**Dendrobium officinale** leaf polysaccharides regulation of immune response and gut microbiota composition in cyclophosphamide-treated mice

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**ABSTRACT**

In this study, the polysaccharides extracted from *Dendrobium officinale* leaf (DOLP) was used in immune deficiency mice to evaluate the bioactivity. Thymus and spleen indices were calculated while the alleviation of the colon and liver histopathological progression was evaluated by H&E staining. The data indicated that DOLP improved immunity status by restoring the gut barrier and atrophy of immune organs. Cytokines levels as marker of inflammation were determined using ELISA in serum and colon. Which proved that DOLP inhibited the expression of pro-inflammatory cytokines (TNF-α, TGF-β1, IL-6, IL-1β) and promoted the expression of anti-inflammatory cytokines (IL-10). Short chain fatty acids (SCFAs) levels and microbial composition in feces were determined using GS and high-throughput sequencing. DOLP improved gut microbiota by increasing the relative abundance of total bacteria and probiotics such as *Bacteroides*, *Lactobacillus* and *Lachnospiraceae*. Therefore, DOLP has potential effect for the treatment of chronic immune diseases.

**Introduction**

The immune system plays an important role in protecting human health. When it is damaged by congenital or acquired reasons, it causes immune deficiency, which lead to a series of diseases, such as failure to thrive, generalized erythematous rash, and recurrent gastrointestinal and respiratory tract infections, including episodes of *Pneumocystis pneumonia* infection and *Candida albicans* fungemia (Somekh et al., 2021), and inflammation is the most common symptom of these diseases. Inflammation is a kind of disease of the immune system (Garrett, Gordon, & Glimcher, 2010), that occurs early in the course of many diseases, such as diabetes (Sun et al., 2020), cancer (Watanabe et al., 2021), and liver cirrhosis (Tang et al., 2021). Although the inflammation produced is a beneficial self-protective immune response, body may also attack its own tissues sometimes due to excessive inflammation (Zhang, Wu et al., 2020; Zhang, Zhang et al., 2020). Several studies have recently revealed the specific relationship between immune responses and gut microbiota (Benitez-Páez et al., 2016). Modulation of the gut microbiota composition in a certain way was found to repair immune deficiency indirectly warranting for exploitation as target in immunomodulation drug development (Ye, Xu, & Liu, 2021). Zhang et al. found that *Dendrobium officinale* Kimura & Migo (*Orchidaceae*) polysaccharides had protective effect against dextran sulfate sodium induced colitis via alleviating gut microbiota dysbiosis (Zhang, Wu et al., 2020; Zhang, Zhang et al., 2020).

*Dendrobium(D.) officinale* is a traditional medicine used for the treatment or prevention of several diseases, such as cancer (Xie, Feng, Farag, Sun, & Shao, 2021), inflammation (Li, Wu et al., 2020; Li, Yue et al., 2020), and intestinal barrier damaged (Liang et al., 2019). Typically, most studies focused on the stem of *D. officinale*, but the leaf of it has similar yield and active substances compared with stem. Liu et al. found that the polysaccharides extract from stem and leaf had some difference in structure and activities (Liu et al., 2020). For instance, the monosaccharide ratio of mannose and glucosamine was 3:1 in stem and 14.5:1 in leaf, otherwise, the polysaccharides came from stem showed better anti-gastric cancer activity compared with leaf. Therefore, it is important to distinguish the activities of polysaccharide in different parts correctly. In recent years, studies have increasingly illustrated that...
**D. officinale** leaf exhibit therapeutic effects on a number of diseases, including cancer (Liu et al., 2020), and inflammation (Yang et al., 2020) warranting for its further investigation of its ameliorative and protective effects in immunodeficiency diseases.

**D. officinale** leaf is rich in several bioactive compounds including flavonoids with an antioxidant, antidiabetic and anti hyperlipidemic actions (Aoi, Iwasa, & Marunaka, 2021), alkaloids with anti-cancer effect (Bai et al., 2021) and polysaccharides known to reduce inflammation and regulate blood sugar level (Liu et al., 2020). Compared to other plant metabolites, polysaccharides are recognized for their higher safety level in addition to their potential actions (Barbosa & de Carvalho Junior, 2021). Owing to their macromolecular polymeric nature, polysaccharides can hardly be absorbed directly in stomach or small intestine, and are mostly subjected to fermentation via intestinal microbiota in the colon (Benitez-Páez et al., 2016; Elshahed, Miron, Aprotosoaie, & Farag, 2021). After fermentation, polysaccharides can influence the composition and metabolism of gut microbiota directly, and to further influence gut and health indirectly status (Fang, Hu, Nie, & Nie, 2019). Considering **D. officinale** polysaccharides (DOLP) possess anti-inflammatory effects, it is likely to demonstrate curative effects in improving immune deficiency.

In this study, determination of DOLP beneficial effects in immune deficiency, and further its action mechanisms were presented for the first time. The effect of DOLP was determined in an immune deficiency disease model induced by cyclophosphamide in a mice model. The potential effects of DOLP under long-term effects were determined via monitoring the change in microbial structural composition and associated metabolism via SCFAs both *in vivo* and *in vitro* and suggestive to be examined further clinically in humans in the future.

**Material and methods**

**Chemicals and reagents**

The leaves of *D. officinale* were obtained from a local market in Wenzhou, Zhejiang Province, China. Cyclophosphamide monohydrate (Cys) (BR, 97%), and Levamisole hydrochloride (LH) (BR, 99%) were purchased from Shanghai Yuanye Bio-Technology Co., Ltd. (Shanghai, China). Acetic acid, propionic acid, butyric acid, valeric acid, mannose, ribose, rhamnose, glucosamine, glucuronic acid, galacturonic acid, galactosamine, glucose, galactose, xylose, arabinose and fucose were all purchased from Changchun Huili Biotechnology Co., Ltd. (Changchun, China). All other chemicals and reagents were analytical reagent grade and purchased from Sigma-Aldrich (Shanghai, China).

**Preparation of DOLP**

Preparation of DOLP was carried out following the protocol of (Yang et al., 2020) with modification. Briefly, leaves of *D. officinale* were dried at 60 °C till complete dryness. After that, 50 g dried leaves were pulverized into powder and screened it with 40 mesh, the powder was further soaked in 500 ml 85% (v/v) ethanol for 12 h, and the sediment was extracted thrice using 1500 ml deionized water (1:30, g/ml, 80–85 °C) with 2 h. The solution was concentrated using rotary evaporators at 60 °C until the volume reached 500 ml and then precipitated by the addition of 2000 ml absolute ethyl ethanol. Precipitate was then dissolved using 500 ml distilled water and further purified by removing its protein content using the Sevage method (Yang, Wang, Li, & Yu, 2015) with 125 ml solution of (chloroform: n-butyl alcohol = 5:1). Solution was further subjected to precipitation using 2000 ml absolute ethyl ethanol and further centrifuged at 5000 × g for 10 min. Precipitate was finally washed with 85% ethanol, lyophilized using freeze dryer (BTP-3XL, SP Virtis Benchtop Pro, St. Louis, Mo, USA) in ~80 °C and stored at 4 °C till further analysis.

**Monosaccharide composition and FT-IR analysis of DOLP**

The monosaccharide composition was determined using precolumn derivatization HPLC method as previous described with some modifications (Yang et al., 2020). 5 ml trifluoroacetic acid (TFA, 2 M) was added to 100 mg DOLP and air seal with N2 for 1 min. After that, the sample was stored in 120 °C for 2 h, and then, 1 ml sample mixed with 1 ml methyl alcohol after cool down to room temperature. Dried the mixture with N2 in 70 °C thrice by adding methyl alcohol repelition to removed the TFA completely, 1 ml NaOH (0.3 M) was added to residual material and dissolved it to gained the DOLP hydrolysate. 400 µl hydrolysate was mixed with 400 µl PMP methanol solution and stored in 70 °C for 2 h, cooled the liquid to room temperature and added 400 µl HCl (0.3 M). Trichloromethane was extracted thrice in the volume 1.2 ml and centrifuged at 12,000 g for 20 min to obtain the supernatant. The derivative supernatant was filtered using 45 nm (Millipore; Bedford, Mass, USA) membrane and analyzed using HPLC (U3000, Thermo-Fisher, Waltham, MA, USA) with C18 column (5 µm, 4.6 mm × 250 mm, ThermoFisher, Waltham, MA, USA) and detected using an ultraviolet detector at 245 nm. The HPLC analysis conditions were as follows: column temperature, 30 °C; mobile phase, 0.1 M phosphate buffer and acetonitrile; flow rate, 1.0 ml min⁻¹. The standard solution of monosaccharides was a mixture of each monose in concentration of 0.2 mg/ml. The peak area of the standard solution was compared with that of the sample to calculate the content of monosaccharide in the sample.

2 mg DOLP was ground with 200 mg KBr powder and then pressed into pellets for FT-IR analyzed (IS-50 FT-IR, Nicolet; Thermo Fisher Scientific, Waltham, MA, USA). The FTIR spectrum was obtained in frequency range of 400 – 4000 cm⁻¹.

**Animals and experimental design**

48 Male BALB/c mice (five-week-old, 18 ± 0.5 g body weight) were purchased from the Shanghai Slack Laboratory Animal Co., Ltd. (Shanghai, China). Mice were maintained in an environment free of pathogen (20 ± 2 °C, 55 ± 10 % humidity) on a 12 h cycle of light dark. All experimental and care procedures of animals used in this study were performed in accordance with the relevant guidelines and regulations, and approved by the Animal Use and Care Committee of Zhejiang University of Technology.

All the mice were divided into 6 groups randomly (n = 8): control group (NC), model group (MC, Cys-treated), Cys + DOLP-treated with low-dose group (LD, 50 mg/kg/d), Cys + DOLP-treated with high-dose group (HD, 200 mg/kg/d), Cys + positive control (PC, LH-treated, 40 mg/kg/d) and normal mice treated with high-dose DOLP group (HLD, 200 mg/kg/d). After 7 days of adaptation, except the mice in NC and HLD groups, all others were subjected to intraperitoneal injection with 300 µl stroke-physiological saline solution which contained Cys at a dose of 80 mg/kg body weight (BW) every three days, while NC and HLD groups were intraperitoneally i.p. injected with 300 µl stroke-physiological saline solution at the same time. LD were treated with 50 mg/kg/d DOLP solution, and HD, HLD groups were treated with 200 mg/kg/d DOLP deionized water solution by intra gastric administration respectively in a volume of 200 µl, whereas PC group was treated with 200 µl LH solution of 40 mg/kg/d which dissolved in deionized water, and the NC, MC Groups were treated using 200 µl of normal saline once a day for 4 weeks. Fresh feces were collected on the 27th day post treatment, and sacrificed on the next day. Nearly 10 cm colon tissue was excised and stored at ~20 °C for further analysis.
**Assay of spleen and thymus indices**

In the 28th day, all the mice were weighted and anesthetized with 4 % chloral hydrate in the dose of 0.1 ml/10 g body weight by intraperitoneal injection. After blood collection, mice were killed by folding necks, with thymus and spleen excised and weighed. Spleen and thymus indices were calculated using the formula of (Xie et al., 2019):

\[
\text{Organ index} = \frac{\text{weight of organ (mg)}}{\text{weight of mouse (g)}}
\]

**Determinations of liver function and inflammatory cytokines**

After the mice were anesthetized with 4 % chloral hydrate, abdominal cavity of mice were dissected and opened, with blood collected through the abdominal aorta. The blood was stored for 1 h at 4°C and centrifuged at 1000 × g for 20 min to yield serum used to analyze cytokines (IgA, ALT, AST) using Elisa kits. After the mice were autopsied, colon tissues were collected, weighed and homogenized with PBS buffer (pH = 7.4). The tissue homogenate was centrifuged at 4°C, 10,000 × g for 15 min, with supernatants aliquoted for the measurement of inflammatory markers (TNF-α, TGF-β1, IL-6, IL-10, IL-1β) following manufacturer’s protocol using Elisa kits (eBioscience, San Diego, CA, USA) (Liang et al., 2019).

**Histological examination**

After tissues fixation with 4% of paraformaldehyde overnight, colon and liver tissues were embedded in paraffin. Slice paraffin blocks were cut into thin slices (5 μm thickness) for further hematoxylin-eosin (H&E) staining. Evaluation of colon and liver anatomical features were performed using Nikon 80i (Tokyo, Japan). Colon wall integrity, colon cells morphology and inflammatory cell infiltration were evaluated in colon slices. Hepatocyte morphology, inflammatory cell infiltration, were evaluated in liver slices

**Determination of SCFAs levels**

SCFAs levels in feces were determined using gas chromatography mass spectrometry GC (Agilent 6890 N) (Santa Clara, CA, USA), equipped with a flame ionization detector (FID) (Fu et al., 2019). Briefly, 200 mg of feces collected from each mice were diluted using PBS at the ratio of 1:10 (g/ml), vortexed for 2 min and placed at 4°C for 5 min, and repeated for three times. Suspension was then centrifuged at 5000 × g, 5 min, 4°C, with supernatants filtered using 0.22 μm membrane for further analysis. 1 μl sample was used to determine, the oven temperature was increased from 60 to 100 °C at 5 °C/min, and then rise to 145 °C at 10 °C/min.

**16S rRNA sequencing of gut microbiota**

Total genome DNA was extracted using CTAB/SDS method (Schriefer et al., 2018). Concentration and purity of DNA was determined using 1 % agarose gels. The extracted genomic DNA was used as a template for the amplification of 16S rRNA genes. Amplified V3-V4 regions of bacterial 16S rDNA by PCR(ABI Gene Amp® 9700) (ThermoFisher, Waltham, MA, USA), with universal primers 338F (5′-ACTCTACGGGAGGCAGCA-3′) and 806R (5′-GGACTACHVGGGTWTCTAAAT-3′). PCR products of each sample were mixed and then detected using electrophoresis on a 2 % agarose gel, and puriﬁed with AxyPrep DNA Gel Extraction Kit (Axygen, NY, USA). The Illumina PE library (Illumina, San Diego, CA, USA) was used to sequence the libraries.

**In vitro fermentation of DOLP**

The fresh feces of NC group and MC group were collected, in order to reduce the influence of individual differences on the experimental results, the feces of the same group were mixed and then diluted with PBS at a ratio of 1:10 (g/ml). After which, 0.5 ml of the fecal suspension was injected into a sealed fermentation bottle that contains 5 ml of yeast extract, casitone, and fatty acid (YCFA) bacterial medium (do Prado, Minguzzi, Hoffmann, & Fabi, 2021). Different drugs were injected into the corresponding fermentation bottle as shown in Table 1. After incubation at 37 °C and constant humidity for 24 h, fermentation liquid was removed and centrifuged at 5000 × g for 5 min, 4°C, with supernatant collected and filtered using 0.22 μm membrane till further analysis.

**Statistical analysis**

Data were presented as mean ± SD. Graphs were drawn using Graphpad Prism 8 software (GraphPad Software, Inc., La Jolla, CA, USA). A one-way analysis of variance (ANOVA) was utilized to analyze data. *p < 0.05 was considered to be statistically significant, while **p < 0.01 was considered to be statistically highly significant.

**Operational taxonomic units (OTUs) were deﬁned as the minimum of 97% sequence similarity. The alpha diversity was calculated, the OTU table was rareﬁed at an even sampling depth of 7050 sequences/sample.** PCA statistical analysis and mapping were performed with R language.

**Results**

**Monosaccharide composition and FT-IR analysis**

The monosaccharide composition of DOLP was determined by HPLC. As shown in Fig. S1 B, DOLP was a heteropolysaccharides comprising mannose, galacturonic acid, glucose, galactose, and arabinose at a molar ratio of 10.0:1.0:0.9:6.0:6.0:3. From the results, it could be found that more than 77% the monosaccharide components of DOLP were mannose, which was considered have positive effect in inﬂammation in recent research (Shaker et al., 2021).

Fig. S1 C showed the FTIR spectra detected from 400 to 4000 cm⁻¹ of DOLP which displayed characteristic bands at 610, 812, 960, 1092, 1252, 1425, 1597, 1735 and 2932 cm⁻¹. The broad absorption peaks at approximately 3423 cm⁻¹ corresponds to O–H stretching, and the band at 2932 cm⁻¹ belonged to –CH stretching vibration. In addition, the peaks at 1735 and 1597 cm⁻¹ were attributed to the stretching of –C=O. The band at 1425 cm⁻¹ corresponds to –C–H stretching while the band at 1252 cm⁻¹ and 1092 cm⁻¹ indicates –O–H and –C–O stretching. The absorption at 960 cm⁻¹ can be attributed to the β-glucopyranosyl, whereas that at 812 cm⁻¹ can be attributed to manno.pyranose. Finally, the band at 610 cm⁻¹ may be the bending vibration of –C–CO–C– (Yuen, Choi, Phillips, & Ma, 2009).

**Effects of DOLP on mice immunity**

The spleen and thymus, as major immune organs, change in size and quality when the immune system is damaged (Li et al., 2017). Fig. 1A and B showed significant decrease in thymus and spleen indices in the

**Table 1**

| Numbers | Sources of feces | Additive drug in fermentation broth |
|---------|------------------|------------------------------------|
| 1       | NC               | DOLP/mg  | Cys/mg | LH/mg |
| 2       | NC               | 0.0625  | –      | –     |
| 3       | NC               | 0.25    | –      | –     |
| 4       | NC               | –       | 0.1    | –     |
| 5       | NC               | –       | –      | 0.05  |
| 6       | MC               | –       | –      | –     |
| 7       | MC               | 0.0625  | –      | –     |
| 8       | MC               | 0.25    | –      | –     |
MC group compared with the NC group, with organ index to decreased from 1.27 to 0.36 and 4.66 to 4.16, respectively at a percentage decrease of 71.65% and 10.73%. Asides, DOLP (LD and HD treatment groups) displayed an increase in both organ index compared with that of the MC group in a dose-dependent manner, the spleen index increased 25.24% and 42.55% in low or high dose of DOLP compared with MC group and the thymus index increased from 0.36 to 1.07 and 1.31 in two dose, respectively. Likewise, IgA serum level (Fig. 1C) was significantly decreased in the MC group, from 301.74 to 207.85 (mg/ml) compared with normal group, and recovered in the HD and PC groups which risen to 289.50 and 305.15 (mg/ml) respectively. In all examinations, the results of HLD group showed that DOLP had no effects in mice’ immune system while they were healthy. The results demonstrated that treatment with DOLP could effectively

Fig. 1. The immune organ index of thymus (A) and spleen (B), and inflammatory factors in serum (IgA)(C). Effect of different treatments on average body weight (D) and daily diet intake of mice (E). In this study, NC: control group, MC: model group (Cys-treated), LD: Cys + DOLP-treated with low-dose group (50 mg/kg/d), HD: Cys + DOLP-treated with high-dose group (200 mg/kg/d), PC: Cys + positive control (LH-treated, 40 mg/kg/d) and HLD: normal mice treated with high-dose DOLP group (200 mg/kg/d). Data are expressed as mean ± SD (n = 8). The different letters represent significant differences among different groups (p < 0.05).

Fig. 2. Protective effects of different dose (50 mg/kg/d and 200 mg/kg/d) of DOLP on liver tissue. Representative H&E-stained liver tissues in (A) NC, (B) MC, (C) LD, (D) HD, (E) PC, and (F) HLD group of mice. Black arrows indicate liver injury, whereas red arrows indicate inflammatory infiltrate. Effects of DOLP on serum liver markers ALT (G) and AST (H). Data are expressed as means ± SD (n = 8). Different letters represent significant differences among different groups (p < 0.05). (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)
increased indices of thymus and spleen in mice, suggestive that DOLP may have potential to reverse the atrophy of immune organs caused by drug or disease.

The body weight and daily diet of mice were observed to assess the healthy status (Zhan et al., 2020), with Cys injection found to decreased mice appetite leading to weight loss, and with intake of DOLP to reverse such effect (Fig. 1 D and E).

Effects of DOLP on liver function

To further verify the safety level of DOLP, liver tissue and serum cytokines level were inspected to evaluate liver health status. As depicted in Fig. 3 A, D and F, liver tissue structure appeared normal, with cells that were arranged neatly, showing no degeneration or obvious inflammatory cell infiltration. In contrast, in case of MC group, the liver tissue structure was severely abnormal, with numerous areas of necrosis in the lobules, nucleus fragmentation, and cytoplasm staining, as shown by the red arrow (Fig. 3 B). There was a large infiltration of inflammatory cells, mainly neutrophils, as shown by the black arrow. Similar injury was observed in Fig. 3 C and E, in which liver cell was found dropical, with some cell nucleus showing fragmentation accompanied by a small infiltration of inflammatory cells around the artery. The results of serum cytokines showed similar pattern, with and elevated ALT and AST levels post Cys administration suggestive of liver damage. Overall results revealed that the use of DOLP (Fig. 2D) and LH (Fig. 2E) could restore liver damage, with DOLP showing better effects.

Effects of DOLP on inflammatory cytokines

Macrophages release inflammatory cytokines in the process of cellular immunity and the stabilization of inflammatory cytokine networks, which is an important aspect of sustaining immune status. Increased in the release of typical pro-inflammatory cytokines (IL-6, IL-1β, TNF-α and TGF-β1) in MC group mice, whereas the anti-inflammatory cytokines IL-10 was increased from 53.33 to 116.30 (pg/ml), which observed confirming the anti-inflammatory effects of DOLP in Cys treated mice.

As showed in Fig. 3 B, C, D, E, pro-inflammatory cytokines increased clearly in MC group, compared with NC group, the pro-inflammatory cytokines increased from 28.95, 25.06, 94.84, 50.50 to 117.25, 98.91, 227.77, 160.324 (pg/ml) respectively, which proved that severe inflammatory reaction that occurred in the model mice. The use of DOLP and LH demonstrated inhibitory effects on IL-6, IL-1β, TGF-β1 and TNF-α in inflammatory mice, reduced the amount to 58.59, 48.06, 140.98, 83.06 and 50.04, 58.34, 118.72, 76.53 (pg/ml), suggesting that DOLP has a therapeutic potential similar to that of LH. In the contrary, LD and HD groups showed increased IL-10 level compared with MC group (Fig. 3F) at percentage of 161.7% and 270.9%. The HLD group also had higher level of 68.1% compared with NC, which suggests that the DOLP promoted the anti-inflammatory cytokines.

Effects on histological changes of the colon

Microscopic examination of the colon tissue (Fig. 3A) showed normal colon tissue structure in NC and HLD groups, with ordered epithelial cells of the mucosa, and no degeneration or shedding was observed. There was no reduction in the number of crypt, a large number of goblet cells were visible, and with no infiltration of inflammatory cells detected inside the tissue. In MC group, the colon structure was moderately abnormal, with some mucosal epithelial cells being exfoliated with the lamina propria being exposed as shown by the black arrow. The mucosal layer crypt structure disappeared, and there was a proliferation of inflammatory cells and fibrous tissue, as shown by the red arrow (Fig. 3A, MC group). Compared with MC group, significant improvement was observed in case of LD, HD and PC groups. However, some crypt epithelial cells appeared necrotic and deformed in LD group, with pyknotic and hyperstained nuclei, as shown by the black arrow (Fig. 3A, LD group). Additionally, focal infiltration of inflammatory cells in the mucosal layer was observed as shown by the red arrow. The results proved that DOLP could protect against colon injury effectively induced by Cys treatment.

Effects of DOLP on intestinal microbiota metabolism

The effect of DOLP on SCFAs production by microorganisms was assessed via the determination of acetic acid, propionic acid, butyric acid, valeric acid and total SCFAs inside the colon. As showed in Fig. 4A, the intestinal feces of mice in LD and HD groups showed recovery of SCFAs, but were higher in HD group compared to NC group, indicating that DOLP can promote microbial fermentation in vivo. Similar results were observed in the case of HLD group. The concentration of propionic acid reduced from 5.22 to 2.49 mmol/g of MC group compared with NC group and increased to 6.40 mmol/g in HD group and 6.810 mmol/g in HLD group. Samilar variation trend could be seen in butyric acid. The concentration in NC group was 1.00 mmol/g and decreased to 0.39 mmol/g in MC group. And it recovered to 1.49 and

Fig. 3. The histological colon changes examined by H&E staining (A). The effect of different dose (50 mg/kg/d and 200 mg/kg/d) of DOLP on cytokines level (B) TNF-α, (C) IL-6, (D) IL-1β, (E) TGF-β1, (F) IL-10 in mice colon. Data is presented as mean ± SD (n = 8). The different letters represent significant differences among different groups (p < 0.05).
1.95 mmol/g after high dose DOLP treatment in HD group and HLD group, which proved that the DOLP could help gut microbiota producing more beneficial SCFAs.

**Effects of DOLP on intestinal microbiota composition**

High-throughput sequencing was used to evaluate changes of intestinal bacterial composition among the different treatment groups. Two venn diagrams of OTUs in different groups were presented in Fig. 5A, with an overlap of 532 OTUs among the six groups of samples, while 54, 43, 26, 33, 28, and 30 OTUs were found uniquely presented in NC, MC, LD, HD, PC, and HLD groups, respectively. To confirm that the effects of dose on the regulation of DOLP in intestinal microbiota, we compared the OTUs of NC, LD, and HD groups. Results (Fig. 5B) revealed exclusion of 768 co-owned OTUs, 37 OTUs were presented in the LD and NC groups, while 119 OTUs were presented in the HD and NC groups. Furthermore, similarities in the structure of the entire microbial community was evaluated using principal component analysis (PCA) (Fig. 5C), with the two main principal components to account for 22.6 % and 9.5 % of the total variance. Results showed that the microbial

![Fig. 4](image-url)
Fig. 5. Evaluation of sequencing data revealing that DOLP could modulate the overall structure of intestinal microbiota. (A, B) Venn diagram of OTUs in the different groups, (C) Principal coordinate analysis (PCA) plot. Each dot represents a colonic community, and the percentage of variation explained by each principal coordinate is shown. (D) LEfSe taxonomic cladogram. The colored nodes from inner circle to outer circle represented the hierarchical relationship of all taxa from the phylum to the genus level. (E) Histogram of the linear discriminant analysis (LDA) scores for differentially abundant genera. Different dose (50 mg/kg/d in LD and 200 mg/kg/d in HD, HLD) of DOLP restored the immune deficiency-induced gut microbial dysbiosis at different taxonomic levels in mice. (F) Abundance of the most important genus in each group. (G) Analysis of gut microbiota at the phylum level.
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inflammation is one of its main hallmarks (Huang et al., 2020). Metabolites produced by gut microbiota play a major role in maintaining health and disease development (Harstrøm, Nieuwdorp, & Herrema, 2016). Several studies have revealed that plant polysaccharides can achieve therapeutic effects i.e., immunomodulation etc. by regulating the metabolism of intestinal microorganisms (Barbosa & de Carvalho Junior, 2021). In this study, we investigated the beneficial effects of DOLP on intestinal microbiota, including its ability to influence gut microbiota composition and metabolism, by adopting an immunodeficiency mouse model.

Mice subjected to co-administration of Cys revealed influence on daily diet and body weight (Fig. 1D, E), likely attributed to Cys negative effect on the immune system, thus causing discomfort and a loss of appetite. However, the use of DOLP restored such negative effect, with mice in HLD group showing no differences to NC group, which proved the safety and effectiveness of DOLP. The spleen and thymus are the main immune organs, with drop in organ index means a damage of the immune system. Subsequent anatomical examination revealed a drop in both the immune organ index and IgA index of mice in MC Cys-treated group. The reversal of this was a decrease by DOLP, proving that it has the potential to improve immune deficiency status.

To confirm the safety and effectiveness of DOLP, liver and colon tissue slice and cytokine levels in mice were observed. Results revealed that the liver tissue of normal mice and mice treated with DOLP only were healthy (Fig. 2A, F), while mice treated with Cys had severe liver damage (Fig. 2B). That injury was alleviated to a certain extent after treatment with DOLP (Fig. 2C, D) or LH (Fig. 2E). Comparison of the injury in LD group and HD groups, we found better therapeutic effects of high dose. DOLP were observed as manifested by Yang et al. in a dose-dependent manner, which was same with us. ALT and AST are the two typical indicators of liver status found elevated in case of liver damage i.e., hepatitis or other organ inflammation (Hong et al., 2020). In our study, significant increase in ALT and AST levels were observed in MC group (Fig. 2G, H), and in accordance to other reports (Li et al., 2020). The treatment of DOLP could inhibit the increase of ALT and AST levels, which proved that DOLP has potential protective effects on Cys-induced inflammation. And the protective effect of DOLP on liver may due to the reduction of oxidative stress response and invasion of inflammation cells. Colon tissue analyses (Fig. 3A) showed similar results to that of liver with Cys treatment showing damaged colonic epithelial tissue, with severe inflammation, and the colon inflammation to show recovery with DOLP and LH treatments. In particular, high doses of DOLP showed the same therapeutic effects as LH, which proved the effectiveness of DOLP. To investigate the possible bio-activity of DOLP further in vivo, protein fraction was further extracted from colon tissue for immunoenalysis revealing that DOLP could not only inhibit the production of pro-inflammatory cytokines (Fig. 3B-E), but rather promote the secretion of anti-inflammatory cytokines (Fig. 3F) suggestive that DOLP may affect the body’s immunity by affecting Th1 cells and Th2 cells (Darvayor, Shiri, Amirghofran, & Kamali-Sarvestani, 2021).

In colon, macromolecules such as polysaccharides, proteins could be fermented by gut microbiota to yield SCFAs (Fang et al., 2019), with SCFAs to maintain the integrity of gut barrier by regulating the expression of tight junction proteins, including claudin-1, occludin, zonula and occludens-1 (Harstrøm et al., 2016). SCFAs can also protect gut epithelium via improving the expression of mucin 2 and modulating immune response and oxidative stress (Elshahed et al., 2021). Consequently, SCFAs play important roles in stabilizing the intestinal environment. In our study (Fig. 4A), SCFAs were found distinguishing among the different groups associated by changes in microbiota composition (Fig. 5E). In HD group, a significant increase in acetic and butyric acid levels were observed (Fig. 4A), concurrent with increase in lactic acid bacteria from sequencing results (Fig. 5F). Besides, it was obvious that the relative abundance of microbiota especially Bacteroidales in MC group showed reduction compared with normal mice, and leading to a decrease in total SCFAs (Fig. 5A), suggestive that decrease of intestinal microbiota is one of the focal manifestations of inflammatory diseases. In contrast, butyric acid showed marked increase in HLD group, which had beneficial effect in inflammation caused by Bacteroidales. In both LD and HD groups, fecal SCFAs reduced significantly compared with NC group, and to reach normal levels in a dose-dependent manner. The results showed that DOLP could regulate SCFAs production concurrent with an improvement of the immune deficiency status. In addition, an in vitro fermentation experiment was designed in this study to determine whether DOLP could be utilized directly by intestinal microbiota or requires an additional in vivo transformation process before being utilized. The results (Fig. 4B) revealed that DOLP, like other polysaccharides, can be utilized directly by intestinal microbiota and to yield...
SCFAs (Wu et al., 2021), thereby regulating physiological activity. The higher the level of polysaccharides (Group 1,2,3), the higher SCFAs in a dose-dependent manner and consistent with in vivo results. Moreover, results of in vitro fermentation of feces from different sources (Group 1,6) were different infering that the microbiota structure in the infected mice was different from that in the normal mice.

One of the mechanism through which DOLP to function in immune modulation is via altering the structure of intestinal microbiota through fermentation, and to play a protective role against immune deficiency. In order to determine the specific strains affected by DOLP, we assessed changes of microbiota composition in the different treatment groups at phylum (Fig. 5G) and genus (Fig. 5F) levels. The results of principal component analysis (Fig. 5C) demonstrated that microbiota composition of MC group was significantly different from that of NC group, with DOLP and LH treatment found to restore such changes imposed by Cys administration. However, only with DOLP treatment that microbiota community structure was different from both NC and MC groups, which may be due to that DOLP promoted the reproduction of specific bacterial community as revealed from LDA analysis (Fig. 5E). The results showed that there were 10 dominant groups in HLD Group, in which Bacteroidetes, Bacteroidales, Bacteroidia and Prevotellaceae had major impact on the dominant community. Likewise, microbiota which had main influence on NC MC groups belonged to Clostridiales, Clostridia and Ruminococaceae, respectively. Studies revealed that Bacteroides play an important role in polysaccharides metabolism and in disease treatment (Qiao et al., 2020), and that change of Bacteroides in the PC and LHD groups as dominant communities in this study likely accounts for their role in the treatment of inflammation (Fig. 5F). Meanwhile, the dominant communities in HD group were Firmicutes and Lactobacillus, which may be due to the combined effects of Cys and DOLP. Bacteroides and Lachnospiraceae are both SCFAs producing bacteria, with previous studies revealing that the abundance of Bacteroides in patients with inflammation to be reduced (Xie et al., 2019; Ying et al., 2020), whereas Lachnospiraceae are protective strains for inflammatory bowel disease (Zeng et al., 2020). DOLP treatment showed increase in the abundance of the two families and to account for the polysaccharides anti-inflammatory actions. Overall, results conclude that DOLP has prebiotic potential that could promote the growth of beneficial probiotic species that has yet to be examined in humans and clinical trials.

Conclusion

In this study, the immune deficiency model was established to study the effects of polysaccharides isolated from D. officinale leaf on gut microbiota and homeostasis. The results indicated that DOLP had therapeutic effects on Cys-treated immune compromised mice. In vitro fermentation experiment results displayed that DOLP could be directly fermented by microorganisms in the gut, and to alleviate inflammatory response by regulating bacterial community structure and promoting the release of SCFAs. Sequencing analysis revealed that DOLP could restore the decreased abundance of bacteria caused by Cys administration to include probiotics such as Bacteroides, Lactobacillus and Lachnospiraceae. In a safety context, DOLP could not only restore the intestinal barrier damage caused by Cys comparable to the positive control drug LH, but also to further recover the liver damage caused by Cys administration posing it as hepatoprotective agent and to be tested against other toxins i.e., aflatoxins etc. According to these results, we conclude that DOLP has the potential to be used as prebiotics in food, which could reduce inflammation and regulates the immune system by regulating gut bacteria of potential to be used in the future as a therapeutic agent or ancillary agent in immune diseases.

Compliance with ethics requirements

All Institutional and National Guidelines for the care and use of animals (fisheries) were followed.

All animals used in this study were cared for in accordance with the Guidelines for the Care and Use of Laboratory Animals published by the United States National Institute of Health (NIH, Publication No. 85–23, 1996). This experiment was approved by the ethics committee of Zhejiang University of Technology (Animal application approval number 20201025072), China.

Declaration of Competing Interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

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Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.fochx.2022.100235.

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