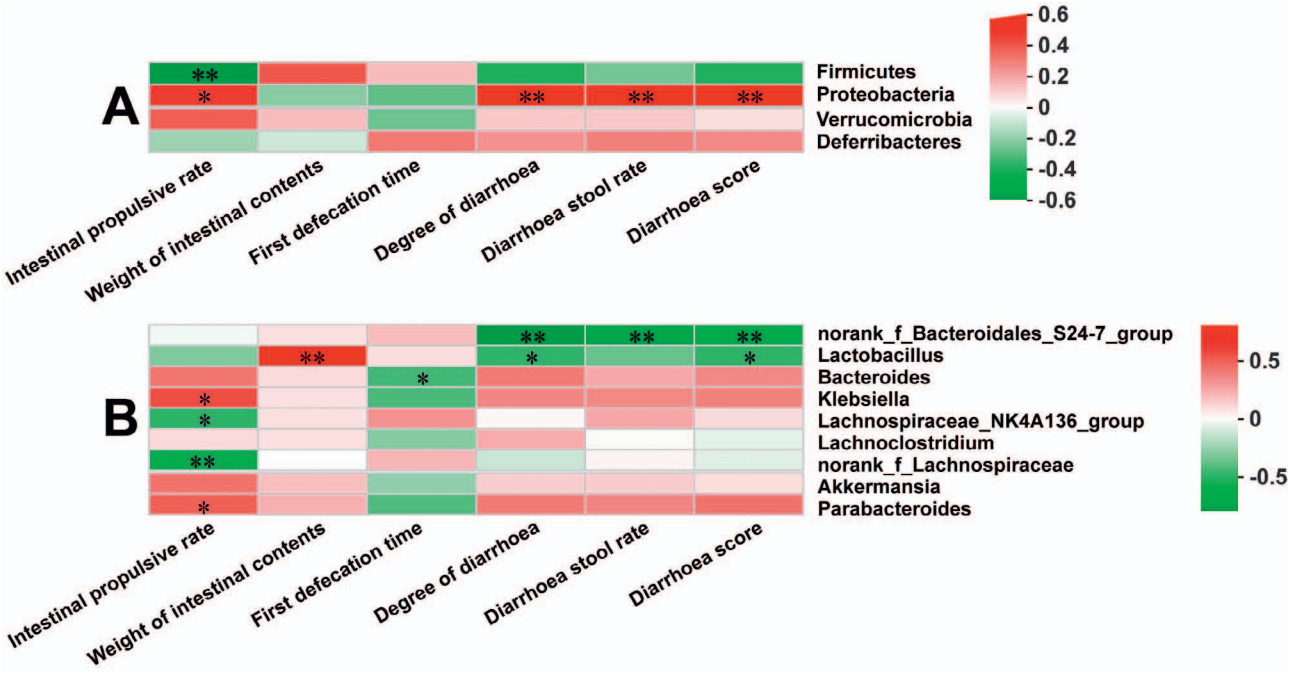

Fig. 5. Pearson's correlation analysis between diarrhoea symptoms and species. (**A**) Phylum. (**B**) Genus. $^{*}P < 0.05$ and $^{**}P < 0.01$.

Akkermansia, and *Parabacteroides* and diarrhoea is scarce, which remains to be further studied. SCFAs, which are the main metabolites of the gut microbiota, energize colonic epithelial cells; they are thought to be useful in the experimental and clinical treatment of intestinal diseases (Cui et al., 2021). Here, we found that LD significantly restored the observed reductions in acetic, propionic, isobutyric, butyric and valeric acids in mice with diarrhoea. Overall, these results suggest that LD can alleviate CO-induced dysbiosis in the gut microbiota.

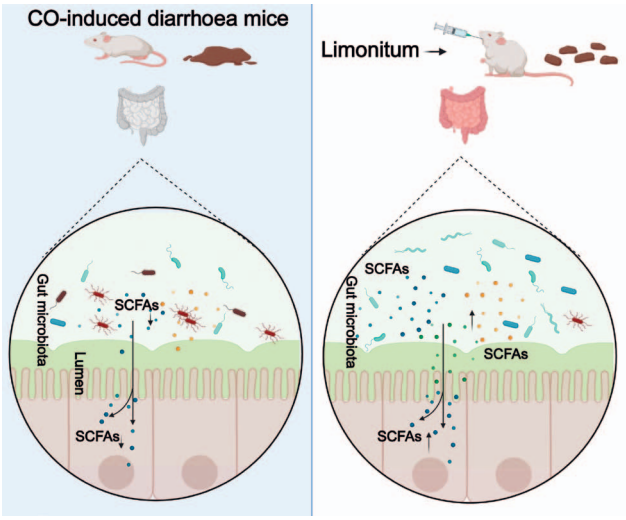

Fig. 6. Schematic diagram of Limonitum-regulated gut microbiota in mice with CO-induced diarrhoea.

In conclusion, the oral administration of LD could exert considerable benefits to CO-induced diarrhoea in a dose-dependent manner, and the therapeutic effects that the high dosage of LD (2.5 g/kg) presented were similar to those of LH. These effects may relate to the restoration of gut microbiota (Fig. 6). Our findings strongly indicate that Limonitum represents a promising agent for the treatment of diarrhoea.

Acknowledgements

Y. L. Ma, B. F. Yan contributed equally to this work.

Disclosure of conflict of interest

The authors declare no conflict of interest.

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