Research Article

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Rapeseed bee pollen alleviates chronic non-bacterial prostatitis *via* regulating gut microbiota

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Abstract

BACKGROUND: Rapeseed bee pollen has been recognized as a critical treatment for chronic non-bacterial prostatitis (CNP) and it also can modulate gut microbiota and improve gut health. This study aimed to explore the anti-prostatitis effects of rapeseed bee pollen with or without wall-disruption, and to investigate the connection between this treatment and gut microbiota.

RESULTS: The results reveal that rapeseed bee pollen can effectively alleviate chronic non-bacteria prostatitis by selectively regulating gut microbiota, with higher doses and wall-disrupted pollen showing greater efficacy. Treatment with a high dose of wall-disrupted rapeseed bee pollen (WDH, 1.26 g kg^{-1} body weight) reduced prostate wet weight and prostate index by approximately 32% and 36%, respectively, nearly the levels observed in the control group. Wall-disrupted rapeseed bee pollen treatment also reduced significantly ($p < 0.05$) the expression of proinflammatory cytokines (IL-6, IL-8, IL-1β, and TNF-α), as confirmed by immunofluorescence with laser scanning confocal microscope. Our results show that rapeseed bee pollen can inhibit pathogenic bacteria and enhance probiotics, particularly in the Firmicutes-to-Bacteroidetes (F/B) ratio and the abundance of *Prevotella* (genus).

CONCLUSION: This is the first study to investigate the alleviation of CNP with rapeseed bee pollen through gut microbiota. These results seem to provide better understanding for the development of rapeseed bee pollen as a complementary medicine.

Supporting information may be found in the online version of this article.

Keywords: chronic non-bacterial prostatitis; gut microbiota; rapeseed bee pollen; wall-disruption

INTRODUCTION

Over the past decade, studies of the gut microbiota have made significant progress in establishing an intricate connection with human health. The gut microbiota is a highly symbiotic community comprising around 100 trillion bacterial and archaeal cells within the human body.[1] A stable gut microbiota provides numerous health benefits, including reducing the prevalence of obesity, limiting harmful byproduct accumulation, and aiding in the recovery from inflammation.[2,3] Fecal microbiota transplantation (FMT) has emerged as a promising therapeutic strategy in clinical trials. In 25 studies involving 234 patients with ulcerative colitis, FMT has been shown to restore gut microbiota diversity, richness, similarity, and certain aspects of composition.[4] However, the imbalanced gut microbiota has been implicated in over 100 different diseases and disorders, including inflammatory bowel disease, colorectal cancer, obesity/metabolic syndrome, type 2 diabetes mellitus, breast cancer, urologic pelvic pain syndrome, and prostatitis.[5,6]

Prostatitis is one of the most common urinary tract diseases among men under 50, and it can significantly impact their quality of life. Patients often experience symptoms such as urinary frequency, urgency, difficulty with urination, and complications such as neurasthenia and sexual dysfunction.[7] Chronic non-bacterial prostatitis (CNP) is the most common prostatitis type, accounting for approximately 5–10% of cases in men under 50.[8] It has diverse causes, including inflammation, hormones, neurons, the prostate, and gut microbiota.[9] Previous studies have suggested that gut microbiota may be a promising therapeutic target for CNP patients. For instance, research on CNP patients revealed distinct alterations in the abundance and diversity of gut microbiota, including wider clustering of PCoA, decreased α-diversity, overrepresented taxa, and 12 under-represented taxa (e.g., *Prevotella*).[10] Research has also demonstrated that the gut microbiota of rats with chronic non-bacterial prostatitis is significantly different from that of healthy

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rats, with the former exhibiting a significant increase in harmful bacteria and a decrease in beneficial bacteria.[11]

Rapeseed bee pollen is the male gametophyte of the flowering rape plant collected by worker honeybees. It is a rich source of proteins, unsaturated fatty acids, vitamins, polysaccharides, phenolics, and flavonoid glycosides.[12] However, the complex cell-wall structure of bee pollen often poses challenges to efficient digestion and absorption. Our previous research findings indicate that the deliberate disruption of cell walls in rapeseed bee pollen leads to an obvious increase in the release of various nutrients, including amino acids, fatty acids, proteins, crude fats, reducing sugars, β-carotene, calcium, iron, zinc, and selenium.[12] Moreover, wall disruption can improve the digestion and absorption efficiencies of nutrients in rapeseed bee pollen.[13] Rapeseed bee pollen has been a nutritional food and traditional medicine for centuries. It has been shown to possess antioxidant, anti-tyrosinase, and anti-inflammatory properties,[14] and protects cells from abnormal melanogenesis.[15] Recent studies have shown increased interest in using rapeseed bee pollen or pollen extracts to combat and treat prostate disease in patients with CNP. For example, rapeseed bee pollen extracts have been found to ease CNP symptoms significantly by lowering mitofusin-1 (Mfn1) levels in the posterior lobes of the prostate.[16] In non-bacterial prostatitis rats, supercritical CO_2 extracts of rapeseed bee pollen could suppress the expression levels of DHT, 5α-reductase, and cyclooxygenase-2 (COX-2).[17] The occurrence of flavonoids in rapeseed bee pollen also conspicuously decreased the secretion of prostate-specific antigen in prostate cancer cells and performed anti-androgenic effects.[18] Moreover, clinical trials have demonstrated the effectiveness of Pule'an capsules, which contain rapeseed bee pollen, in relieving symptoms for 93.3% of patients with obstructive benign prostatic hyperplasia (BPH).[19] This drug has been regarded extensively as an ideal treatment for prostate patients with obstructive symptoms and CNP.[20] These findings reveal that rapeseed bee pollen or extracts can demonstrate reliable therapeutic effects on prostate disease. Studies suggest that rapeseed bee pollen and its extracts can modulate gut microbiota composition and improve gut health. For example, rapeseed bee pollen extract increased the abundance of beneficial bacteria, such as *Lactobacillus* and *Bifidobacterium*, in the guts of mice with induced colitis.[21,22] Rapeseed bee pollen extract was able to modulate the gut microbiota composition and improve the symptoms of type 2 diabetes in rats.[23] However, it has remained unclear whether wall-disrupted rapeseed bee pollen can better alleviate prostate disease via gut microbiota.

In this study, we aimed to investigate the therapeutic effects of wall-disrupted and not-disrupted rapeseed bee pollen on prostate disease using a CNP rat model. We evaluated the prostate index and histomorphometry of rat prostate tissues. In addition, inflammatory cytokines (IL-6, IL-8, IL-1β, and TNF-α) in prostate tissues and serum were determined using enzyme-linked immunosorbent assay (ELISA), and the expression levels of inflammatory cytokines in prostate tissues were further observed in confocal laser scanning microscope. Finally, gut microbiota compositions were analyzed through high-throughput sequencing of the V4 region of bacterial 16S-rDNA-capture.

MATERIALS AND METHODS

Materials

Avertin, 2-methyl-2-butanol, formaldehyde, absolute ethyl alcohol, xylene, formaldehyde, agarose gel, bovine serum albumin (BSA), and polybutadiene-styrene (PBS) were purchased from Sigma-Aldrich Chemical Company (St Louis, MO, USA); Phosphate buffer, phosphate-buffered saline (pH 7.4) and λ-carrageenan were purchased from Sinopharm Co., Ltd (Shanghai, China).

Cole's hematoxylin solution, eosin Y solution, ethylene diamine tetra acetic acid (EDTA) pH 8.0, anti-IL-6 rabbit pAb, rabbit anti-IL-8/CXCL8 antibody, anti-IL-1 beta mouse mAb, rabbit anti-TNF alpha antibody (bs-10802R), Cy3 conjugated goat anti-rabbit IgG (H + L), DAPI (4′,6-diamidino-2-phenylindole), anti-fade mounting medium were obtained from Abcam (Cambridge, MA, USA). Rat interleukin 6 (IL-6) ELISA kit, rat interleukin 8 (IL-8) ELISA kit, rat interleukin 1β (IL-1β) ELISA kit, and rat tumor necrosis factor α (TNF-α) ELISA kit were purchased from Abcam.

Rapeseed bee pollen samples

Rapeseed bee pollen samples (Supporting Information, Fig. S1) were collected from Xining apiaries (Qinghai province) established by the Modern Agro-industry Research System during the flowering season of 2020. The plant origin and purity were detected by DNA barcoding, with purity over 97%.[24] Fresh rapeseed bee pollen (1200 g) was freeze-dried in a vacuum (LGJ-18S, Songyuan Huaxing Technology Development Co., Ltd, Beijing, China) to a constant weight, and stored at −18 °C until use.

Wall-disruption treatment

The preparation of wall-disruption rapeseed bee pollen was carried out according to the reported method with slight modifications.[12] Five grams of rapeseed bee pollen was added to 50 mL of ultrapure water (Merck Millipore, Darmstadt, Germany) in a 100 mL beaker and stirred with a glass rod; then the suspensions were treated with a combination of ultrasonication and high-shear technique (US-HS, 25000 × g, 50 s). The intermittent ultrasound (1000 W, 80 Hz, 20 °C) further broke the pollen wall for 4 h (DCTZ-1000, Hongxianglong Biotechnology Co., Ltd, Beijing, China). Finally, the rapeseed bee pollen solution was freeze-dried in a vacuum (LGJ-18S, Songyuan Huaxing Technology Development Co., Ltd). Wall-disrupted rapeseed bee pollen was stored at −18 °C until use.

Animal model

A total of 48 male Sprague–Dawley (SD) rats (weighting 190–220 g) were purchased from Beijing Vital River Laboratory Animal Technology Co., Ltd. (Beijing, China) (licenses: 1100111911041531; animal use licenses: SYXK(JING) 2019–0041). All animals were kept under controlled conditions of 25 ± 2 °C and relative humidity of 55 ± 5%. The animals were kept in ambient conditions with free access to food and water in a 12 h dark–light cycle. The experimental Animal Welfare Committee of the Chinese Academy of Agricultural Sciences approved ethical requirements (permit number: IARCAAS-2019-003). Rats were randomly divided into six groups (n = 8 rats/group), including the control group, model group, low (NL) or high (NH) dose group of non-disruption, and low (WDL) or high (WDH) dose group of wall-disruption rapeseed pollen.

For the model rats, chronic non-bacteria prostatitis (CNP) was induced by the administration of λ-carrageenan according to the reported method with slight modification. λ-carrageenan powder was heat-sterilized and then mixed in sterile normal saline (1% w/v). Except for the control group, the sterile λ-carrageenan suspension (50 uL, 1% w/v) was injected into the right and left lobes of the ventral prostate. The same surgical procedures were followed for the control group. Instead of λ-carrageenan, 50 µL of sterile normal saline was injected into both the right and left lobes. For low-dose groups, 0.63 g kg^{-1} body weight (bw) was used as the

dosage for an adult rat. For the high-dose group, a twofold dosage (1.26 g kg^{-1} bw) was used. Body weights, prostate weights, and stool consistency were recorded daily for each animal. All rats were euthanized on the final day of the study (15th day). Prostate tissues were harvested and weighed instantly. Then the prostate index was calculated as the ratio of the prostate weight to the total body weight.

Histomorphometry of rat prostate

Prostate histomorphometry was performed using hematoxylin and eosin (H&E) staining, according to the previous method with minor modification.[16] Briefly, prostate specimens were stored in 10% formaldehyde and further mounted on paraffin blocks. Five-micrometer-thick sections were cut from the paraffin-embedded specimens using a Leica RM2255 Microtome (Leica Biosystems, Leica, Germany). Then, the tissue sections were deparaffinized using xylene, dehydrated in a gradient of alcohol solutions, and stained with H&E. Normal parameters of prostate histomorphometry were defined from the prostate biopsies of the normal control group. Glandular epithelium, glandular structure, and glandular space were observed using a Nikon eclipse Ci microscope with a Nikon digital sight DS-Fi2 system (Nikon, Tokyo, Japan).

Quantitative determination of inflammatory cytokines

The levels of inflammatory cytokines IL-6, IL-8, IL-1β, and TNF-α in serum and prostate tissue were detected by ELISA according to the manufacturer protocols. Each serum sample was collected in a serum separator tube (BD Biosciences, Rockville, MD, USA). The samples were allowed to clot for 2 h at room temperature. Then, the clots were removed by centrifugation at approximately 1000 × g for 20 min. Each prostate tissue sample was rinsed with ice-cold PBS (0.01 mol L^{-1}, pH = 7.4) to remove excess blood thoroughly. the prostate samples were weighed and homogenized in ice-cold PBS by an electric homogenizer. Finally, the homogenates were centrifuged for 5 min at 5000 × g to obtain the supernatants. All ELISA assays were performed according to the manufacturer's instructions. Each assay was repeated three times. Data were analyzed, and statistical significance was accepted at $P < 0.05$.

Immunofluorescence visualization of inflammatory cytokines

Immunofluorescence visualization of inflammatory cytokines in prostate tissue was performed using confocal laser scanning microscopy. Rat prostates were dissected and fixed in 10% buffered formalin for 24 h, then equilibrated and dehydrated in 30% sucrose/10 mM PBS overnight at 4 °C. The prostate tissues were embedded in OCT media for frozen sectioning. The 8 μm thick paraffin sections were deparaffinized with xylenes and rehydrated. Antigen retrieval was performed by microwave treatment (Galanz, Guangdong, China) for 10 min in EDTA buffer (pH 8.0). Slices were washed in phosphate-buffered saline (pH 7.4) and then incubated with 2% bovine serum albumin in phosphate-buffered saline for 30 min to block unspecific binding. Additional washes followed slices in phosphate-buffered saline. Finally, the primary antibody was added and incubated overnight at 4 °C. Slides were then washed with PBS and incubated with a secondary antibody at room temperature for 50 min. The nuclei were counter-stained with 4′,6-diamidino-2-phenylindole (DAPI) in the dark at room temperature for 10 min. For visualization, inflammatory cytokines scans were undertaken on a Nikon Eclipse Ti confocal laser scanning microscope with DS-U3 imaging system (Nikon, Tokyo, Japan). Immunofluorescence images were randomly taken for data analyses in CaseViewer 2.0 (Panoramic 250/MIDI, 3D Histech, Budapest, Hungary).

Analysis of gut microbiota in rectal feces

On the 15th day, all rats were euthanized. Whole rectal feces were harvested and stored immediately at −80 °C in liquid nitrogen. Then, the samples were thoroughly mashed with liquid nitrogen for DNA extraction. Total genome DNA from samples was extracted using CTAB/SDS kits (Beijing Novogene Biological Technology Co., Ltd, Beijing, China). All extracted DNA samples were performed for analysis of microbiota composition by (Beijing Novogene Biological Technology Co., Ltd). To put it simply, the variable regions V3-V4 of the bacteria 16S rDNA were amplified following the previous research.[25] Polymerase chain reaction (PCR) products were detected by operating electrophoresis on 2% agarose gel and then purified with Zymoclean gel DNA recovery kit (Zymo Research, Irvine, CA, USA), following the manufacturer's instructions. The Qubit 3.0 Fluorometer (Life Technologies, Carisbad, CA, USA) was used to detect the concentration of DNA samples accurately. Library preparation was performed using the NEBNext Ultra II DNA Library Prep kit (New England Biolabs E7370S/L, Ipswics, MA, USA). Finally, the library preparations were sequenced on an Illumina Hiseq 2500 platform, and 250 bp paired-end reads were generated.

Alpha diversity is an indication of diversity within a sample and was calculated using Mothur software (V. 1.35.0), including a species richness estimator (Chao 1) and diversity indices (the Shannon and Simpson indices). Beta diversity is an indication of differences in composition across samples and was calculated in QIIME (v.1.9.1). Sequences with more than 97% similarity were clustered into operational taxonomic units (OTUs) using the non-parametric ANOVA (Kruskal–Wallis rank sum test). All other statistical analyses between the model and pollen groups were performed with GraphPad Prism version 7.01 (GraphPad Software Inc., La Jolla, CA, USA). A P value < 0.05 was considered to be statistically significant.

RESULTS

Prostate wet weight and prostate index

Prostatitis can cause changes in the prostate's tissue structure, resulting in an increase in its wet weight. Measuring prostate wet weight can aid in the diagnosis of prostatitis and evaluation of its severity. The prostate index reflects the ratio of prostate gland size to body weight and is used to assess prostatic hyperplasia severity and treatment efficacy.[26] Table 1 shows the changes in prostate wet weight and prostate index among the control group, model group, and pollen groups. Our results indicated a significant increase in both prostate wet weight and prostate index in the model group, in comparison with the control group, indicating the occurrence of prostatitis by λ-carrageenan. The prostatic index and wet weight decreased significantly with the addition of bee pollen. Notably, the prostate wet weight ranged from 0.69 ± 0.07 g per rat in the control group to 0.47 ± 0.05 g per rat in the wall-disruption high dose (WDH) group, dropping approximately 32%, close to the control group. Moreover, the prostate index of the WDH group decreased by nearly 36%, compared with the model group. Overall, treatment with high-dose pollen and wall-disruption pollen can decrease the levels of prostate wet weight and prostate index.

Table 1. The prostate wet weight and prostate index of rats

Groups	Prostate wet weight (g)	Prostate index (%)
Control	0.42 ± 0.02[a]	1.09 ± 0.12[a]
Model	0.69 ± 0.07	2.31 ± 0.16
NL (non-disruption low dose)	0.56 ± 0.06[a]	1.68 ± 0.20[a]
NH (non-disruption high dose)	0.49 ± 0.03[a]	1.49 ± 0.12[a]
WDL (wall-disruption low dose)	0.52 ± 0.04[a]	1.57 ± 0.13[a]
WDH (wall-disruption high dose)	0.47 ± 0.05[a]	1.47 ± 0.15[a]

Note: Control, the normal rat group; Model, the introduced rat group of chronic non-bacteria prostatitis.
Abbreviations: NL, the model group with low dose non-disruption pollen; NH, the model group with high-dose non-disruption pollen; WDL, the model group with low dose wall-disruption pollen; WDH, the model group with high-dose wall-disruption pollen.

Prostate histomorphometry

Prostate histomorphology provides a comprehensive understanding of prostatitis lesions and serves as a basis for further treatment. Tissue samples are commonly stained using hematoxylin and eosin (H&E) prior to review by a pathologist. As shown in Fig. 1, pathological changes are evident in the prostate tissues of the different groups of rats. The control group's tissue sections (Fig. 1(A)) exhibited a normal prostate gland appearance, with clear lobules and intact glandular epithelium. In contrast, the model group's tissue sections (Fig. 1(B)) displayed a decrease in the number of prostate glands, and glandular cavity widening due to inflammation, along with inflammatory cell infiltration (black arrow), connective tissue hyperplasia (yellow arrow), and multiple necrotic foci of an eosinophilic filamentous network (red arrow). Interestingly, all pollen groups alleviated CNP based on histomorphological observations (Fig. 1(C)–(F)). After pollen treatment, the most significant changes observed in prostate histomorphology were the reduction of inflammatory cell infiltration and narrowed necrotic foci areas (Fig. 1(C)–(F)). The WDH group's prostate section (Fig. 1(F)) demonstrated a remarkable decrease in the number of inflammatory cell infiltration and necrotic foci and lacked prostatic hyperplasia, resembling normal tissue. Low-dose groups (Fig. 1(C),(E)) still slightly presented inflammatory cell infiltration (black arrow), connective tissue hyperplasia around the glands (yellow arrow), and glandular epithelium thinning (green arrow) in comparison with high-dose groups (Fig. 1(D),(F)). This suggests a dose-dependent effect of rapeseed bee pollen on prostate hyperplasia and inflammation.

Determination of inflammatory cytokines

Previous studies have suggested that prostate carcinoma patients with cachexia often have high levels of inflammatory cytokines, including TNF, IL-6, and IL-8.[27] The pathogenesis of CNP is closely related to the excessive and prolonged production of inflammatory cytokines such as IL-6, IL-8, IL-1β, and TNF-α.[28] Figure 2 shows the expression levels of these cytokines in the prostate tissue and blood serum of rats with CNP. The results indicate significant differences in the levels of these inflammatory factors in the different groups of rats. Specifically, the levels of IL-6, IL-8, IL-1β, and TNF-α were substantially lower in the WDH group compared to the model group, by almost 30%. Moreover, the wall-disruption

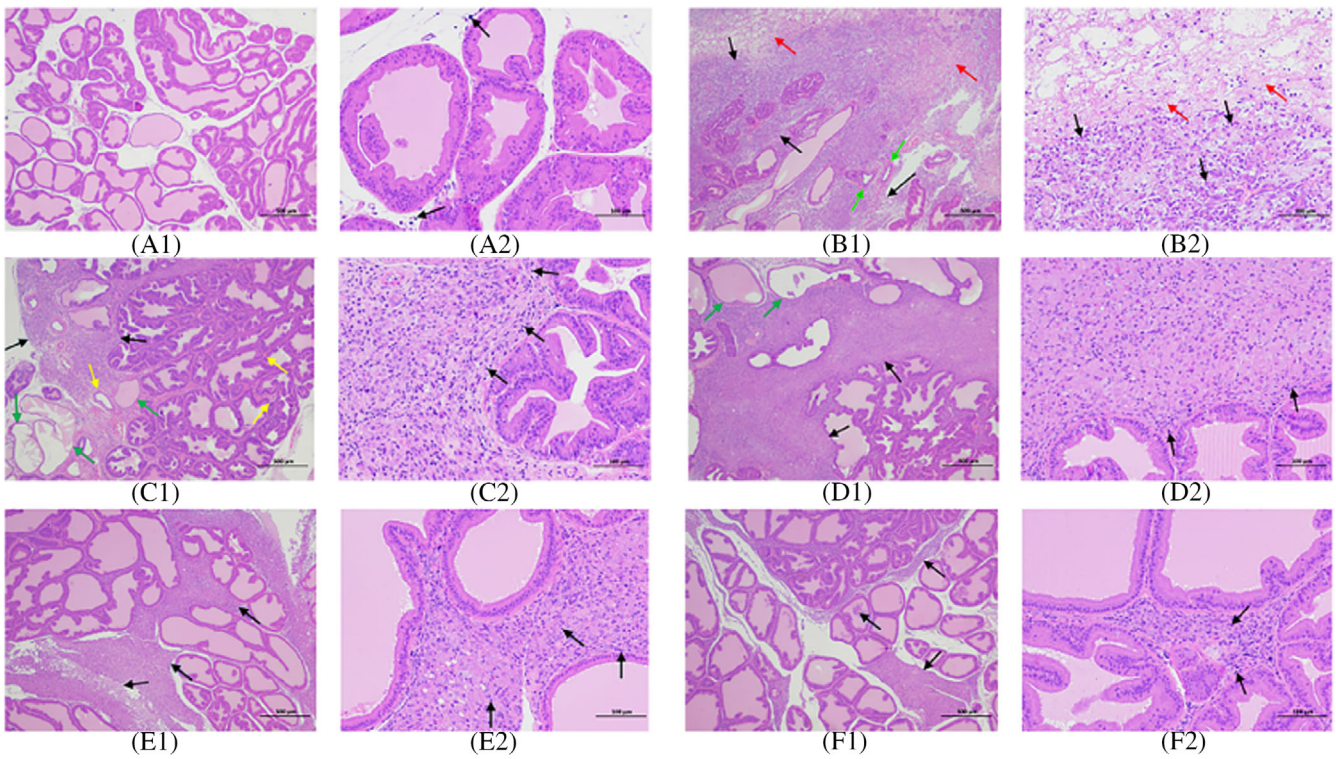

Figure 1. Pathological changes in prostate tissue in rats in each group. (A) Control group; (B) model group; (C) non-disruption low-dose rapeseed bee pollen group; (D) non-disruption high-dose rapeseed bee pollen group; (E) wall-disruption low-dose rapeseed bee pollen group; (F) wall-disruption high-dose rapeseed bee pollen group. 1, 40 times magnification view; 2, 200 times magnification view.

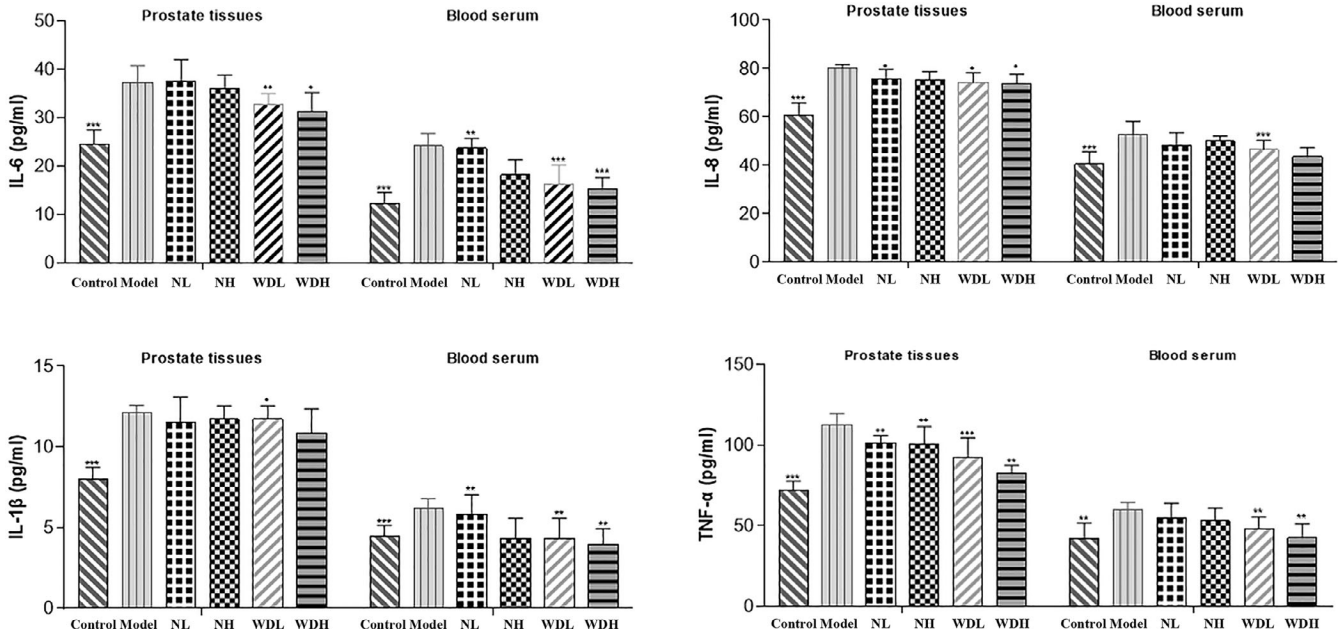

Figure 2. The expression levels of IL-6, IL-8, IL-1β and TNF-α in rats in each group. Control, control group; Model, model group; NL, non-disruption low-dose rapeseed bee pollen group; NH, non-disruption high-dose rapeseed bee pollen group; WDL, Wall-disruption low-dose rapeseed bee pollen group; WDH, Wall-disruption high-dose rapeseed bee pollen group. The values were presented as mean ± standard deviation ($n = 8$). $P < 0.05$ was considered statistically significant.

groups had lower levels of all inflammatory factors in comparison with the non-disruption groups.

Inflammatory cytokines expression using confocal laser scanning microscopy

Confocal immunofluorescence imaging using confocal laser scanning microscopy provides a high-resolution and multicolor imaging platform for the real-time visualization and analysis of prostate tissues. It has the potential to offer additional diagnostic information due to its high sensitivity and specificity. Figure 3 illustrates the differences in inflammatory cytokine expression in prostate tissues. Red fluorescence indicates an inflammatory signal, indicating an exacerbation of CNP, while blue fluorescence represents a normal cellular state, stained with a counterstain for cell nuclei. The model group (Fig. 3(B)) markedly revealed the highest red fluorescence, indicating that prostate tissue exhibited the highest expression levels of IL-6, IL-8, IL-1β, and TNF-α, especially the expression levels of IL-1β. All inflammatory cytokine expressions in bee pollen groups (Fig. 3(C)–(F)) were lower than in the model group (Fig. 3(B)) as indicated by red fluorescence. High-dose groups of rapeseed bee pollen (Fig. 3(D),(F)) presented the lower level of IL-6, IL-8, IL-1β, and TNF-α than the low-dose group (Fig. 3(C),(E)). Remarkably, the lowest expression levels of all inflammatory cytokines were found in the WDH group (Fig. 3(F)), as indicated by the appearance of blue fluorescence and the absence of red fluorescence. Confocal immunofluorescence imaging analysis suggested that the WDH group (Fig. 3(F)) exhibited a cellular state similar to the control group (Fig. 3(A)).

Gut microbiota diversity of rat feces

This study explored the microbiome compositions of rat feces using high throughput 16S rRNA sequencing. In total, 3 067 954 usable raw reads were obtained from 48 samples. A total of 3 067 954 usable raw reads were obtained from 48 samples, which were then filtered and clustered at a 97% similarity threshold into 936 ± 85 OTUs per sample. As shown in Supporting Information, Fig. S2, rarefaction curves and species accumulation curves of all samples both gradually reached a stable state, indicating the sampling depth and the adequacy of the sampling efforts. Moreover, all sample coverages were over 99.56%, reflecting that sequencing reads were sufficient for statistical analysis.

Alpha diversity is an intuitive index to evaluate microbial diversity. Four indexes (Shannon, Simpson, Chao1, and richness) were calculated to evaluate the microbial diversity (Fig. 4(A)). The Chao1 and richness indices estimate community richness, whereas the Shannon and Simpson indices consider both community richness and evenness. Thus, the Shannon and Simpson indices can more comprehensively reflect the microbial structure than the Chao1 and richness indices. As can be seen (Fig. 4(A)), the gut microbiota among the six groups presented wide variations in alpha diversity. For the model group, the administration of λ-carrageenan increased the richness (Chao1) and diversity (both Shannon and Simpson indices) of the gut microbiome. The Simpson and Shannon indices were also slightly reduced in the rapeseed bee pollen groups, with the indices of the WDH group approaching those of the control group.

To profile the overall pattern of microbial communities, non-metric multidimensional scaling (NMDS) was used to reveal dissimilarities in microbial composition at the OTU level, as shown in Fig. 4(B). The NMDS results revealed a broad and variable clustering for the microbiome of the model group; in comparison, there was a tight clustering for the microbiome of other groups. Moreover, the gut microbial structures of rapeseed bee pollen groups were distinguished from that of the model group. Like the results for alpha diversity, the gut microbial structure of the WDH group was closer to that of the control group.

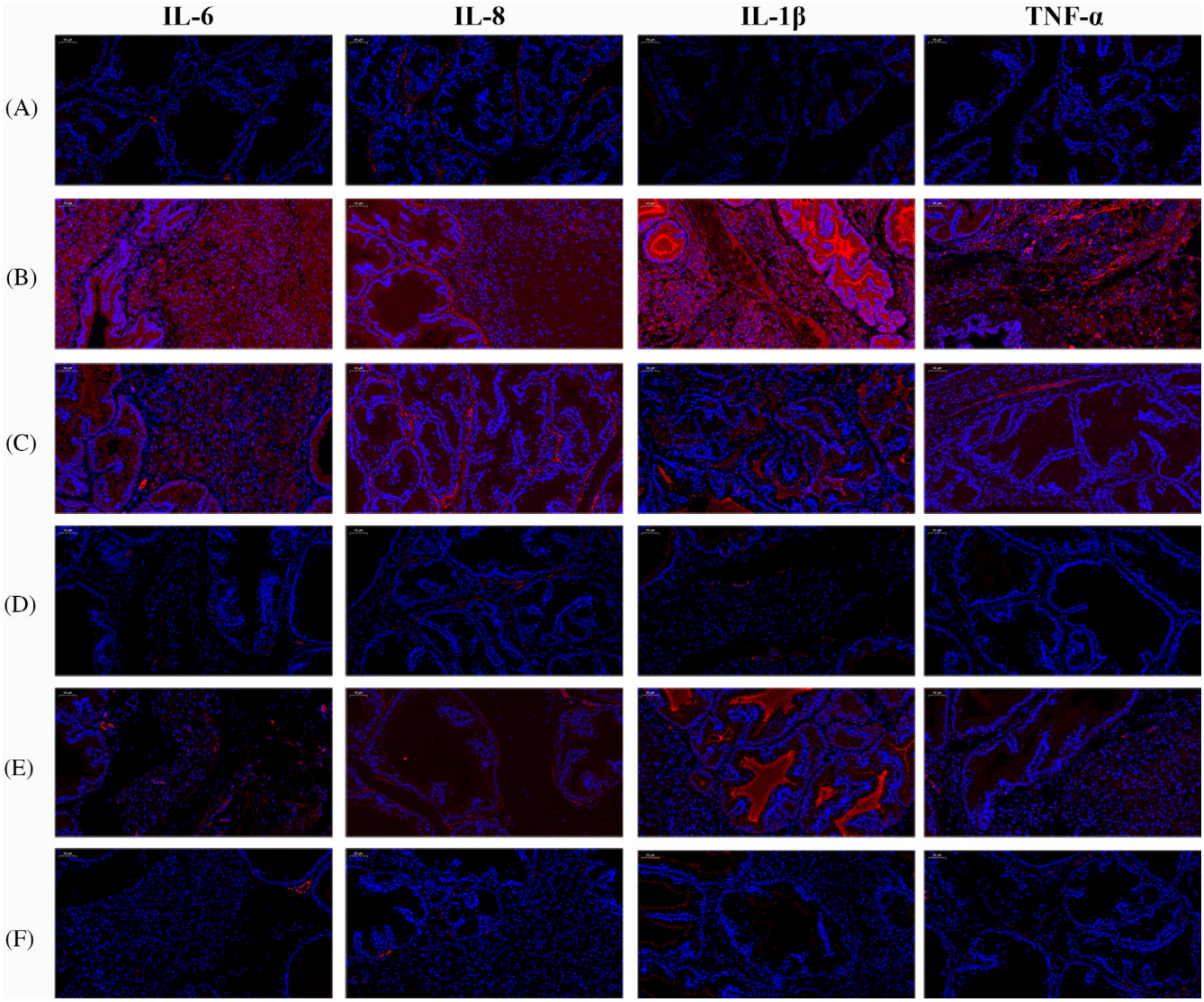

Figure 3. Inflammatory cytokine expression analysis using confocal immunofluorescence imaging. (A) Control group; (B) model group; (C) non-disruption low-dose rapeseed bee pollen group; (D) non-disruption high-dose rapeseed bee pollen group; (E) wall-disruption low-dose rapeseed bee pollen group; (F) wall-disruption high-dose rapeseed bee pollen group. Confocal images demonstrating tissue distribution of target inflammatory cytokines (red); DAPI stained cell nucleus are blue under UV excitation (blue). Phase contrast images are merged for tissue structure. Immunofluorescence (IF) of IL-6, IL-8, IL-1β, and TNF-α were shown in all prostate of rats; the scale bar indicating 50 μm applies to all micrographs.

Gut microbial compositions of rat feces

Figure 5(A) presents the significant differences in the relative abundance of gut microbiota at the phylum level. Among the six groups, the top four phyla accounted for approximately 97% of the total sequences, including Firmicutes, Bacteroidetes, Actinobacteria, and Proteobacteria, respectively. In the model groups, the relative abundance (75.44%) of Firmicutes was more than 7.55% higher than those of the other groups. Compared with the model group, the relative abundance of Bacteroidetes in the non-disruption and WDH group increased by 16.6% and 21.45%, respectively. Previously, the Firmicutes to Bacteroidetes ratio (F/B) can be related to disease occurrence.[29] An increased F/B ratio has been suggested as a marker for gut dysbiosis.[29] The F/B ratio in the model group was 3.38, and the four groups that consumed rapeseed bee pollen reduced significantly, with the F/B ratio ranging from 1.98 to 1.27, similar to the F/B ratio of 2.07 in the control group. The results suggested that oral administration of rapeseed bee pollen can reverse gut dysbiosis and retrieve the normal gut state.

The relative abundance of gut microbiota at the genus level displayed wide variations, as shown in Fig. 5(B). *Lactobacillus* and *Prevotella* were the two most prevalent genera among the six groups examined. In the model group, there was a significant decrease in gut microbiota, including *Bacteroidales_S24-7_group_ge*, *Prevotella_9*, *Prevotellaceae_UCG-001*, and *Turicibacter*, compared with the control group. In the WDH group, the relative abundance of *Prevotella* approached the highest at 22.4%, which was constituted by *Prevotella_9* (12.5%), *Prevotella_1* (3.2%), *Prevotellaceae_NK3B31_*group (2.4%), *Prevotellaceae_UCG-001* (1.2%), *Prevotellaceae_unclassified* (0.9%) and *Alloprevotella* (2.2%). The relative abundances of *Prevotella_1* and *Prevotella_9* in the WDH group were approximately 3.4 times those in the model group.

Figure 5(C) shows a cladogram to depict significantly enriched taxa using linear discriminant analysis (LDA). Only taxa with an

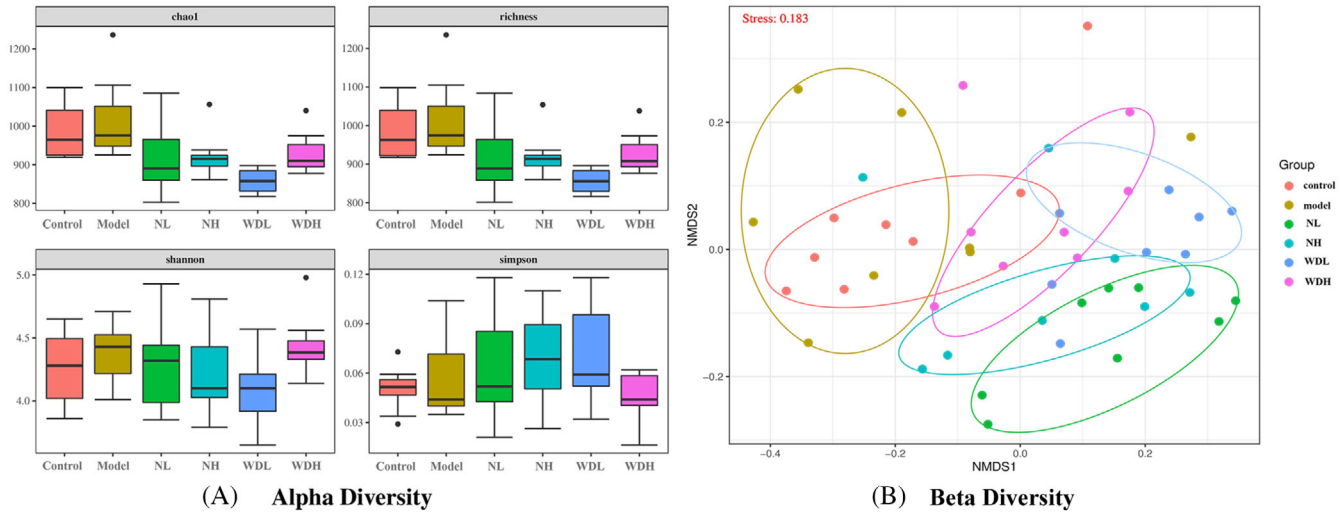

Figure 4. Effect of rapeseed bee pollen on gut microbiota structure in rats with prostatitis. Control, control group; Model, model group; NL, non-disruption low-dose rapeseed bee pollen group; NH, non-disruption high-dose rapeseed bee pollen group; WDL, wall-disruption low-dose rapeseed bee pollen group; WDH, wall-disruption high-dose rapeseed bee pollen group. A. Chao1, Richness, Shannon and Simpson indices shared by six groups of prostate samples. B. The NMDS score plot of the gut microbiota.

LDA > 2 are represented in the cladograms to better specify all of these clades as distinct classes. In the model group, the *Lactobacillaceae* h, i, and j were obviously dominant, belonging to Firmicutes. In contrast, in WDH group, the abundance of *Prevotella* increased significantly.

DISCUSSION

Gut microbiota is potentially critical in maintaining host health.[18] In a healthy state, gut microbiota can modulate the availability of ingested nutrients and the consequent efficiency in energy harvesting to contribute a balance with the host's metabolism and immune system.[30] However, an imbalance in the taxonomic composition of gut microbiota indicates gut microbiota dysbiosis, leading to multiple diseases. Emerging evidence suggests that gut microbiota dysbiosis is involved in inflammatory status.[31,32] For example, gut microbiota dysbiosis can aggravate systemic inflammation in ischemic stroke patients,[33] diabetes mellitus, and related metabolic diseases.[34,35] Dysbiosis is characterized by a reduction in beneficial bacteria and an increase in conditional pathogenic bacteria, which interact with the plasma membrane receptor to generate proinflammatory mediators.[31] Recent studies have also linked gut microbiota dysbiosis to prostatitis,[36,37] the most common urological disease in men worldwide. Rapeseed bee pollen has been used for many decades to treat CNP. In this study, we compared the effect of non-wall-disruption and wall-disruption rapeseed bee pollen on rats with CNP, and evaluated the relation between prostatic inflammation and gut microbiota.

Our results revealed that rapeseed bee pollen possesses significant anti-inflammatory properties for CNP. Prostate wet weight and prostate index are indicators of intertissued pathological structure. Our results show that pollen treatments significantly decreased the prostate index (Table 1), which is consistent with research by Chen *et al.*[38] Compared with the model group, pollen treatments significantly reduced the inflammatory cells in prostate tissue, presenting clearer glandular structures (Fig. 1(C)–(F)). IL-6, IL-8, IL-1β, and TNF – α are known as proinflammatory cytokines to promote inflammation in prostate tissue. Advanced prostate carcinoma is accompanied by increased serum levels of TNF-α, IL-1β, IL-6, and IL-8. The present study showed that pollen treatments could decrease the levels of inflammatory cytokines IL-6, IL-8, IL-1β, and TNF – α (Fig. 2). Confocal immunofluorescence analysis showed down-regulated expression of inflammatory cytokines in all pollen treatment groups (Fig. 3), consistent with the previous research,[39] which found that bee pollen could alleviate abacterial prostatitis in rats by reducing IL-8 levels.

Our findings also revealed that rapeseed bee pollen could modulate gut microbiota. Pollen treatments lowered alpha diversity (Fig. 4(A)) and increased the aggregate degree of overlap in the microbial community structure between bacterial populations (Fig. 4(B)). Some natural products have shown a similar trend in gut community diversity, including *Lentinula* erodes-derived polysaccharide, and Chinese yam polysaccharide.[40] Rapeseed bee pollen seems to benefit for reducing the alpha diversity of CNP. Furthermore, rapeseed bee pollen can inhibit pathogenic bacteria and enhance probiotics. For example, the F/B ratio in WDH group decreased significantly, as indirectly suggested by the up-regulation of Bacteroidetes expression and down-regulation of Firmicutes expression (Fig. 5(A)). This effect is similar to that seen with high fiber intake.[3] The Firmicutes-to-Bacteroidetes ratio was also lower in the androgen deprivation therapy (ADT) for standard treatment of locally advanced or metastatic prostate cancer.[41] On the other hand, in comparison with the abundance of *Prevotella* in the control group, the rapeseed bee pollen groups, especially WDH group, increased the abundance of *Prevotella* (genus), which was most over-represented with an LDA greater than 2 (Fig. 4(B)). *Prevotella* was significantly reduced in CNP patients.[11,36] Compared with recent studies, this is the first study to confirm that rapeseed bee pollen increased the abundance of *Prevotella*. *Prevotella* is a genus of anaerobic gram-negative bacilli colonizing the gastrointestinal tract, oral cavity, and vaginal tract. *Prevotella* is associated with plant-rich diets and may have a positive effect on host metabolism, optimizing energy intake and protecting against inflammation.[42] Our study revealed that gut microbiota significantly changed in CNP rats, which was similar to the findings in CNP patients.[36,37] Thus, targeting the gut microbiota with rapeseed bee pollen may be a promising therapeutic approach for early intervention to prevent damage to the prostate. We plan

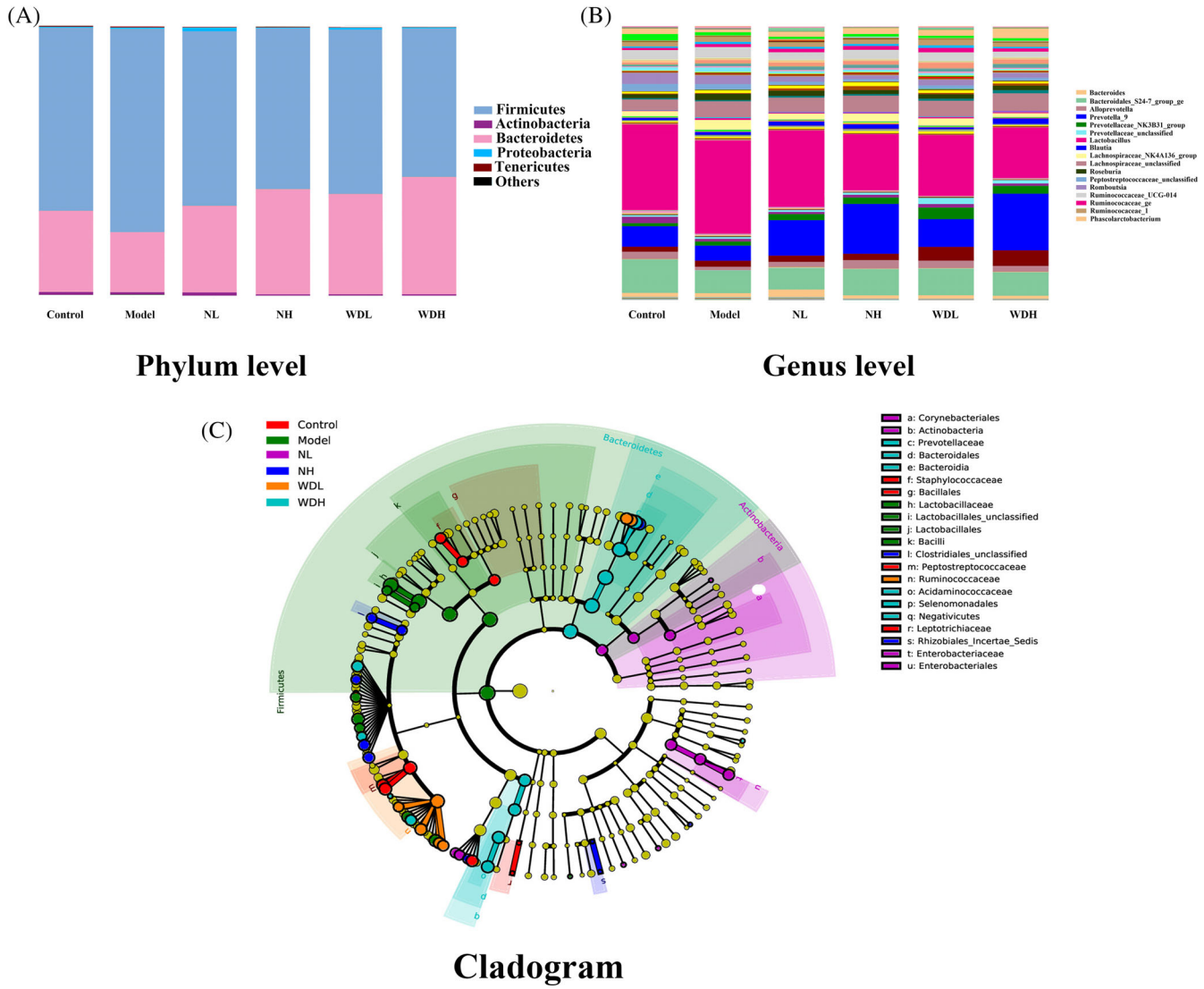

Figure 5. Effect of rapeseed bee pollen on different gut microbial abundances in rats with prostatitis. Control, control group; Model, model group; NL, non-disruption low-dose rapeseed bee pollen group; NH, non-disruption high-dose rapeseed bee pollen group; WDL, wall-disruption low-dose rapeseed bee pollen group; WDH, wall-disruption high-dose rapeseed bee pollen group. (A) Composition and abundance distribution histograms of each group at the phylum level. (B) Composition and abundance distribution histograms of each group at the genus level. (C) A cladogram, by clustering, can distinguish between high-abundance and low-abundance OTUs, and reflect the difference in community composition among all the groups.

to continue investigating the mechanism of gut microbiota and prostatitis for CNP patients.

The wall-disruption high-dose group has shown the best anti-CNP effects. The results revealed that WDH group could significantly decrease the prostate wet weight and the prostate index (Table 1), which is consistent with a previous report.[38] Moreover, the WDH group showed the inflammation reduction in the prostate tissue, with recovering glandular structure and lobular architecture (Fig. 1), and down-regulated levels of inflammation-associated cytokines (Figs. 2 and 3). In addition, the wall-disruption high dose rapeseed bee pollen promoted the healthy functioning of gut microbiota (Figs. 4 and 5). Considering the resistance of pollen wall to chemical, physical, and biological degradation, the augmented nutrient release,[12] and enhanced digestion and absorption rates[13] of wall-disruption bee pollen seem to play an important role in promoting anti-inflammatory activity. We therefore recommend wall-disruption rapeseed bee pollen for anti-prostatitis in clinical trials.

CONCLUSION

Our results reveal that rapeseed bee pollen can alleviate chronic non-bacteria prostatitis effectively by selectively promoting gut microbiota. This is the first report that the alleviation may result from decreasing the F/B ratio and increasing the abundance of *Prevotella* genus in the gut. We recommend wall-disruption rapeseed bee pollen as a complementary and alternative medicine for prostatitis patients.

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CONFLICTS OF INTEREST

The authors declare that there are no conflicts of interest.

DATA AVAILABILITY STATEMENT

The authors will supply the relevant date in response to reasonable requests.

SUPPORTING INFORMATION

Supporting information may be found in the online version of this article.

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