

# Chrysanthemum extract mitigates high-fat diet-induced inflammation, intestinal barrier damage and gut microbiota disorder†

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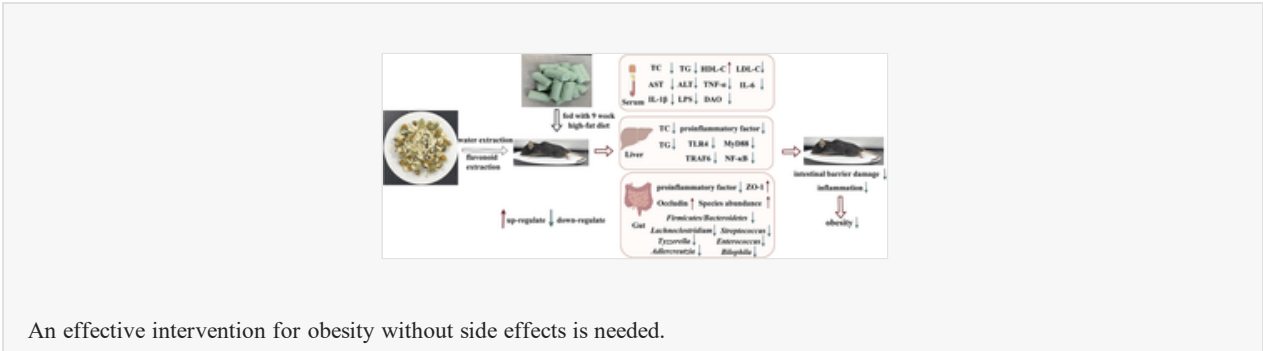
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## Abstract

An effective intervention for obesity without side effects is needed. Chrysanthemum may be the preferred choice due to its influence in the improvement of glycolipid metabolism. This study assessed the efficacy of chrysanthemum and its flavonoids in mitigating high-fat diet (HFD) induced obesity, focusing on the integrity of the intestinal barrier, inflammation, and gut microbiota. Fifty male C57BL/6J mice were divided into 5 groups randomly: normal control (NC), HFD, HFD with chrysanthemum aqueous extract (CM), HFD with a low-dose flavonoid extract of chrysanthemum (FLL), and HFD with a high-dose flavonoid extract of chrysanthemum (FLH). The results showed that after 9 weeks of intervention with CM, FLL and FLH, the body weight and blood lipid levels of mice were reduced. The chrysanthemum treatment regimens down-regulated the gene expression and protein levels of TLR4, MyD88, TRAF6 and NF- $\kappa$ B, upregulated the gene expression levels of ZO-1 and occludin, and decreased the levels of LPS and diamine oxidase (DAO) in the serum. With CM, FLL and FLH, the levels of the inflammatory factors IL-1 $\beta$ , TNF- $\alpha$ , and IL-6 were decreased, and the abundance of pernicious bacteria *Lachnospirillum*, *Streptococcus* and *Enterococcus* was decreased. Notably, the purified chrysanthemum flavonoid extract showed greater effects as compared to the CM. The study demonstrated that chrysanthemum extracts could achieve anti-obesity effects by strengthening the intestinal barrier function, relieving inflammation and modulating the gut microbial composition.

## Introduction

Obesity and being overweight are highly common chronic conditions globally, with a strong link to the development of hyperlipidemia, non-alcoholic fatty liver disease (NAFLD) and cancer.<sup>1,2</sup> Studies have shown evidence that obesity is associated with a mild chronic inflammatory state in metabolically active organs, including the liver and intestine.<sup>3</sup> Furthermore, consumption of excess dietary fat is postulated to facilitate low-grade chronic inflammation.<sup>4</sup> HFD-induced lipid accumulation leads to increased lipid uptake by hepatocytes and subsequent disruption of metabolic homeostasis,<sup>5</sup> whereas loss of metabolic homeostasis and tissue damage lead to NAFLD dominated by steatosis and inflammation.<sup>6</sup>

Research indicates that NAFLD frequently involves persistent low-level inflammation, typically resulting from lipid accumulation. Chronic HFD can reduce intestinal tight-junction protein expression, damage the intestinal mucosal barrier, and elevate oxidative stress levels in the body, resulting in disruption of the gut barrier function.<sup>7–9</sup> Intestinal barrier dysfunction may result in the presence of elevated circulating bacterial components, such as lipopolysaccharides (LPSs) due to the crossing of the gut barrier and entering the systemic circulation.<sup>10</sup> Toll-Like Receptor 4 (TLR4) serves in recognizing and activating upon LPS interaction. Once TLR4 recognizes and binds to LPS, it elicits the activation of the myeloid differentiation primary response protein 88 (MyD88) signalling pathway, a cascade of molecular interactions that ultimately trigger nuclear factor kappa-B (NF- $\kappa$ B) and pro-inflammatory cytokines.<sup>11–14</sup> This inflammatory response to LPSs increases the risk of inflammation-related diseases, including NAFLD and irritable bowel syndrome.<sup>15</sup>

Evidence supports that the gut microbiota is crucial for maintaining intestinal barrier integrity and facilitating immune responses within the gastrointestinal tract, and plays a key role in various types of diseases caused by obesity.<sup>16–18</sup> *Firmicutes* and *Bacteroides* constitute the predominant phyla within the human gut microbiota. An elevated ratio of *Firmicutes* to *Bacteroides* (F/B) is a potential biomarker for gut dysbiosis, particularly in the realm of obesity, contributing to associated metabolic disorders.<sup>19,20</sup> Therefore, improvement of gut microflora dysbiosis is a novel target for the mitigation of obesity-related diseases.

Current treatments and interventions for obesity are still limited, despite a growing understanding of the disease. Thus, an immediate necessity exists for the advancement of novel effective therapeutic interventions. *Chrysanthemum morifolium* Ramat. is a traditional Chinese botanical tea with potential medicinal applications.<sup>21</sup> Chrysanthemums exhibit various effects, such as antioxidant,<sup>22</sup> hypolipidemic<sup>23</sup> and hypoglycemic effects.<sup>24</sup> However, the regulatory effects and the underlying mechanisms of chrysanthemum on obesity are unclear. This research is intended to explore the impact of chrysanthemum extract on obesity from the perspectives of intestinal barrier damage, inflammation and the gut microbiota by studying the effects in the context of a mouse model with obesity. Our study is important for defining the utility of chrysanthemum and its components in the development of related lipid-lowering products.

## Materials and methods

### Preparation of CM and FL

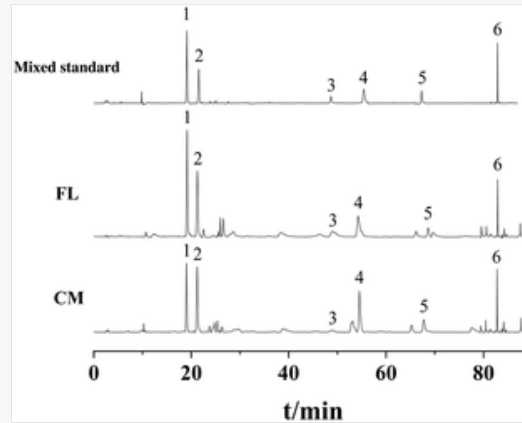
The processing of Hang white chrysanthemum samples (supplied by Yifutang Tea Co., Tonglu County, Zhejiang, China) was carried out in line with our prior study, albeit with some suitable adjustments.<sup>25</sup> Chrysanthemum was decocted with ultrapure water (g : v = 1 : 10) for 30 min, and then filtered through a gauze after cooling. Next the residue was added to water and continued to be decocted for half hour, with the process repeated three times. Finally, the extract was concentrated by rotary evaporation at 50 °C to 200 ml, part of which was lyophilized to chrysanthemum aqueous extract (CM) and the other part was used for isolation and purification.

A modified published protocol<sup>26,27</sup> used AB-8 macroporous resin to purify the aqueous extract of chrysanthemum, using 95% ethanol for rinsing 2 column volumes and concentrating the chrysanthemum extract and adsorbing from the AB-8 macroporous resin. After 3.5 hours, 3 column volumes were eluted with 60% ethanol to obtain an alcohol-containing total flavonoid eluate, which was freeze-dried to obtain the purified flavonoid extract (FL).

### HPLC analysis

Mixed standard solutions composed of cynaroside, luteolin, apigenin, acacetin, kaempferol and quercetin (Shanghai Yuanye Bio-Technology Co., Ltd), as well as CM and FL were subjected to a Shimadzu LC-20A high performance liquid chromatograph (HPLC). The mobile phases consisted of 0.1% phosphoric acid (A) and acetonitrile (B) with varying gradient concentrations: from 0 to 11 minutes, 90–82% A; from 11 to 35 minutes, 82% A; from 35 and 45 minutes, 82–80% A; from 45 to 50 minutes, 80–74% A; from 50 and 55 minutes, 74% A; from 55 to 60 minutes, 74–70% A; from 60 to 65 minutes, 70% A; between 65 and 70 minutes, 70–67% A; from 70 to 75 minutes, 67–90% A; from 75 to 80 minutes, 90% A; and from 80 to 85 minutes, 10% A (Fig. 1).

**Fig. 1**



HPLC chromatogram print of the mixed standard solutions – CM and FL. 1, cynaroside, 2, luteolin, 3, apigenin, 4, quercetin, 5, acacetin, and 6, kaempferol.

## Measurement of total antioxidant capacity

FRAP and ABTS were used to calculate the total antioxidant capacity in accordance with the procedure outlined in the kit instructions (Nanjing Jiancheng Biotechnology Research Institute Co., Ltd).

## Animal experiments

Fifty 8-week-old male C57BL/6 mice [SYXK (Zhejiang) 2022-0004] were procured from Hangzhou Ziyuan Experimental Animal Technology Co., Ltd (Hangzhou, China). All experimental procedures were conducted under the approval number ([2023] 001) of the Animal Ethics Committee of China Jiliang University. They were housed in a standard animal facility at a temperature of  $23 \pm 2$  °C, under 12 hour alternating light and dark conditions, and at a relative humidity of  $50 \pm 20\%$ . After acclimatization, mice were randomized into 5 groups. The normal control (NC) group was fed a maintenance diet (Jiangsu Xietong Biological Engineering Co., Ltd), while the remaining mice were assigned to an HFD regimen (D12492, Jiangsu Xietong Biological Engineering Co., Ltd) in the absence or presence of the supplement. CM received chrysanthemum aqueous extract ( $200 \text{ mg kg}^{-1}$ ), FLL received the total flavonoid extract at a low dose ( $150 \text{ mg kg}^{-1}$ ), and FLH received the total flavonoid extract at a high dose ( $200 \text{ mg kg}^{-1}$ ). The chosen dose was determined in accordance with earlier research on CM,<sup>28,29</sup> FLL and FLH.<sup>30</sup> The dose of CM and FL was approximately 16.2 mg per kilogram of body weight in humans, or 0.97 g of CM and FL for a person weighing 60 kg. With an average yield of 13.8% for CM and 12.9% for FL, this corresponds to a daily consumption of 7–7.5 g of chrysanthemum, which is within the range of 5–10 g recommended by the Chinese Pharmacopoeia.

## Oral glucose tolerance test (OGTT)

The mice underwent a 12-hour fasting period, followed by gavage with a 20% glucose solution ( $2 \text{ g kg}^{-1}$ ). Subsequently, glucose levels were measured at 0, 15, 30, 60, and 120 minutes using a second drop of blood from the tail vein. Glucose tolerance curves were plotted and the area under the curve (AUC) was determined.

## Measurement of serum and liver biochemical indices

After a 9-week feeding regimen and following a 12 hour fast, the mice were euthanized by subjecting to inhalation of carbon dioxide. Blood samples were collected from mice *via* the orbital route and centrifuged. Serum levels of

triglycerides (TGs), total cholesterol (TC), high-density lipoprotein cholesterol (HDL-C), low-density lipoprotein cholesterol (LDL-C), albumin aminotransferase (ALT) and albumin aminotransferase (AST) as well as the liver levels of TC and TGs were measured following the kit instructions (Nanjing Jiancheng Biotechnology Research Institute Co., Ltd). Moreover, ELISA (Shanghai Enzyme-linked Biotechnology Co., Ltd) was used for the determination of serum TNF- $\alpha$ , IL-6, IL-1 $\beta$ , diamine oxidase (DAO) and LPS levels.

### Histopathological staining

Liver and ileal tissues were collected and promptly immersed in a 4% paraformaldehyde fixative, and then embedded within paraffin, sectioned and deparaffinized. The liver was stained with hematoxylin–eosin (H&E).<sup>31</sup> Additionally, oil red O staining was also used on the liver tissue cryosections.<sup>32</sup> The ileum was stained with the H&E stain to visualize the sectioned tissues under a microscope.

### Immunohistochemistry of colon tissue

Paraffin-embedded sections of colon tissue were prepared using the method of a previous study.<sup>33</sup> After deparaffinizing, the sections were placed in 3% H<sub>2</sub>O<sub>2</sub>, incubated in a light-free setting at ambient temperature for a duration of 25 minutes, and 3% BSA was added for blocking. Then, primary antibodies for ZO-1 or occludin were added to the sections for overnight incubation followed by 1 hour of incubation with secondary antibodies at ambient temperature. Subsequently, a DAB chromogenic development solution was added. Hematoxylin was used for counterstaining. The sections were washed in water, dehydrated, embedded in xylene, and photographed under a microscope.

### Real-time PCR

The total RNA from the liver and ileum was extracted using the TRIzol reagent and transformed into single-stranded cDNA *via* the SuperScript First-Strand Synthesis System (Yeasen Biotechnology (Shanghai) Co., Ltd, Shanghai, China). RT-PCR was performed using the Blue qPCR SYBR Master Mix. The sequences of primer pairs are provided in ESI Table 1.<sup>†</sup> Among the endogenous reference genes was 3-phosphoglyceraldehyde dehydrogenase (GAPDH).

### Western blotting

Total protein was extracted from the liver by employing RIPA buffer, which was supplemented with a blend of protease inhibitors (Thermo Pierce, 78445). Sample (60  $\mu$ g) aliquots were subjected to SDS-PAGE and subsequently blotted onto PVDF membranes. These membranes were incubated in TBST buffer with 5% bovine serum albumin (BSA) for blocking purposes before being exposed to the primary antibodies, including TLR4 (Thermo Fisher 48-2300, 1:100), MyD88 (Abcam ab219413, 1:1000), TRAF6 (Abcam ab40675, 1:5000), p-NF- $\kappa$ B and NF- $\kappa$ B (CST 3033, 1:1000), and GAPDH (Abcam ab181602, 1:10000), for overnight incubation at 4 °C. After washing in TBST membranes, the samples were incubated in a secondary antibody for one hour at ambient temperature. After washing, the membranes were incubated with ECL (Thermo Pierce, 34075) at ambient temperature for 1 min. Specific protein expression levels were normalised to GAPDH.

### 16S rDNA gene sequences

The colons of the mice were dissected to collect their contents, and fecal samples were dispatched to Mingke Biotechnology (Hangzhou) Co., Ltd for DNA extraction (TransGen AP221-02) and 16S rRNA-based high-throughput sequencing. Sequences were analyzed for taxonomic annotation of distinctive sequences and microbial diversity using USEARCH (version 10.0).

### Statistical analysis

The data were displayed as mean  $\pm$  standard deviation. The body weight, blood glucose, serum biochemical indicators, q-PCR, WB, and 16S rRNA data were analyzed using one-way ANOVA with Duncan's test. HPLC and antioxidant capacity levels were analyzed using paired Student's *t*-test, utilizing SPSS 20.0 software and the plotting with Origin2024. Values represented by distinct letters indicate statistically significant differences ( $p < 0.05$ ).

## Results

Analysis of flavonoid compounds and antioxidant capacity in CM and FL

HPLC analysis showed six flavonoids in CM and FL, with cynaroside, luteolin, and apigenin increasing significantly after purification (Table 1). Correspondingly, enhanced antioxidant capacity, as indicated by increased FRAP and ABTS levels, was demonstrated in FL *versus* CM (Table 2).

Table 1

Contents of the compounds in the chrysanthemum extract

| Component (µg per 100 mg) | CM               | FL                            |
|---------------------------|------------------|-------------------------------|
| Cynaroside                | 698.21 ± 17.72   | 864.46 ± 24.48 <sup>a</sup>   |
| Luteolin                  | 398.87 ± 56.57   | 759.03 ± 87.65 <sup>a</sup>   |
| Apigenin                  | 125.31 ± 20.60   | 385.97 ± 46.21 <sup>a</sup>   |
| Quercetin                 | 771.45 ± 64.62   | 657.22 ± 11.78 <sup>a</sup>   |
| Acacetin                  | 441.63 ± 25.67   | 442.44 ± 24.41                |
| Kaempferol                | 425.87 ± 6.30    | 426.02 ± 17.00                |
| Total content             | 2861.34 ± 191.48 | 3535.14 ± 211.53 <sup>a</sup> |

Table Footnotes

<sup>a</sup>A significant variance in the mean values (Duncan's test, *p* < 0.05).

Table 2

Levels of the total antioxidant capacity in chrysanthemum

| Component                  | CM             | FL                          |
|----------------------------|----------------|-----------------------------|
| FRAP (mg g <sup>-1</sup> ) | 0.7723 ± 0.011 | 0.8759 ± 0.018 <sup>a</sup> |
| ABTS (mg g <sup>-1</sup> ) | 1.2305 ± 0.01  | 1.4402 ± 0.010 <sup>a</sup> |

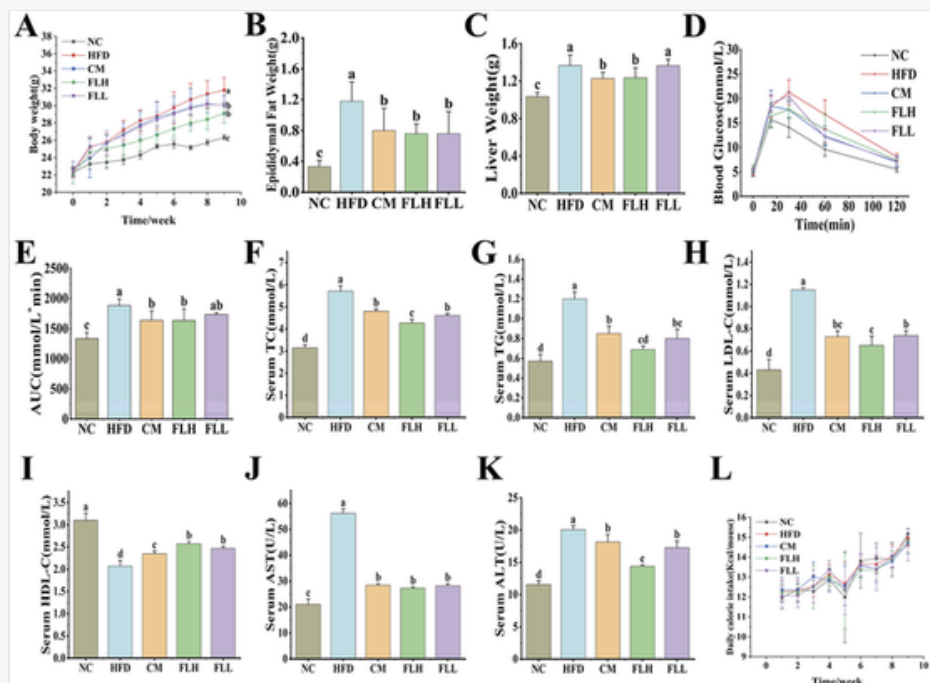
Table Footnotes

<sup>a</sup>A significant variance in the mean values (Duncan's test, *p* < 0.05).

Chrysanthemum relieves HFD-induced body weight increase, hyperglycemia and dyslipidemia

As shown in Fig. 2(A–C), the HFD significantly increased body weight, epididymal fat, and liver weight, while these indicators were significantly inhibited by CM and FL intervention (*p* < 0.05). The AUC for both the CM and FLH groups displayed a notable decrease compared with the HFD group (*p* < 0.05) (Fig. 2D and E).

Fig. 2

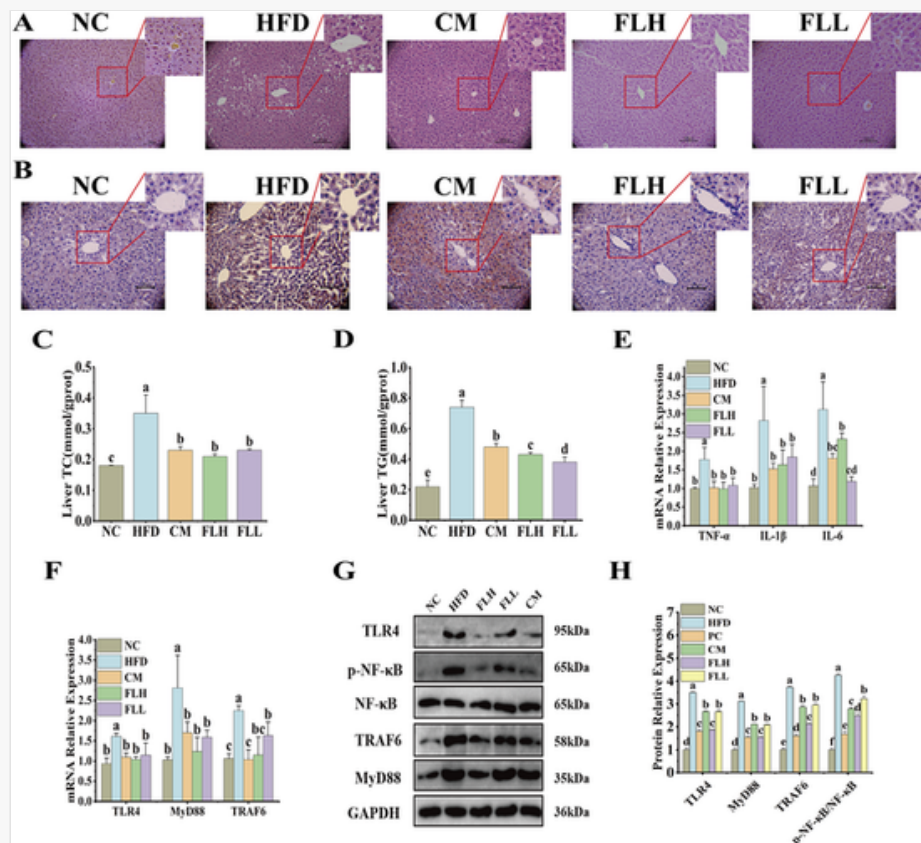


Chrysanthemum relieves HFD-induced obesity in mice. (A) Body weight changes in each group; (B) epididymal fat weight; (C) liver weight; (D) blood glucose was determined at 0, 15, 30, 60, and 120 min after glucose administration; (E) the AUC value (area under the curve) for the glucose tolerance test; (F) serum TC; (G) serum TG; (H) serum LDL-c; (I) serum HDL-c; (J) serum AST; (K) serum ALT; and (L) daily calorie intake. (a–e) Different letters indicate a significant difference in mean values (Duncan's test,  $P < 0.05$ ).

HFD significantly increased TG, TC and LDL-C levels, and reduced the HDL-C level ( $p < 0.05$ ), while CM, FLH and FLL treatments significantly reversed these parameters ( $p < 0.05$ ) (Fig. 2F–I). Importantly, FLH is superior to CM in the attenuation of HFD-induced changes in serum lipids. AST and ALT activities were significantly lower in the CM, FLL and FLH groups compared with the HFD group ( $p < 0.05$ ) (Fig. 2J and K), suggesting chrysanthemum's potential in exhibiting hepatoprotective effects. Daily calorie intake was not significantly different between the groups (Fig. 2L).

### Chrysanthemum alleviates HFD-induced liver fat buildup and inflammatory reactions

The liver lobule structures in the HFD group were disorganised, a large number of vacuoles gathered around the nuclei, and a minimal level of inflammatory cell infiltration was noted (Fig. 3A and B). However, the liver histological morphology in the CM, FLL and FLH groups was almost restored to the normal state, as indicated by the well-defined cells and few and small lipid droplets. Analysis of the liver lipid levels showed the TC (Fig. 3C) and TG (Fig. 3D) values, which were significantly lowered in the CM, FLL and FLH groups compared to the HFD group ( $p < 0.05$ ). Further, CM, FLL, and FLH treatments significantly downregulated the gene expression levels of TNF- $\alpha$ , IL-1 $\beta$ , and IL-6 compared with the HFD group (Fig. 3E) ( $p < 0.05$ ). Additionally, a significant reduction in TLR4, MyD88, and TRAF6 gene and protein expression levels was observed in CM, FLL or FLH-treated mice compared with the HFD group (Fig. 3F and H) ( $p < 0.05$ ), suggesting that these treatments could reverse the trends and lower the HFD-induced increase in the ratio of the TLR4/NF- $\kappa$ B signalling pathway (Fig. 3G and I). FLH was markedly superior to CM in the attenuation of HFD-induced variations in the TLR4/NF- $\kappa$ B signalling pathway.

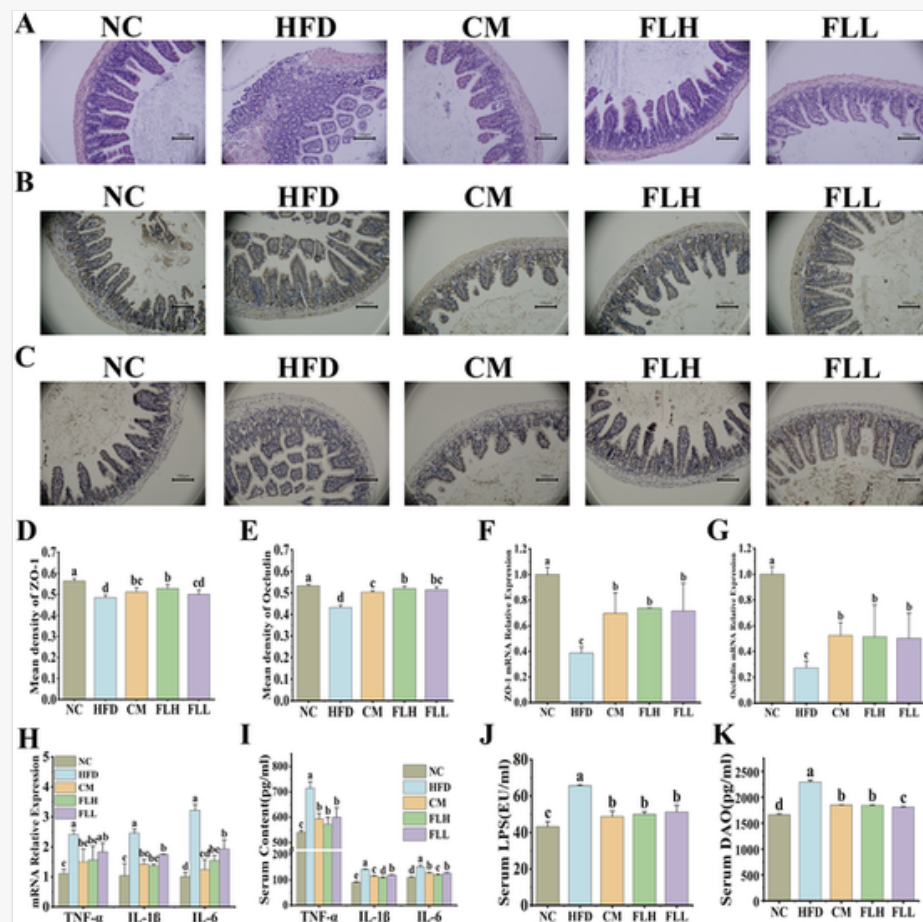


Chrysanthemum alleviates HFD-induced hepatic lipid accumulation and inflammatory responses. (A and B) H&E and Oil-Red-O staining of the livers, respectively ( $\times 200$ , scale bar = 100  $\mu$ m); (C and D) hepatic TC and TG, respectively; (E) mRNA relative expression of the inflammatory response genes in the mouse liver tissue; (F) mRNA relative expression of the inflammatory response genes in the TLR4 signalling pathway genes in the liver tissue; (G) western blot image; and (H) the expression levels of proteins associated with the TLR4 signalling pathway. (a–f) Different letters indicate a significant difference in mean values (Duncan's test,  $P < 0.05$ ).

## Chrysanthemum ameliorates HFD-induced intestinal damage

Examination of the intestinal tissue revealed that the HFD group had disordered edges of the villi, the apical part of the villi was detached, and the squamous epithelium presented inflammatory cell infiltration (Fig. 4A). However, the histological morphology of the ileal region in the CM, FLL, and FLH groups was similar to the NC group, with the ileal tissue structure intact and clear, and the villi were neatly arranged and did not show any pathological changes. The immunohistochemistry of the ileal tissue showed that ZO-1 and occludin protein expression levels were significantly reduced in the HFD group relative to the NC group (Fig. 4B–E), which was consistent with the trend of the corresponding gene transcript mRNA levels (Fig. 4F and G). In contrast to the HFD group, ZO-1 and occludin protein and gene expression levels significantly improved in the CM, FLL, and FLH groups ( $p < 0.05$ ) (Fig. 4B–G). TNF- $\alpha$ , IL-1 $\beta$ , and IL-6 (Fig. 4H) gene expression levels in the ileal tissues were significantly greater in the HFD group compared to the CM, FLL, and FLH groups ( $p < 0.05$ ) and remained consistent with the levels in the serum (Fig. 4I). Correspondingly, a significant inhibition of serum LPS and DAO levels was found in CM, FLL, or FLH-treated mice compared with those in the HFD group (Fig. 4J and K). Notably, FLH was superior to CM in alleviating the increases of serum inflammatory factors instigated by HFD.

Fig. 4

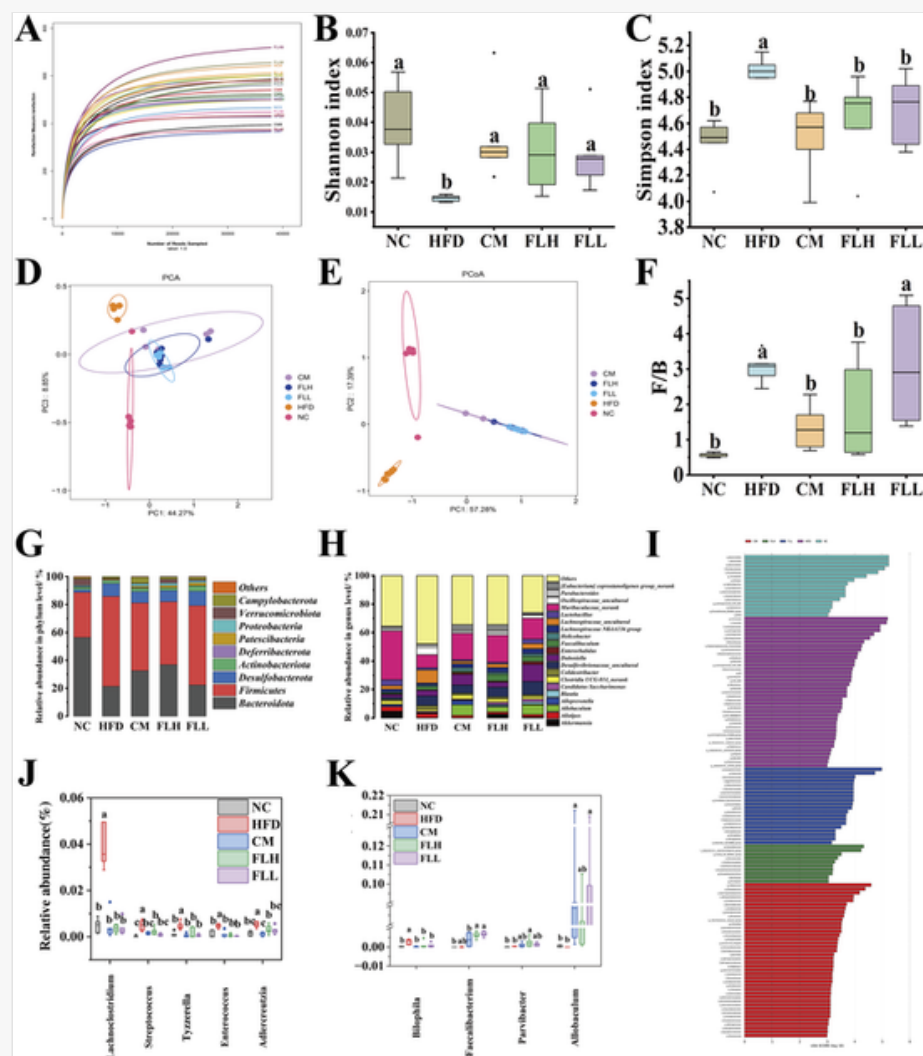


Chrysanthemum relieves HFD-induced intestinal damage. (A) Representative H&E staining of the ileum from the six groups ( $\times 200$ , scale bar =  $100\ \mu\text{m}$ ); (B and C) immunohistochemical analysis of ileum ZO-1 and occludin, respectively ( $\times 200$ , scale bar =  $100\ \mu\text{m}$ ); (D and E) mean optical density of ZO-1 and occludin, respectively; (F and G) mRNA relative expression of ZO-1 and occludin in the ileal tissue, respectively; (H and I) serum LPS and serum DAO; (J) serum TNF- $\alpha$ , IL-1 $\beta$  and IL-6; and (K) mRNA relative expression of TNF- $\alpha$ , IL-1 $\beta$  and IL-1 $\beta$  in the ileal tissue, respectively. (a-e) Different letters indicate a significant difference in mean values (Duncan's test,  $P < 0.05$ ).

## Chrysanthemum-adapted gut microbiota composition of HFD-fed mice

Plotting a curve of rare reads progressively increased and subsequently reached a stable plateau phase, indicating that the OTU coverage was adequate for precisely delineating the microbial makeup within each group (Fig. 5A). Analysis of alpha diversity showed that CM, FLL and FLH interventions could inhibit the HFD-induced decrease in the Shannon index and reduce the HFD-induced increase in the Simpson index ( $p < 0.05$ ) (Fig. 5B and C). Variance and cluster analysis of  $\beta$ -diversity using PCA and PCoA showed that PC1 accounted for 44.27% and PC3 accounted for 8.85% in PCA, and PC1 accounted for 57.28% and PC2 17.39% in PCoA, with clear divisions between NC and HFD (Fig. 5D and E). The microbiota profile gradually approached the NC group after the intervention of CM and FL.

Fig. 5



Chrysanthemum-adapted intestinal microbiota composition of the mice. (A) Sequencing depth; (B) Shannon's index; (C) Simpson's index; (D) PCA analysis; (E) PCoA analysis; (F) ratio of *Firmicutes* to *Bacteroidetes*; (G) relative abundance of bacterial groups at the phylum level; (H) relative abundance of bacterial groups at the genus level; (I) LefSe analysis; (J) relative abundance of the groups at the genus level; and (K) relative abundance of the groups at the genus level. (a–c) Different letters indicate a significant difference in mean values (Duncan's test,  $P < 0.05$ ).

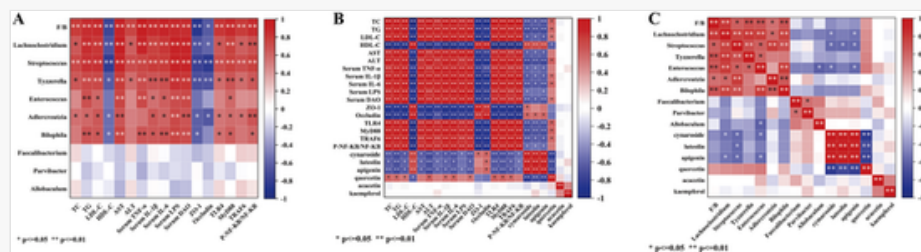
Analysis of bacterial taxonomic composition disparities across the groups was conducted using QIIME2, aiming to understand the fluctuations in microbial community abundance. At the phylum level, (Fig. 5F) the HFD had increased *Firmicutes* and lowered *Bacteroidetes* compared to the NC group. However, CM or FLH treatment could reverse the changes in the two phyla, resulting in a lower ratio of *Firmicutes*/*Bacteroidetes* (F/B) ( $p < 0.05$ ) (Fig. 5F and G).

Fig. 5H shows the species stacking histogram for bacterial relative abundance at the genus level, and LefSe [difference analysis (LDA score  $> 3$ )] based on the intestinal microbiota of each group of mice (Fig. 5I). According to the LefSe analysis, HFD significantly increased the relative abundance of *Lachnoclostridium*, *Streptococcus*, *Tyzzereella*, *Enterococcus*, *Adlercreutzia*, and *Bilophila* compared with NC ( $p < 0.05$ ). Chrysanthemum and its flavonoids significantly decreased the abundance of these flora compared with HFD alone. The relative abundance of *Faecalibacterium*, *Parvibacter* and *Allobaculum* increased in the CM and FL treatment groups, but was not statistically significant ( $p > 0.05$ ).

## Correlation analysis

To assess the connections between obesity and the gut microbiota, Pearson's correlation analysis was performed for determining significant differences in the genus level gut microbiota, serum biochemical and inflammation factors, gut barrier indices, hepatoprotection-related indicators and flavonoid extracts (Fig. 6). The results showed that cynaroside, luteolin, apigenin and quercetin negatively associated with serum biochemical parameters and inflammatory factors and positively associated with the expression of intestinal barrier-related genes ( $p < 0.05$ ). Furthermore, cynaroside, luteolin, and apigenin negatively associated with *Lachnoclostridium*, *Streptococcus* and *Enterococcus* ( $p < 0.05$ ).

Fig. 6



Correlation of bacterial flora, flavonoids and biochemical indices induced by the HFD. (A) Bacterial flora and biochemical indices; (B) biochemical indices and flavonoids; and (C) bacterial flora and flavonoids – the color of the rectangle in the matrix shows the level of the correlation index (red indicates a positive correlation and blue indicates a negative correlation) \*( $P < 0.05$ ) and \*\*( $P < 0.01$ ).

## Discussion

Obesity arises when there is a discrepancy between the calories consumed and those burned through physical exercise and metabolic processes. The condition is marked by an overabundance of body fat, accompanied by systemic low level chronic inflammation.<sup>34</sup> An HFD leads to excessive caloric intake and thus induces obesity development.<sup>35</sup> Chrysanthemum has shown effective intervention against obesity and safety in experimental mouse models. As a daily dietary supplement, it has the potential to improve metabolic health.<sup>23,24</sup> Here, we found that chrysanthemum had significant ameliorative effects on obesity parameters by modulating intestinal barrier damage, inflammation and the gut microbiota.

Animal studies indicate that HFD-fed obese mice usually show signs of hyperlipidemia, increased weight gain, impaired glucose metabolism, and liver steatosis and injury.<sup>36</sup> In this study, we demonstrated that CM and FL were effective in mitigating the HFD-stimulated increase in body weight and epididymal fat weight, and ameliorated glucose metabolism disorders. Elevated levels of lipids are a typical symptom of metabolic disorders associated with obesity.<sup>37</sup> Chrysanthemum treatment mitigated the HFD-induced rise in TG, TC, and LDL-C levels and the reduction in the HDL-C level. The FLH treatment group showed superior results compared to the CM group, probably because of the high content of luteolin favoring the improvement of dyslipidemia.<sup>38</sup> This study showed that HFD caused liver damage with elevated serum AST and ALT activities, indicative of liver pathology.<sup>39</sup> However, the serum AST and ALT levels with CM or FL treatment were found to be reduced compared to the HFD group, indicating that chrysanthemum may possess the ability to enhance liver function to some extent.

During obesity development, excessive lipid accumulation will cause adipocytes to release pro-inflammatory factors and induce macrophage infiltration, resulting in systemic chronic inflammation.<sup>40</sup> The study found that HFD-induced elevation of serum inflammation responses could be attenuated by chrysanthemum treatment. Latest research indicates that mice with diet-induced obesity demonstrate mild inflammation in the intestines, potentially caused by an influx of immune cells and an increase in cytokines.<sup>41</sup> Cytokine-triggered breakdown of the intestinal epithelial tight junction barrier during pathophysiological conditions leads to immune system activation and tissue inflammation, playing a crucial role as a causative and/or contributory element in diverse intestinal and systemic disorders.<sup>42</sup> Intestinal inflammation along with impaired intestinal barrier function could be critical factors in the onset of liver damage and metabolic disturbances.<sup>43</sup> Here, the transcriptional levels of inflammatory factors were increased in the ileal tissues of the HFD group. Meanwhile, elevated serum levels of LPS and DAO, which serve as indicators of gut permeability,<sup>44</sup> were accompanied by reduced expression of intestinal ZO-1 and occludin, suggesting that HFD induces intestinal dysfunction. However, these indicators could be improved by CM and FL treatments, supporting the ability of chrysanthemum to relieve HFD-induced intestinal inflammation and intestinal barrier dysfunction. Furthermore, FL showed a greater effect than CM in reducing serum inflammatory factors, which could be credited to the elevated content of anti-inflammatory components such as coumarin glycosides, lignans and other flavonoids in FL.<sup>45,46</sup>

The liver serves as a vital site for the synthesis and breakdown of lipids in the body and for the occurrence of inflammatory responses.<sup>47</sup> LPSs, released from enteric microorganisms induced by chronic HFD, translocate across the

damaged intestinal barrier and travels to the liver through the portal vein, triggering an innate immune response.<sup>48</sup> An important pattern-recognition receptor, TLR4, detects LPSs and triggers the inflammatory response in NAFLD. Within the liver, two distinct TLR4 signal transduction pathways exist: the MyD88-dependent and independent pathways. Compelling evidence suggests that the MyD88-dependent pathway is predominantly activated in response to liver injury. In the context of steatohepatitis, MyD88 serves as a crucial link, bridging TLR4 with the downstream pro-inflammatory signalling cascade, including NF- $\kappa$ B. After NF- $\kappa$ B is activated, inflammatory cytokines are released. Therefore, targeting the TLR4-MyD88-NF- $\kappa$ B signaling axis for inhibition emerges as a potentially valuable therapeutic avenue to address inflammation stemming from NAFLD.<sup>49</sup> Here, we showed that CM and FL significantly inhibited HFD-induced increase in the expression levels of TLR4, Myd88 and TRAF6, and the ratio of p-NF- $\kappa$ B/NF- $\kappa$ B in hepatic tissues, indicating a reduction in the activation of TLR4/Myd88/NF- $\kappa$ B signalling by chrysanthemum.

Developing obesity and metabolic syndromes involves the gut microbiota.<sup>50</sup> Studies reveal a strong link between the F/B ratio and the development of gut inflammation and obesity.<sup>51</sup> The present study showed a significant increase in F/B levels in the HFD group in. In contrast, in the CM or FLH group of mice, the F/B values were significantly decreased. It was suggested that the incidence of obesity-related metabolic syndrome could be reduced by chrysanthemum and its flavonoids.<sup>52</sup> The so-called beneficial bacteria may aggravate hepatic lipid accumulation and steatosis as reported in recent studies.<sup>53</sup> Although an increase in the abundance of the acetic acid SCFA-producing *Lachnospirillum* has been shown to mitigate harmful effects of obesity,<sup>54</sup> some studies suggest that *Lachnospirillum* may contribute to obesity by negatively affecting cyclic levels of acetate and by participating in the biosynthesis of harmful lipid compounds.<sup>55</sup> Research suggests that high levels of *Streptococcus* can increase harmful substances in the blood (such as LPSs), which in turn triggers an inflammatory response.<sup>56</sup> High levels of *Tyzzarella* have an effect on endogenous metabolites such as fatty acyl and amino acid derivatives.<sup>57</sup> Studies have shown that *Adlercreutzia* and *Bilophila* positively correlated with total fat gain.<sup>58,59</sup> In this study, chrysanthemum and its flavonoids significant decreased HFD-induced *Lachnospirillum*, *Streptococcus*, *Tyzzarella*, *Adlercreutzia* and *Bilophila* relative abundance. Notably, the *Faecalibacterium*, *Parvibacter* and *Allobaculum* abundance was also significantly elevated by chrysanthemum and its flavonoids. *Faecalibacterium* and *Allobaculum* produce the SCFA – butyric acid, which in turn stimulates epithelial cell proliferation, plays an important role in improving intestinal barrier integrity, and has the potential to inhibit LPS release and thus manifest anti-obesity activity.<sup>60,61</sup>

A comparable inverse correlation was observed between *Parvibacter* abundance and serum and hepatic lipid levels in obese mice.<sup>62</sup> These study findings indicate that *Lachnospirillum*, *Streptococcus*, *Tyzzarella*, *Adlercreutzia* and *Bilophila* elevated flora exhibiting a positive correlation with serum lipids as well as inflammation. Conversely, CM and FL demonstrated the capacity to alleviate this change, suggesting that CM and FL may play a role in mitigating HFD-induced obesity by improving the composition of the intestinal flora in mice.

Polyphenols and flavonoids have been shown to play a role in regulating lipid metabolism.<sup>63–65</sup> Research indicates that cynaroside ameliorates LPS-induced inflammation and multi-organ injury.<sup>66</sup> The study showed that luteolin inhibits the development of adipose tissue and hepatic steatosis by suppressing lipogenesis and reducing inflammatory cytokines, while increasing biliary sterol excretion.<sup>67</sup> Furthermore, apigenin was observed to exert a restraining effect on gut dysbiosis and metabolic deterioration in mice with obesity.<sup>68</sup> The present study demonstrated a significant association between cynaroside, luteolin and apigenin and serum biochemical parameters and inflammatory factors. Furthermore, these compounds exhibited a positive correlation with the expression of intestinal barrier-related genes and the abundances of *Lachnospirillum*, *Streptococcus*, *Enterococcus*, indicating that chrysanthemum and flavonoids have the potential to ameliorate inflammation, intestinal barrier dysfunction, and microbial dysbiosis in obese mice *via* bioactive compounds such as cynaroside, luteolin and apigenin.

Although we uncovered a possible beneficial part of the gut flora in the modulation of obesity by chrysanthemum, for further confirmation, fecal transplantation combined with sterile mouse experiments will need to be carried out in the future.

## Conclusions

Chrysanthemum could inhibit body weight gain, alleviate dyslipidaemia, inhibit TLR4/MyD88/NF- $\kappa$ B inflammation signalling, and reshape the intestinal microbiota composition in mice, which ultimately lead to the improvement of obesity. Importantly, chrysanthemum flavonoid extracts after purification exhibited a stronger effect in lowering blood lipids and inflammation. Taken together, this research contributes to the study of dietary interventions to prevent and treat obesity and its related metabolic diseases.

## Abbreviations

|                 |                                                       |
|-----------------|-------------------------------------------------------|
| NC:             | Normal control                                        |
| HFD:            | High-fat diet                                         |
| CM:             | Chrysanthemum extract                                 |
| FL:             | Chrysanthemum flavonoid extract                       |
| OGTT:           | Oral glucose tolerance test                           |
| TC:             | Total cholesterol                                     |
| TG:             | Triglyceride                                          |
| LDL-C:          | Low-density lipoprotein cholesterol                   |
| HDL-C:          | High-density lipoprotein cholesterol                  |
| ALT:            | Alanine aminotransferase                              |
| AST:            | Aspartate aminotransferase                            |
| H&E:            | Hematoxylin and eosin                                 |
| FRAP:           | Ferric ion reducing antioxidant power                 |
| ABTS:           | Total antioxidant capacity assay kit with ABTS method |
| GAPDH:          | Glyceraldehyde-3-phosphate dehydrogenase              |
| ZO-1:           | Tight junction protein 1                              |
| TLR4:           | Toll-like receptor 4                                  |
| MyD88:          | Myeloid differentiation primary response gene 88      |
| TRAF6:          | TNF receptor-associated factor 6                      |
| TNF- $\alpha$ : | Tumor necrosis factor                                 |
| IL-6:           | Interleukin-6                                         |
| IL-1 $\beta$ :  | Interleukin-1 $\beta$                                 |
| LPS:            | Lipopolysaccharide                                    |
| DAO:            | Diamine oxidase                                       |
| BSA:            | Blood serum albumin                                   |
| DAB:            | 3,3'-Diaminobenzidine.                                |

## Author contributions

Yiwei Yuan: conceptualization, data curation, investigation, supervision, visualization, and writing – original draft. Yu Zhang: methodology and formal analysis. Zhenyu Wang: writing – review and editing and formal analysis. Mark Christian: writing – review and editing. Qianqian Xu: writing – review and editing and conceptualization. Ruili Pan: writing – review and editing, Chunli Hu: project administration. Peinan Zhu: resources. Jin Zhao: conceptualization, funding acquisition and project administration. Xiaojun Li: resources.

## Data availability

The data supporting this article have been included as part of the ESI.<sup>†</sup>

## Conflicts of interest

The authors declare that they have no known competing financial interests.

## Acknowledgements

This research was funded by the Science and Technology Research Project of Zhejiang Provincial Technology Special Commissioner Project (SKT20241106), the Jiande Agricultural and Social Development Plan Project (2024NS10), the Quzhou Science and Technology Research Competitive Project (2023k098), the Zhejiang Provincial Team Technology

Special Commissioner Project (2025-2029 Kaihua Tea Industry Upgrading), the Hangzhou Technology Special Commissioner Project (20231122t48 and 20231122t59), and the Zhejiang Province Key Research and Development Project (2020C02045). We appreciate the technical support provided by the Hangzhou Doctoral Innovation Station in Hangzhou Tongge Tang Herbal Slices Co., Ltd and extend our gratitude to Zhejiang Yifutang Tea Co., Ltd for supplying the Chrysanthemum materials and to Mingke Biotechnology (Hangzhou) Co., Ltd for their assistance in the analysis of 16S rDNA sequencing.

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