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Virofree Associates with the Modulation of Gut Microbiomes and Alleviation of DSS-Induced IBD Symptoms in Mice

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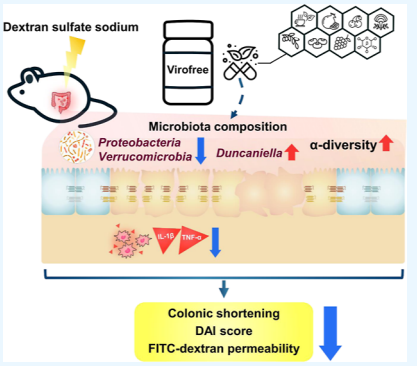
ABSTRACT: Inflammatory bowel disease (IBD) is a chronic, nonspecific inflammation of the intestines that primarily comprises Crohn's disease and ulcerative colitis. The incidence and prevalence of IBD have been increasing globally, highlighting the significance of research and prophylactic interventions. Virofree, a mixture of various botanical extracts (including grapes, cherries, olive leaves, marigolds, green tea, and others), has shown significant potential in disease prevention. This study examined the effects of Virofree on intestinal inflammation and the gut microbiota in mice using a dextran sulfate sodium (DSS)-induced model. The mice showed no adverse reactions when administered Virofree. Virofree administration reduced the disease activity index as indicated by amelioration of DSS-induced symptoms in the mice, including weight loss, diarrhea, and rectal bleeding. Regarding the gut microbiota, Virofree intervention modulated the DSS-induced decrease in gut microbial diversity; the Virofree group showed no increase in the phyla *Proteobacteria* or *Verrucomicrobia* while displaying an increase in the genus *Duncaniella*, bacteria that may have protective properties. These findings suggest that Virofree may have a direct or indirect impact on the composition of the gut microbiota and that it can alleviate the imbalance of the microbiome and intestinal inflammation caused by DSS treatment.



1. INTRODUCTION

Inflammatory bowel disease (IBD), including Crohn's disease and ulcerative colitis, is a chronic inflammatory disorder that affects the gastrointestinal system and progresses in an unpredictable and persistent manner. The worldwide prevalence of IBD has increased in recent decades. Due to the prolonged and progressive nature of IBD along with the high cost associated with its management, the rise in IBD cases represents a growing socioeconomic burden.[1,2] Historically, IBD has been considered a condition primarily affecting residents of the Western Hemisphere. However, research in the past decade has shown a rise in the incidence of IBD in emerging industrialized nations in Asia such as China and India, countries with populations exceeding one billion.[3] The etiology of IBD is multifactorial, involving genetic predisposition, compromised epithelial barriers, alterations in the gut microbiota, dysregulation of the immune system, and environmental factors.[4]

In response to environmental stress, plants produce polyphenols that comprise a diverse family of secondary metabolites known as phytoalexins.[5] This broad category includes flavonols, quercetin, catechins, daidzein, and stilbenoids, compounds that are widely present in various fruits, vegetables, and beverages and are an integral part of human nutrition. The increasing interest in dietary polyphenols and the production and consumption of polyphenol-rich foods has been driven by their health-promoting properties. Dietary polyphenols are recognized for their ability to regulate intracellular signaling pathways and prevent oxidative damage that has been implicated in the development of degenerative disorders and chronic inflammation. Additionally, polyphenols can modulate the gut microbiota, the intestinal immune response, and the production of molecular mediators of inflammation.[6−8] Numerous studies have suggested a correlation between dietary polyphenols and a reduction in the severity of symptoms associated with IBD.[9,10] Moreover, it is widely acknowledged that the intestinal microbiota plays crucial roles in maintaining intestinal homeostasis and in the pathogenesis of IBD. Alterations and disturbances of the gut microbiota have been associated with the initiation and progression of IBD. However, the classification of these alterations as primary or secondary as well as the elucidation of the underlying mechanisms remain to be determined.[11,12]

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Currently, the main pharmacological approaches for managing IBD include the use of azathioprine, glucocorticoids, 5-aminosalicylates, and cyclosporine. Unfortunately, long-term administration of these pharmaceutical agents can have significant adverse side effects, highlighting the importance of exploring alternative therapeutic approaches derived from natural resources and functional foods.[13,14] Notably, in vitro and in vivo models using isolated compounds have been employed to study the impact of specific polyphenols on gut inflammation.[15,16] However, this strategy has some inherent drawbacks, including the high cost of purchasing large quantities of purified compounds and the understanding that the food matrix and its interactions with other nutritional components can significantly influence the bioavailability and bioactivity of dietary polyphenols.[17,18] Therefore, the present research was designed to investigate whether Virofree, a supplement that consists of multiplant extracts, has the ability to modulate the composition of the microbiota and thereby alleviate the severity of colitis.

2. MATERIALS AND METHODS

2.1. Reagents and Chemicals. Virofree was supplied by Geninova Biotech Inc. and contains a combination of plant-derived compounds in specific proportions. Each 1000 mg of the supplement contains 250 mg of grape seed extract, 180 mg of Acerola cherry extract, 160 mg of olive leaf extract, 90 mg of marigold extract, 80 mg of green tea extract, 80 mg of pomegranate extract, 80 mg of yeast beta-glucan, and 80 mg of soya bean extract. These extracts are rich sources of active ingredients, such as quercetin, hesperidin, genistein, daidzein, and resveratrol. The colitis-inducing agent, dextran sulfate sodium (DSS) with a molecular weight ranging from 36,000 to 50,000 g per mole, was purchased from MP Biomedicals (Aurora, OH, USA). All other required reagents were obtained from Sigma-Aldrich unless otherwise stated.

2.2. Animal Experimental Design. DSS-induced colitis was stimulated in male Institute of Cancer Research (ICR) mice following previously described methods.[19,20] The mice were obtained from the BioLASCO Experimental Animal Center in Taipei, Taiwan. The study protocols were approved by the Institutional Animal Care and Use Committee of the National Taiwan University to ensure ethical compliance. The mice were housed under controlled environmental conditions at a temperature of 25 ± 1 °C, relative humidity of 50%, and a 12 h light−dark cycle. Following a 1 week adaptation period, the mice were randomly allocated among five groups (n = 12) as follows: a Control (Ctrl) group receiving a normal diet; a 0.6% Virofree group receiving a diet containing 0.6% Virofree; a DSS group receiving a normal diet along with a 2% DSS solution, and two experimental groups with DSS-induced colitis receiving diets containing either 0.2 or 0.6% Virofree. The application of DSS involved two cycles of a 2% DSS solution (MP Biomedicals, LLC, Illkirch, France), with a 7 day treatment period followed by a 14 day interval of deionized water only. The mice had ad libitum access to food and water. The sample diets were administered as a pretreatment 1 week prior to the commencement of the experiment. The dosage of Virofree was based on previous studies.[21] Throughout the experimental period, the mice were regularly assessed for body weight and the disease activity index (DAI) that was determined based on clinical manifestations including weight loss, stool consistency, and the presence of hematochezia. Figure 1A provides an overview of the animal protocol employed in this study. All mice were euthanized with CO_2 and dissected at the end of the study. Blood samples were obtained through cardiac puncture and centrifuged to separate the serum. The liver, kidneys, and spleen were promptly collected, weighed, and photographed. Fresh fecal pellets (approximately 100 mg per mouse) were aseptically collected in sterile tubes and rapidly frozen using liquid nitrogen. All samples were stored at −80 °C for subsequent analysis.

2.3. Intestinal Permeability In Vivo. Intestinal permeability was assessed by measuring the amount of FITC-dextran in the bloodstream following oral administration, as previously described.[22,23] In brief, each mouse was administered 400 mg/kg of FITC-dextran (molecular weight, 4 kDa; Sigma-Aldrich) via gavage. A blood sample obtained 2 h later was initially centrifuged (10,000g at 4 °C) for 10 min, and the serum was collected and placed into a 96-well microplate. The concentration of FITC-dextran was measured through spectrophotofluorometry at an excitation wavelength of 485 nm and an emission wavelength of 528 nm.

2.4. Biochemical Analysis. Blood samples were obtained from the left ventricle after anesthesia was administered at the end of the experiment. Subsequently, the samples were combined with 10 μL of heparin sodium and centrifuged at 1000 × g, 4 °C for 10 min. The resulting serum was then stored at −80 °C until further use. The levels of aspartate transaminase (AST) and alanine transaminase (ALT) were assessed at the National Laboratory Animal Center in Taipei, Taiwan, utilizing a 7080 Biochemical Analyzer (Hitachi, Tokyo, Japan), following the manufacturer's instructions.

2.5. Measurement of Proinflammatory Cytokines. Colonic homogenates were assayed to quantify the levels of pro-inflammatory cytokines, specifically TNF-α (cat. no. 88-7324) and IL-1β (cat. no. 88-7013) using a commercially available ELISA kit (Invitrogen, Waltham, MA, USA) and an ELISA reader (BioTek Instruments, Winooski, VT, USA) according to the manufacturer's protocol. Normalization of cytokine levels was performed relative to the colon tissue protein concentrations.

2.6. Histological Analyses. Colonic tissues were fixed in 10% buffered formalin for overnight fixation, dehydrated in a series of ethanol solutions, and prepared for embedding in paraffin. Sections measuring 4−5 μm in thickness were obtained, deparaffinized, rehydrated, stained with hematoxylin and eosin, and assessed using photomicroscopy.

2.7. Gut Microbiota Analysis. The gut microbiota analysis followed a previously described methodology.[22] Genomic DNA of gut bacteria was isolated and purified using an InnuPREP Stool DNA kit following the manufacturer's protocol, with minor modifications. The DNA samples were sent to Biotools Co. Ltd. for fecal microbial composition analysis using the 16S amplicon sequencing technique. PCR was employed to amplify the 16S rRNA gene from each DNA sample, targeting 10 conserved regions (V3−V4) and nine hypervariable regions (V1−V9). The obtained sequencing reads from the Illumina HiSeq2500 platform (250 bp) were clustered into amplicon sequence variants at a 97% identity threshold, representing taxonomic units corresponding to a bacterial species or genus. Microbial data analyses encompassing α- and β-diversity were conducted using Quantitative Insights into Microbial Ecology (version 1.9.1) software.

2.8. Statistical Analysis. The data were reported as the mean ± standard error of the mean (SEM). One-way ANOVA followed by Duncan's multiple comparison test was conducted to detect significant differences between groups at the accepted

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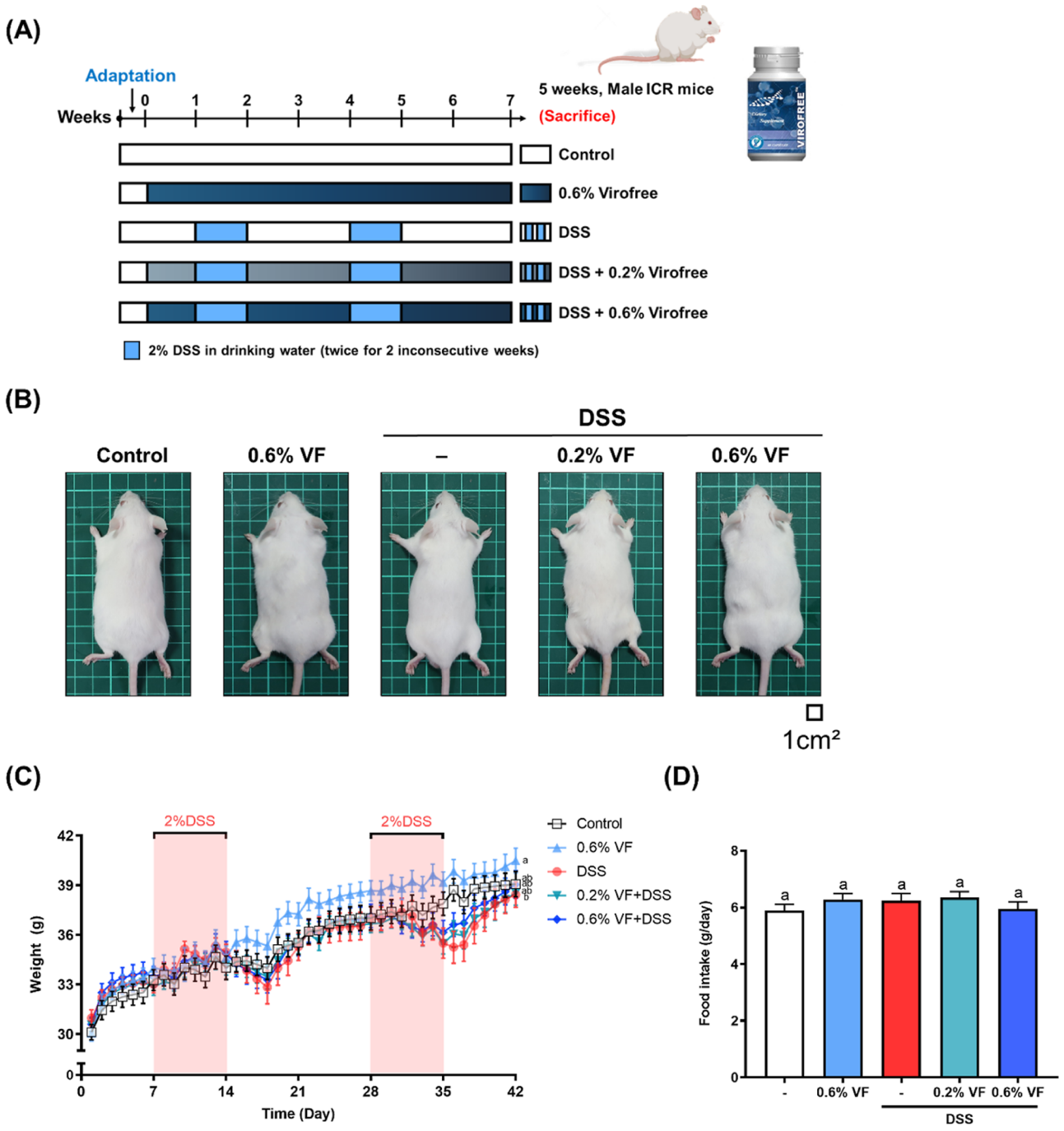

Figure 1. Effects of Virofree on the profile, body weight, and food intake of DSS-induced ICR mice. Mice were fed a normal diet with or without Virofree for 6 weeks. A 2% DSS solution was orally administered in deionized water for two cycles with a 2 week rest between cycles. The control group was given deionized water only. (A) Experimental procedure. (B) Representative photographs of each group of mice after sacrifice. (C) Body weight changes after intervention with 2% DSS in water. (D) Daily food intake. Each bar represents the mean ± SEM (n = 12). p-Values were determined through a one-way ANOVA using Duncan's multiple comparison test. Values labeled with different letters were significantly distinct ($p < 0.05$) among groups.

significance level of $p < 0.05$. Data points exceeding two times the standard deviation were excluded as outliers. Variation in sample sizes was observed due to the requirement of adequate tissue and serum for all experiments. There was a minimum of three specimens per group.

3. RESULTS

3.1. Effects of Virofree and DSS on the General Well-Being of Mice. The administration of Virofree was well tolerated by the ICR mice as they exhibited no discernible systemic toxicity or side effects, as indicated by metrics such as body weight, general appearance (Figure 1B–D), and organ histology (Figure 2A). Acute exposure to 2% DSS resulted in weight loss and hematochezia in the mice; however, these symptoms improved during the recovery periods, facilitated by the provision of tap water. By the 7 week mark, there were no statistically significant differences between the groups. The DSS group exhibited significantly elevated AST and ALT levels

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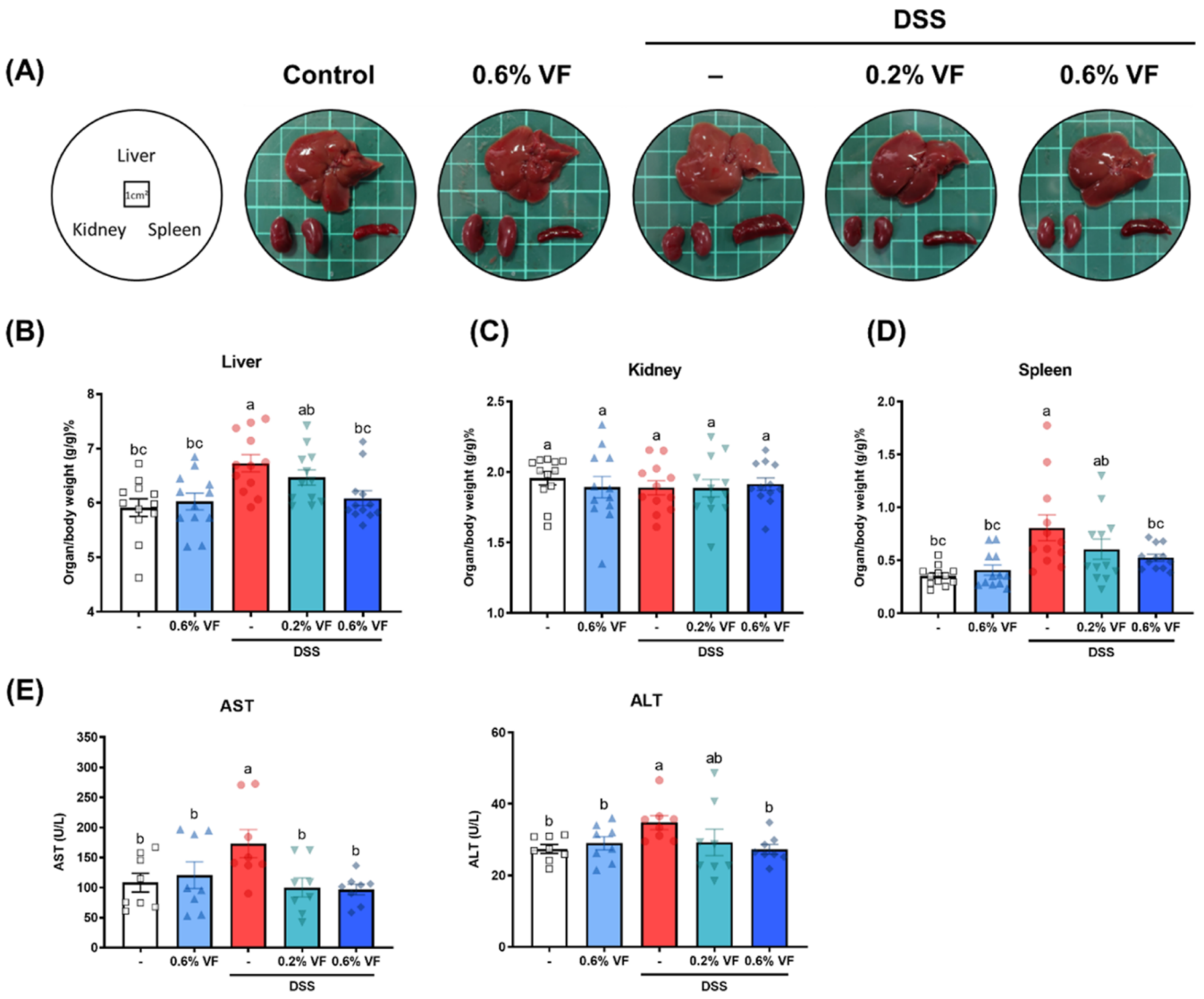

Figure 2. Effects of Virofree on the appearance of organs, weight, and serum biochemical parameters in DSS-induced mice. (A) Representative photographs of organs of mice in each group. The relative weights of the (B) liver, (C) kidney, and (D) spleen. (E) Liver function analyses were carried out with serum. Each bar represents the mean ± SEM (n = 8−12). p-Values were determined through a one-way ANOVA using Duncan's multiple comparison test. Values labeled with different letters were significantly distinct ($p < 0.05$) among groups.

compared to the other groups. Furthermore, the liver weights were significantly reduced in the group treated with 0.6% Virofree ($p < 0.05$) compared to the DSS and control groups that did not receive Virofree. However, there were no significant differences in renal weights. Systemic inflammation was indicated by a notable increase in the spleen weight; this was substantially reduced in the Virofree-treated cohort (Figure 2B−E).

3.2. Virofree Ameliorated Chronic Colitis Induced by DSS. To examine the chemoprophylactic implications of Virofree on chronic colitis, we conducted a study involving mice with DSS-induced chronic colitis. Pronounced inflammation induced by DSS in experimental subjects is typically accompanied by a significant decrease in body weight. The results (Figure 3A) indicated that Virofree at doses of 0.2 and 0.6% effectively mitigated the decrease in body weight associated with DSS-induced inflammation. Additionally, we observed a significant reduction in the level of DAI, a clinical parameter that indicates the severity of colitis, in groups treated with the aforementioned concentrations of Virofree (Figure 3B,C). Moreover, Virofree at concentrations of 0.2 and 0.6% attenuated DSS-induced colon shortening (Figure 4A,B).

We evaluated intestinal permeability by orally administering FITC-labeled dextran, a large glucose polymer that remained undigested and unabsorbed by the control mice. Our findings suggested that DSS treatment significantly increased intestinal permeability, as indicated by elevated serum levels of FITC-labeled dextran in the DSS-induced group compared to the control group. However, serum levels of FITC-labeled dextran were significantly reduced in the 0.2 and 0.6% Virofree groups (Figure 4C). Histopathological evaluation of colon sections from DSS-treated mice revealed the loss of goblet cells, crypt distortion, and severe mucosal epithelial injury. The administration of Virofree at concentrations of 0.2 and 0.6% significantly ameliorated the DSS-induced epithelial damage (Figure 4D). These results demonstrated the efficacy of Virofree in alleviating DSS-induced physiological aberrations and symptoms in ICR mice.

3.3. Inhibitory Effect of Virofree on Pro-Inflammatory Responses in DSS-Induced Mice. During inflammation, the

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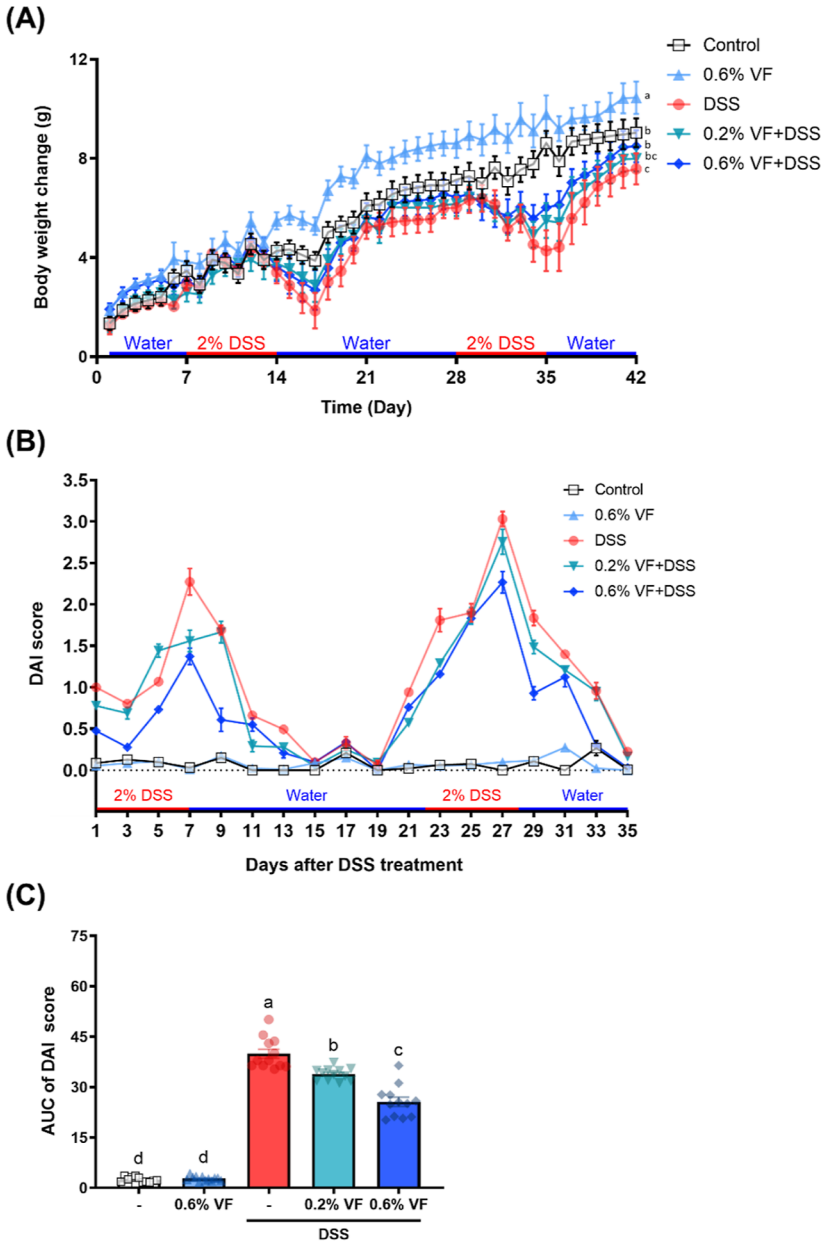

Figure 3. Effects of Virofree on DAI in DSS-induced ICR mice. (A) Body weight change, (B) DAI score, and (C) AUC of DAI score. Each bar represents the mean ± SEM ($n = 12$). p-Values were determined through a one-way ANOVA using Duncan's multiple comparison test. Values labeled with different letters were significantly distinct ($p < 0.05$) among groups.

increased secretion of pro-inflammatory cytokines triggers NF-κB activation, leading to structural changes in the intestinal barrier, increased gut permeability, and compromised mucosal barrier integrity. Considering the crucial role of pro-inflammatory cytokines in the development of colitis, an ELISA assay was conducted to determine their concentrations in colon homogenates from mice with induced colitis. As shown in Figure 5A,B, the colonic homogenates from the DSS group exhibited significant elevation in the levels of pro-inflammatory cytokines (TNF-α and IL-1β) compared to the control group. In contrast, a significant reduction was observed in the 0.6% Virofree treatment groups. Furthermore, spleen weight, which is often correlated with the severity of inflammation and anemia in colitis,[19] was significantly decreased in the 0.6% Virofree group, suggesting a potential Virofree-induced reduction in macrophage infiltration in the spleen (Figure 2D).

3.4. Virofree Affected the Composition of Gut Microbiota in Colitic Feces. Disturbed gut microbiota in patients with IBD can lead to a decrease in the gut microbiome diversity. Alpha diversity is used to describe the diversity of an ecological community. The Shannon and Simpson indices measure species evenness. The Menhinick and Margalef indices were for species richness. The diversity indices showed a significant reduction in the DSS group compared with the control group. However, with the intervention of DSS and Virofree, the Shannon, Simpson, and Menhinick indices exhibited upward trends with increasing dosage (Figure 6A–C). Conversely, when employing the Margalef species richness indices, while the trends in each group paralleled those of the evenness indices, notable differences in microbiota richness among the groups were absent, except for the 0.6% Virofree group and DSS group (Figure 6D).

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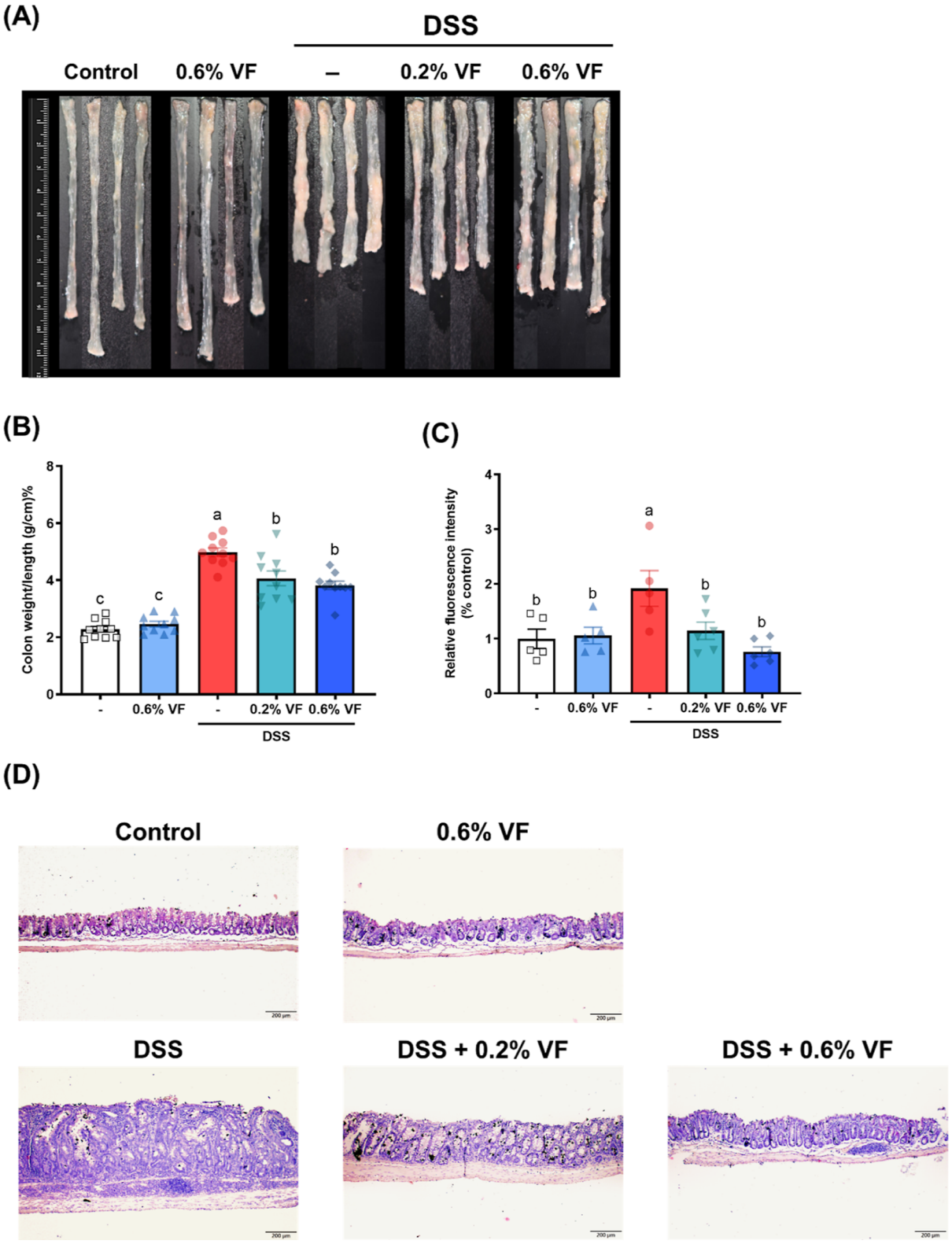

Figure 4. Effects of Virofree on colon appearances, weight/length ratio, intestinal permeability, and morphology in DSS-induced ICR mice. (A) Macroscopic views of the colon of each group. (B) Colon weight/length ratio in each group. (C) Gut permeability was assessed by measuring the levels of FITC-dextran in serum after oral gavage of 4 kDa FITC-dextran for 2 h. (D) Representative images of H&E staining of the colon tissues. (100× magnification, 200 μm) Each bar represents the mean ± SEM (n = 6−12). p-Values were determined through a one-way ANOVA using Duncan's multiple comparison test. Values labeled with different letters were significantly distinct ($p < 0.05$) among groups.

Beta diversity is a key concept explaining the relationship between local community assemblages; in other words, it helps us to determine the intersite differences between groups based on their microbial composition. Principal component analysis (PCA) is a dimension reduction method used for complex microbial data. By extracting the most significant microbial

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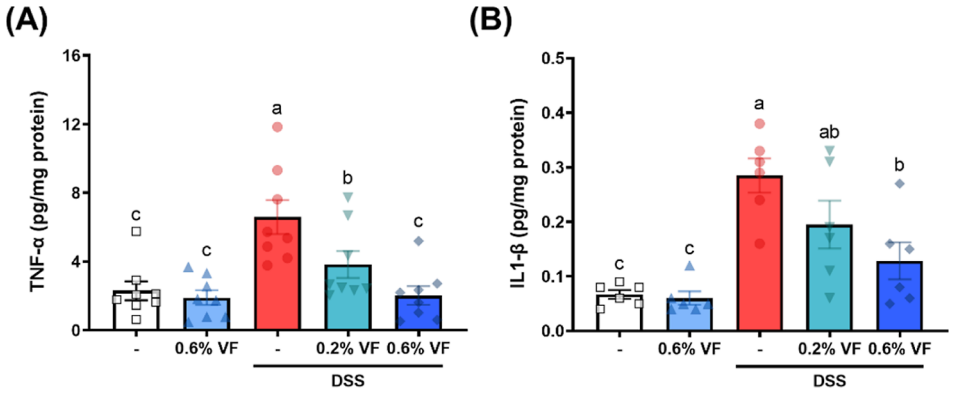

Figure 5. Effects of Virofree on the cytokine content in the colon tissues in DSS-induced ICR mice. The levels of (A) TNF-α and (B) IL-β in colorectal tissues. Each bar represents the mean ± SEM (n = 6−8). p-Values were determined through a one-way ANOVA using Duncan's multiple comparison test. Values labeled with different letters were significantly distinct ($p < 0.05$) among groups.

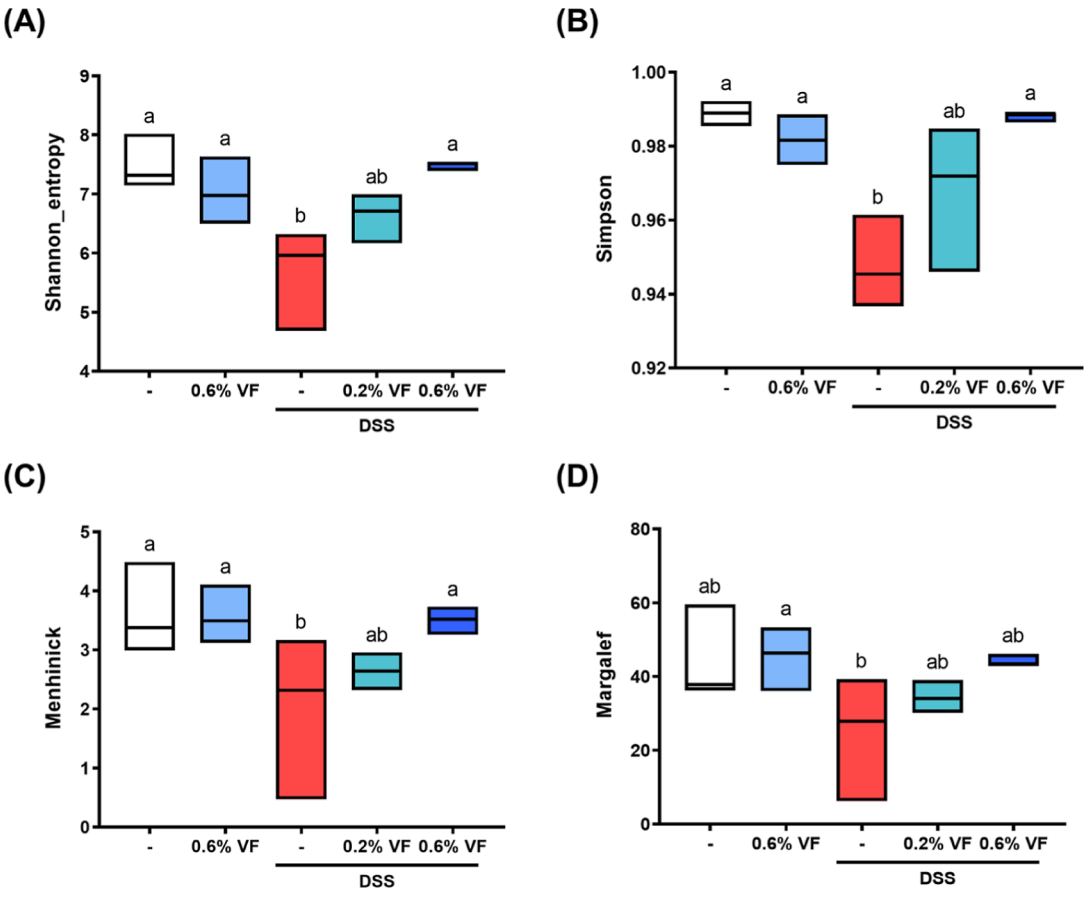

Figure 6. Effects of Virofree on alpha diversity indices of gut microbiota in DSS-induced ICR mice. (A) Shannon's diversity index, (B) Simpson's diversity index, (C) Menhinick's richness index, and (D) Margalef's richness index. Each box plot represents the median, interquartile range, minimum, and maximum values; n = 3 per group. p-Values were determined through a one-way ANOVA using Duncan's multiple comparison test. Values labeled with different letters were significantly distinct ($p < 0.05$) among groups.

features from each group, PCA can visualize results on a two-dimensional graph reflecting the differences between groups and the similarities within groups. As shown in Figure 7A, PC1 and PC2 accounted for 35.28 and 26.87% of the variation in intestinal microbial composition, respectively. The DSS group's community was significantly separated (the top right area in the figure), while the control group and the group treated with a high dose of Virofree alone overlap (the left side of the figure). Interestingly, the DSS-induced groups treated with low and high doses of Virofree overlapped (bottom of the figure). Constrained Ordination is an ordering method based on correspondence analysis that combines a multivariate regression analysis with environmental factors. It is also known as multivariate direct gradient analysis. Its main purposes are to reflect the relationship between species and environmental factors, to detect the relationship among environmental factors, samples, and bacteria, and to identify important environmental driving factors that affect the distribution of samples. In Figure 7B, the communities of the DSS-induced mouse groups are significantly separated in the top right area. The DSS-induced

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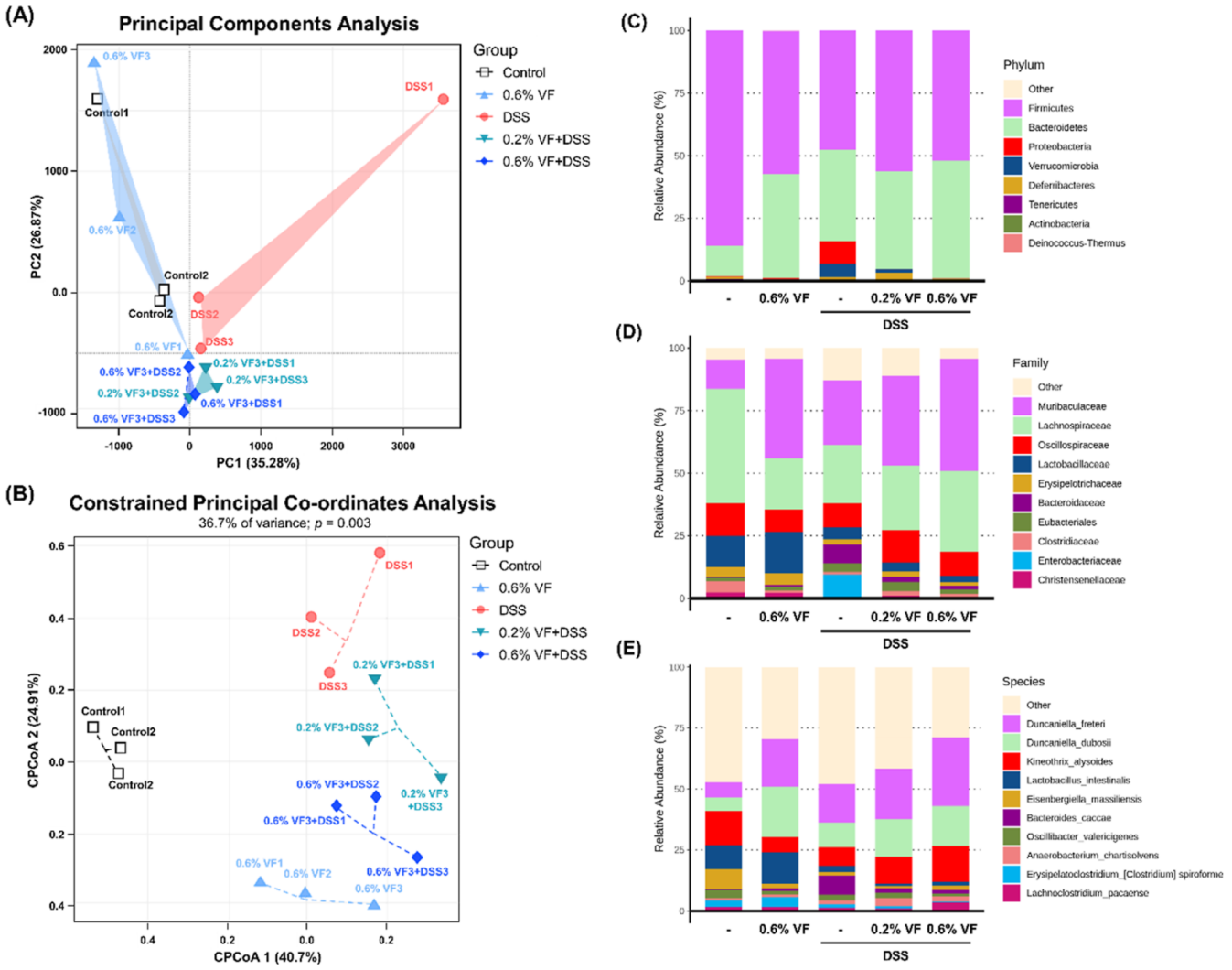

Figure 7. Effects of Virofree on the beta diversity of gut microbiota in DSS-induced ICR mice. Gut microbiota composition in feces was analyzed by 16S rRNA gene sequencing analyses (n = 3 for each group). The plots shown were generated using (A) PCA of the correlation matrix and (B) constrained PCoA.

groups treated with 0.2 and 0.6% Virofree gradually move closer to the bottom, where the group treated with 0.6% Virofree alone is located. This suggests that treatment with Virofree may have had a direct or indirect impact on the composition of the intestinal microbiota and ameliorated the imbalance in the intestinal microbiota caused by the DSS induction.

3.5. Virofree Regulated the Compositions of Gut Microbiota Compositions in Colitic Feces. The gut microbiota of mice is primarily composed of the phyla *Firmicutes* and *Bacteroidetes*, which typically account for up to 90% of the relative abundance of gut microbes under normal circumstances.[24] However, in this study, the DSS-induced group had an increased presence of the phyla *Proteobacteria* and *Verrucomicrobia*, whereas the DSS-induced groups treated with low and high doses of Virofree did not exhibit such increases in these phyla (Figure 7C). At the family level, the DSS group showed a higher proportion of *Enterobacteriaceae* and *Bacteroidaceae*, while the proportions of *Enterobacteriaceae* and *Bacteroidaceae* were decreased in the DSS-induced groups treated with low and high doses of Virofree, similar to those of the control group and the group given a high dose of Virofree alone (Figure 7D). The proportions of the species *Duncaniella_freteri* and *Duncaniella dubosii* were lowest in the control group but tended to increase in the groups treated with Virofree, as shown in Figure 7E. In addition, the proportion of *Bacteroides caccae* in the DSS-induced group was significantly higher than that in the other groups.

To explore the interrelationships among environmental factors, samples, and the microbial community, constrained ordination was used to identify the most influential environmental factors affecting the sample distribution. This method, based on canonical correspondence analysis, integrates correspondence analysis and multivariate regression analysis by including environmental factors in the regression analysis. Figure 8 shows that the control group and the group given a high dose of Virofree alone exhibited negative correlations with inflammatory factors and the degree of intestinal damage, while the DSS-induced group was more dispersed outward, indicating a higher correlation with intestinal ulcer factors. The relationship between the species *Duncaniella* and *B. caccae* was also more distributed in the lower right area, indicating a higher degree of correlation between these two species and gut inflammation.

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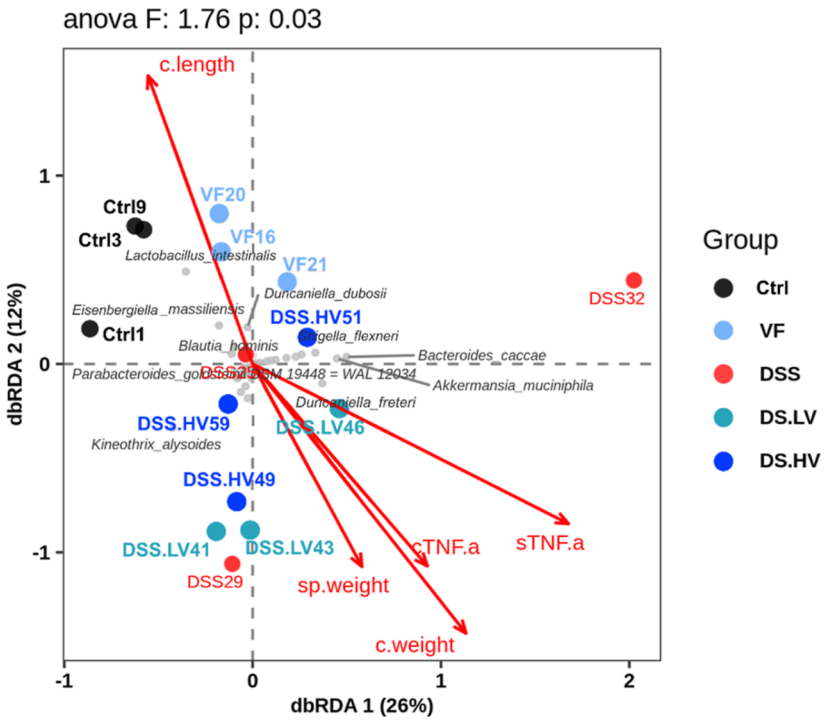

Figure 8. Correlation between gut microbiota and colitis parameters. Distance-based redundancy analysis was used to analyze the correlations at the species level. The lengths of the arrows in the diagram indicate the degree of impact of environmental factors on bacterial species. The angle between arrows represents the correlation between environmental factors, with acute angles indicating a positive correlation and obtuse angles indicating a negative correlation. Gray dots represent species, with the top 10 contributing species names displayed by default. Colored dots represent sample points labeled by the group.

4. DISCUSSION

IBD is a chronic inflammatory disorder of the intestinal tract and is the most important health-threatening disease in the world. However, the present treatment strategies for IBD are limited, with many side effects and associated poor prognosis.[25,26] Therefore, there is an urgent need to explore safe therapeutic approaches and additional effective strategies for managing IBD. Virofree is composed of quercetin, hesperidin, genistein, daidzein, and resveratrol, compounds that possess potent antioxidant and anti-inflammatory activities. Based on previously unreported clinical observations, the pharmacodynamic properties of Virofree include prevention and therapeutic interventions for influenza, adjunct treatment for radiotherapy/chemotherapy, reduction in the frequency of asthma attacks, anti-inflammatory effects, and facilitation of genotoxic repair.[21] Consuming a diet rich in various phytochemicals such as polyphenols, polymethoxyflavones, stilbenoids, and flavonoids can have beneficial effects that include modulation of the intestinal microbiota and prevention of various inflammatory diseases.[27−31] Moreover, there is increasing evidence concerning the beneficial effects of plant extracts in managing colitis.[32−34]

In this study, we evaluated the protective roles of Virofree against IBD and modulation of the role of the microbiota in DSS-induced colitis. Our results showed that dietary administration of Virofree for 7 weeks markedly attenuated colitis in DSS-treated mice (Figures 3 and 4). These results were consistent with a previous study that reported the combined effects of dietary anthocyanins, flavonols, and stilbenoids in mitigating IBD in mice.[35] Interestingly, an innovative discovery within the scope of our research suggests that oral administration of Virofree, which shows chemoprophylactic potential against colitis, may partly function by modulating the composition of the gastrointestinal microbiota and reducing the production of pro-inflammatory cytokines in the colonic tissues (Figures 5−7).

Gut microbiota dysbiosis can cause colon chronic inflammation due to factors such as loss of beneficial microbes, breakdown of the gut barrier, immune dysregulation, production of harmful substances, and alteration of metabolites.[36] Under normal circumstances, *Firmicutes* and *Bacteroidetes* constitute up to 90% of the relative abundance of human gut microbiota.[24] While the gut microbiota of humans and mice are not completely identical, any direct extrapolation to human health should be approached cautiously, and further research is necessary to determine if similar effects are observed in humans. However, it is worth noting that many foundational principles of mammalian biology, including aspects of the gut microbiota, are conserved between mice and humans.[37] Interestingly, in this study, the DSS-induced group had higher proportions of the phyla *Proteobacteria* and *Verrucomicrobia*. A recent study reported that as the dose and duration of DSS increased (0, 1, 2, and 3% for 12 consecutive days), the levels of the phyla *Proteobacteria* and *Verrucomicrobia* also increased.[38] However, in this study, the DSS-induced group treated with low and high doses of Virofree did not show such increases in the phyla *Proteobacteria* or *Verrucomicrobia* (Figure 7D). In this study, at the family level, the DSS group had a higher abundance of *Enterobacteriaceae* and *Bacteroidaceae*. Previous research studies have also observed a higher proportion of *Bacteroidaceae* in DSS-induced mice.[38,39] Additionally, despite the variation in clinical cases, patients with IBD commonly exhibit a decrease in the abundances of *Faecalibacterium*, *Clostridiales*, *Lactobacillus*, and *Bifidobacterium*, along with an increase in the abundances of

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Enterobacteriaceae and *Escherichia coli*.[40−42] In the present study, the proportions of *Enterobacteriaceae* and *Bacteroidaceae* in the DSS-induced group treated with low and high doses of Virofree were decreased, similar to the control group and the group given a high dose of Virofree alone (Figure 7E). At the species level, the proportions of *Duncaniella_freteri* and *Duncaniella dubosii* were the lowest in the control group, but there was a trend of an increase in the Virofree treatment groups (Figure 7F). Recent research has indicated that *Duncaniella* is a bacterium abundantly present in the mouse intestine and that it plays a protective role in DSS-induced colitis.[43,44] Furthermore, *B. caccae* in the DSS-induced group was notably higher than that in other groups. This species of bacterium is believed to be associated with immune responses related to IBD.[45] Recent studies have highlighted the potential protective role of genus *Akkermansia* against colitis. Specifically, *Akkermansia muciniphila*, a resident of the human gut, has been observed to have beneficial effects on the intestinal barrier function and inflammatory responses.[46,47] It is pertinent to mention that certain botanical extracts have shown to increase the abundance of this particular bacteria, fortifying the gut barrier and potentially ameliorating symptoms of IBD.[48,49] Although our study witnessed a rise in the *Duncaniella* genus, future research could delve into the potential synergies or complementary impacts of Virofree on other advantageous genera, such as *Akkermansia*.

A constrained ordination analysis was further conducted to investigate the interactions between environmental factors, samples, and bacterial communities and to identify the most significant environmental factors affecting the distribution of the samples. As shown in Figure 8, the control group and the group given a high dose of Virofree alone exhibited negative correlations with inflammatory factors and the degree of intestinal damage. These groups tended toward the upper left and upper areas of the ordination diagram, while the microbiota in the DSS-induced group was more dispersed in the middle and peripheral areas of the plot (bottom and right), showing higher correlations with intestinal ulcer factors. The relationship between the species *Duncaniella* and *B. caccae* was more prominent in the lower right of the ordination plot. In summary, these findings suggest that the administration of Virofree has the potential to improve gut microbiota. However, further rigorous experimentation is needed to determine whether Virofree directly or indirectly regulates the microbiome and characterize its impact on specific bacterial communities.

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Author Contributions

The study was designed by W.S. Lin, W.C. Cheng, and M.H. Pan. The study was conceived and the manuscript was written by W.S. Lin, W.C. Cheng, and M.H. Pan. W.C. Cheng conducted most of the experiments. All authors have reviewed and approved the final version of the manuscript. W.-S.L. and W.-C.C. contributed equally to this study and are cofirst authors.

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Notes

The authors declare no competing financial interest.

ABBREVIATIONS

ALT, alanine transaminase; AST, aspartate transaminase; dbRDA, distance-based redundancy analysis; DAI, disease activity index; DSS, dextran sulfate sodium; ELISA, enzyme-linked immunosorbent assay; FITC, fluorescein isothiocyanate-dextran; IBD, inflammatory bowel disease; IL-1β, interleukin-1β; PCA, principal components analysis; PCoA, principal coordinates analysis; PCR, polymerase chain reaction; SEM, standard error; TEER, *trans*-epithelial electrical resistance; TJs, tight junctions; TNF-α, tumor necrosis factor-α

REFERENCES

(1) Wright, E. K.; Ding, N. S.; Niewiadomski, O. Management of inflammatory bowel disease. *Med. J. Aust.* **2018**, *209* (7), 318−323.

(2) Xavier, R. J.; Podolsky, D. K. Unravelling the pathogenesis of inflammatory bowel disease. *Nature* **2007**, *448* (7152), 427−434.

(3) Song, E. M.; Yang, S. K. Natural history of inflammatory bowel disease: a comparison between the East and the West. *Intest. Res.* **2022**, *20* (4), 418−430.

(4) Ramos, G. P.; Papadakis, K. A. Mechanisms of disease: Inflammatory bowel diseases. *Mayo Clin. Proc.* **2019**, *94* (1), 155−165.

(5) Bhattacharya, A.; Sood, P.; Citovsky, V. The roles of plant phenolics in defence and communication during Agrobacterium and Rhizobium infection. *Mol. Plant Pathol.* **2010**, *11* (5), 705−719.

(6) Fraga, C. G.; Croft, K. D.; Kennedy, D. O.; Tomás-Barberán, F. A. The effects of polyphenols and other bioactives on human health. *Food Funct.* **2019**, *10* (2), 514−528.

(7) Cardona, F.; Andrés-Lacueva, C.; Tulipani, S.; Tinahones, F. J.; Queipo-Ortuño, M. I. Benefits of polyphenols on gut microbiota and implications in human health. *J. Nutr. Biochem.* **2013**, *24* (8), 1415−1422.

(8) Rana, A.; Samtiya, M.; Dhewa, T.; Mishra, V.; Aluko, R. E. Health benefits of polyphenols: A concise review. *J. Food Biochem.* **2022**, *46* (10), No. e14264.

(9) Shapiro, H.; Singer, P.; Halpern, Z.; Bruck, R. Polyphenols in the treatment of inflammatory bowel disease and acute pancreatitis. *Gut* **2007**, *56* (3), 426−436.

(10) Caban, M.; Lewandowska, U. Polyphenols and the potential mechanisms of their therapeutic benefits against inflammatory bowel diseases. *J. Funct.Foods* **2022**, *95*, 105181.

(11) Shan, Y.; Lee, M.; Chang, E. B. The gut microbiome and inflammatory bowel diseases. *Annu. Rev. Med.* **2022**, *73*, 455−468.

(12) Lee, M.; Chang, E. B. Inflammatory bowel diseases (IBD) and the microbiome-searching the crime scene for clues. *Gastroenterology* **2021**, *160* (2), 524−537.

(13) Mijan, M. A.; Lim, B. O. Diets, functional foods, and nutraceuticals as alternative therapies for inflammatory bowel disease: Present status and future trends. *World J. Gastroenterol.* **2018**, *24* (25), 2673−2685.

(14) Aslam, N.; Lo, S. W.; Sikafi, R.; Barnes, T.; Segal, J.; Smith, P. J.; Limdi, J. K. A review of the therapeutic management of ulcerative colitis. *Ther. Adv. Gastroenterol.* **2022**, *15*, 175628482211381.
(15) Martin, D. A.; Bolling, B. W. A review of the efficacy of dietary polyphenols in experimental models of inflammatory bowel diseases. *Food Funct.* **2015**, *6* (6), 1773−1786.
(16) Romier, B.; Schneider, Y. J.; Larondelle, Y.; During, A. Dietary polyphenols can modulate the intestinal inflammatory response. *Nutr. Rev.* **2009**, *67* (7), 363−378.
(17) Liu, R. H. Dietary bioactive compounds and their health implications. *J. Food Sci.* **2013**, *78*, A18−A25.
(18) Zhang, L.; Virgous, C.; Si, H. Synergistic anti-inflammatory effects and mechanisms of combined phytochemicals. *J. Nutr. Biochem.* **2019**, *69*, 19−30.
(19) Chassaing, B.; Aitken, J. D.; Malleshappa, M.; Vijay-Kumar, M. Dextran sulfate sodium (DSS)-induced colitis in mice. *Curr. Protoc. Im.* **2014**, *104*, 15.25.11−15.25.14.
(20) Hu, Q.; Yuan, B.; Wu, X.; Du, H.; Gu, M.; Han, Y.; Yang, W.; Song, M.; Xiao, H. Dietary Intake of Pleurotus eryngii Ameliorated Dextran-Sodium-Sulfate-Induced Colitis in Mice. *Mol. Nutr. Food Res.* **2019**, *63* (17), No. e1801265.
(21) Doan, L. H.; Chu, L. W.; Huang, Z. Y.; Nguyen, A. T.; Lee, C. Y.; Huang, C. L.; Chang, Y. F.; Hsieh, W. Y.; Nguyen, T. T. H.; Lin, C. H.; et al. Virofree, an herbal medicine-based formula, interrupts the viral infection of delta and omicron variants of SARS-CoV-2. *Front. Pharmacol* **2022**, *13*, 905197.
(22) Lin, W. S.; Chueh, T. L.; Nagabhushanam, K.; Ho, C. T.; Pan, M. H. Piceatannol and 3′-hydroxypterostilbene alleviate inflammatory bowel disease by maintaining intestinal epithelial integrity and regulating gut microbiota in mice. *J. Agric. Food Chem.* **2023**, *71* (4), 1994−2005.
(23) Zakostelska, Z.; Kverka, M.; Klimesova, K.; Rossmann, P.; Mrazek, J.; Kopecny, J.; Hornova, M.; Srutkova, D.; Hudcovic, T.; Ridl, J.; et al. Lysate of probiotic Lactobacillus casei DN-114 001 ameliorates colitis by strengthening the gut barrier function and changing the gut microenvironment. *PLoS One* **2011**, *6* (11), No. e27961.
(24) Magne, F.; Gotteland, M.; Gauthier, L.; Zazueta, A.; Pesoa, S.; Navarrete, P.; Balamurugan, R. The Firmicutes/Bacteroidetes ratio: A relevant marker of gut dysbiosis in obese patients? *Nutrients* **2020**, *12* (5), 1474.
(25) Guo, B. J.; Bian, Z. X.; Qiu, H. C.; Wang, Y. T.; Wang, Y. Biological and clinical implications of herbal medicine and natural products for the treatment of inflammatory bowel disease. *Ann. N.Y. Acad. Sci.* **2017**, *1401* (1), 37−48.
(26) Mao, E. J.; Hazlewood, G. S.; Kaplan, G. G.; Peyrin-Biroulet, L.; Ananthakrishnan, A. N. Systematic review with meta-analysis: comparative efficacy of immunosuppressants and biologics for reducing hospitalisation and surgery in Crohn's disease and ulcerative colitis. *Aliment. Pharmacol. Ther.* **2017**, *45* (1), 3−13.
(27) Dingeo, G.; Brito, A.; Samouda, H.; Iddir, M.; La Frano, M. R.; Bohn, T. Phytochemicals as modifiers of gut microbial communities. *Food Funct.* **2020**, *11* (10), 8444−8471.
(28) Carrera-Quintanar, L.; Roa, R. I. L.; Quintero-Fabián, S.; Sánchez-Sánchez, M. A.; Vizmanos, B.; Ortuño-Sahagun, D. Phytochemicals that influence gut microbiota as prophylactics and for the treatment of obesity and inflammatory diseases. *Mediat. Inflamm.* **2018**, *2018*, 9734845.
(29) Antoniussen, C. S.; Rasmussen, H. H.; Holst, M.; Lauridsen, C. Reducing disease activity of inflammatory bowel disease by consumption of plant-based foods and nutrients. *Front. Nutr.* **2021**, *8*, 733433.
(30) Dvorakova, M.; Landa, P. Anti-inflammatory activity of natural stilbenoids: A review. *Pharmacol. Res.* **2017**, *124*, 126−145.
(31) Maleki, S. J.; Crespo, J. F.; Cabanillas, B. Anti-inflammatory effects of flavonoids. *Food Chem.* **2019**, *299*, 125124.
(32) Farzaei, M. H.; Bahramsoltani, R.; Abdolghaffari, A. H.; Sodagari, H. R.; Esfahani, S. A.; Rezaei, N. A mechanistic review on plant-derived natural compounds as dietary supplements for prevention of inflammatory bowel disease. *Expet Rev. Gastroenterol. Hepatol.* **2016**, *10* (6), 745−758.
(33) Nascimento, R. d. P. d.; Machado, A.; Galvez, J.; Cazarin, C. B. B.; Maróstica Junior, M. R. Ulcerative colitis: Gut microbiota, immunopathogenesis and application of natural products in animal models. *Life Sci.* **2020**, *258*, 118129.
(34) Ng, S. C.; Lam, Y. T.; Tsoi, K. K.; Chan, F. K.; Sung, J. J.; Wu, J. C. Systematic review: the efficacy of herbal therapy in inflammatory bowel disease. *Aliment. Pharmacol. Ther.* **2013**, *38* (8), 854−863.
(35) Scarano, A.; Butelli, E.; De Santis, S.; Cavalcanti, E.; Hill, L.; De Angelis, M.; Giovinazzo, G.; Chieppa, M.; Martin, C.; Santino, A. Combined dietary anthocyanins, flavonols, and stilbenoids alleviate inflammatory bowel disease symptoms in mice. *Front. Nutr.* **2018**, *4*, 75.
(36) Yu, L. C. Microbiota dysbiosis and barrier dysfunction in inflammatory bowel disease and colorectal cancers: exploring a common ground hypothesis. *J. Biomed. Sci.* **2018**, *25* (1), 79.
(37) Nguyen, T. L.; Vieira-Silva, S.; Liston, A.; Raes, J. How informative is the mouse for human gut microbiota research? *Dis. Models Mech.* **2015**, *8* (1), 1−16.
(38) Park, H.; Yeo, S.; Kang, S.; Huh, C. S. Longitudinal microbiome analysis in a dextran sulfate sodium-induced colitis mouse model. *Microorganisms* **2021**, *9* (2), 370.
(39) Xu, H. M.; Huang, H. L.; Liu, Y. D.; Zhu, J. Q.; Zhou, Y. L.; Chen, H. T.; Xu, J.; Zhao, H. L.; Guo, X.; Shi, W.; et al. Selection strategy of dextran sulfate sodium-induced acute or chronic colitis mouse models based on gut microbial profile. *BMC Microbiol.* **2021**, *21* (1), 279.
(40) Pittayanon, R.; Lau, J. T.; Leontiadis, G. I.; Tse, F.; Yuan, Y.; Surette, M.; Moayyedi, P. Differences in gut microbiota in patients with vs without inflammatory bowel diseases: a systematic review. *Gastroenterology* **2020**, *158* (4), 930.
(41) Imhann, F.; Vila, A. V.; Bonder, M. J.; Fu, J.; Gevers, D.; Visschedijk, M. C.; Spekhorst, L. M.; Alberts, R.; Franke, L.; van Dullemen, H. M.; et al. Interplay of host genetics and gut microbiota underlying the onset and clinical presentation of inflammatory bowel disease. *Gut* **2018**, *67* (1), 108−119.
(42) Alam, M. T.; Amos, G. C. A.; Murphy, A. R. J.; Murch, S.; Wellington, E. M. H.; Arasaradnam, R. P. Microbial imbalance in inflammatory bowel disease patients at different taxonomic levels. *Gut Pathog.* **2020**, *12*, 1.
(43) Feng, P.; Li, Q.; Liu, L.; Wang, S.; Wu, Z.; Tao, Y.; Huang, P.; Wang, P. Crocetin prolongs recovery period of DSS-induced colitis via altering intestinal microbiome and increasing intestinal permeability. *Int. J. Mol. Sci.* **2022**, *23* (7), 3832.
(44) Chang, C. S.; Liao, Y. C.; Huang, C. T.; Lin, C. M.; Cheung, C. H. Y.; Ruan, J. W.; Yu, W. H.; Tsai, Y. T.; Lin, I. J.; Huang, C. H.; et al. Identification of a gut microbiota member that ameliorates DSS-induced colitis in intestinal barrier enhanced Dusp6-deficient mice. *Cell Rep.* **2021**, *37* (8), 110016.
(45) Hansen, J. J.; Huang, Y.; Peterson, D. A.; Goeser, L.; Fan, T. J.; Chang, E. B.; Sartor, R. B. The colitis-associated transcriptional profile of commensal Bacteroides thetaiotaomicron enhances adaptive immune responses to a bacterial antigen. *PLoS One* **2012**, *7* (8), No. e42645.
(46) Gu, Z. Y.; Pei, W. L.; Zhang, Y.; Zhu, J.; Li, L.; Zhang, Z. Akkermansia muciniphila in inflammatory bowel disease and colorectal cancer. *Chin. Med. J.* **2021**, *134* (23), 2841−2843.
(47) Bian, X.; Wu, W.; Yang, L.; Lv, L.; Wang, Q.; Li, Y.; Ye, J.; Fang, D.; Wu, J.; Jiang, X.; et al. Administration of Akkermansia muciniphila Ameliorates Dextran Sulfate Sodium-Induced Ulcerative Colitis in Mice. *Front. Microbiol.* **2019**, *10*, 2259.
(48) Zhu, M.; Song, Y.; Xu, Y.; Xu, H. Manipulating Microbiota in Inflammatory Bowel Disease Treatment: Clinical and Natural Product Interventions Explored. *Int. J. Mol. Sci.* **2023**, *24* (13), 11004.
(49) Mo, J.; Ni, J.; Zhang, M.; Xu, Y.; Li, Y.; Karim, N.; Chen, W. Mulberry Anthocyanins Ameliorate DSS-Induced Ulcerative Colitis by Improving Intestinal Barrier Function and Modulating Gut Microbiota. *Antioxidants* **2022**, *11* (9), 1674.

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