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Effects of *Broussonetia papyrifera* leaf extract on the immunity and gut microflora of cyclophosphamide-induced immunosuppressed mice

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Abstract

This experiment was conducted to determine the effects of *Broussonetia papyrifera* leaf water extract on the immunity and gut microflora of cyclophosphamide-induced immunosuppressed mice. Forty female ICR mice were randomly divided into five groups (n = 8): the blank control (BC) group, the cyclophosphamide-induced immunosuppression model (CTX) group, and the low (BPL), medium (BPM), and high (BPH) dose *B. papyrifera* extract treatment groups. *Broussonetia papyrifera* leaf water extract was administered intragastrically to the BPL, BPM, and BPH groups at 2, 4, and 8 g/kg body weight, respectively, once a day for 14 d. From day 12, all except the BC group mice were injected with 50 mg/kg cyclophosphamide once a day for 3 d. Administering *B. papyrifera* leaf extract substantially enhanced the contents of white blood cells, lymphocytes, monocytes, neutrophils, IgG, and IgM, compared with the CTX group. The *B. papyrifera* leaf extract also restored the gut microbiota composition by decreasing the relative abundance of *Lactobacillus,* Lachnospiraceae_NK4A136 group, *Roseburia,* Lachnospiraceae_uncultured, *Lachnoclostridium*, and *Anaerotruncus*, and increasing the relative abundance of Bacteroidales_S24-7 group norank, *Desulfovibrio, Akkermansia, Enterorhabdus, Blautia*, and *Romboutsia* in the cyclophosphamide-induced immunosuppressed mice. These findings suggest that *B. papyrifera* leaf extract can be used as an immunomodulator of the gut microbiota, with the potential to promote animal health.

Keywords: *Broussonetia papyrifera*, cyclophosphamide, gut microbiota, immunological function
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Introduction

Broussonetia papyrifera, the Moraceae plant, is a Chinese medicinal herb valued for its leaves, fruits, seeds, roots, and bark; it can also be used as drug and fodder crop, and is recorded in the *Mingyi Bielu*. In traditional Chinese medicine, the leaves of *B. papyrifera* have the functions of clearing heat, cooling blood, and removing dampness, among others (Zhu *et al*., 2011). Additionally, *B. papyrifera* leaves are rich in terpenoids, volatile oils, lignins, flavonoids, alkaloids, fatty acids, and amino acids, among other naturally- occurring active compounds (Feng *et al*., 2008; Wang *et al*., 2012). In terms of pharmacological activity, Malaník *et al*. (2020) found that *B. papyrifera* relieved the inflammatory response in LPS-stimulated THP-1 cells and exhibited the greatest antioxidant effect, as measured using the cellular antioxidant activity assay. Furthermore, *B. papyrifera* polyphenols efficiently inhibited the catalytic activity of SARS CoV-2 (Ghosh *et al*., 2021). Zhou *et al*. (2020) found that *B. papyrifera*

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leaves regulated the dominant fungal communities of Ascomycota and Basidiomycota in the gut microbiome of grass carp. This research indicates that the *B. papyrifera* has some officinal value.

Cyclophosphamide is an immunosuppressive drug that causes multiple immunosuppressive diseases and organ damage in humans and animals (Miller, 1997; Voelcker, 2020). Ying *et al*. (2021) found that cyclophosphamide could substantially reduce the immunological factor levels of IFN-γ, TNF-α, and IL-6, and increase the abundance of Firmicutes and Proteobacteria and decrease the abundance of Bacteroidetes in cyclophosphamide-treated mice. Therefore, it is possible for the animal immunosuppressive model that is established by cyclophosphamide to be used to investigate the pesticide effect of an immunopotentiator. Thus, *B. papyrifera* leaf extract was fed to cyclophosphamide-induced immunosuppressed mice, and the immune response and gut microbiome were analysed to provide a theoretical basis for the clinical application of this extract in animal production.

Materials and Methods

The experimental protocol of this study was approved by the Animal Ethics Committee of West Anhui University, and the experimental procedures complied with the relevant provisions of the Chinese Guidelines for the Welfare and Ethics of Laboratory Animals. All mice were kept in an environment maintained at 22 ± 2 °C, with the humidity maintained at 50% and 12 h of light per day. All mice had *ad libitum* access to feed and water.

Broussonetia papyrifera leaves were collected from the plantations of the Anhui Baochu Eco-Agriculture Technology Co., Ltd. The leaves were dried in an electrically heated drying cabinet at 60 °C, after which 500 g of leaves were first soaked in 9 L of distilled water and then extracted twice by reflux condensation (60 min at 100 °C). The filtrate was then concentrated to 1 g/mL using a rotary evaporator (RE52AA, Shanghai Yarong) at 60 °C and stored at -20 °C.

Forty female ICR strain mice were divided into the following five groups (n = 8) according to the random number table method: (1) the blank control group (BC), (2) the cyclophosphamide-induced immunosuppressed model group (CTX), (3) the low dose *B. papyrifera* treatment group (BPL), (4) the medium dose *B. papyrifera* treatment group (BPM), and (5) the high dose *B. papyrifera* treatment group (BPH). The BPL, BPM, and BPH treatment groups were administered *B. papyrifera* leaf water extract intragastrically at 2, 4, and 8 g/kg body weight, respectively, and the BC and CTX groups were administered equal amounts of saline intragastrically, once a day, continuously, for 14 d. From day 12, all except the BC group mice were intraperitoneally injected with 50 mg/kg cyclophosphamide once a day, continuously, for 3 d, and the BC group received equivalent normal saline until all the mice were sacrificed. At the end of the trial period, blood samples were collected in anticoagulant vials to calculate the white blood cell (WBC) count using a Mindray Hematology Analyzer (BC-2600vet, Shenzhen, China), and in non-anticoagulant vials for the immediate separation and collection of the serum. Caecal faeces samples were also collected for 16S rDNA sequencing.

The serum IgG and IgM antibodies were measured using enzyme-linked immunosorbent assay (ELISA) kits according to the operating instructions (Lu *et al*., 2019), purchased from the MultiSciences (Lianke) Biotech, Co., Ltd (Hangzhou, China).

Total faecal DNA was extracted using the cetyltrimethylammonium bromide method (Yu *et al*., 2022). The DNA was then diluted uniformly to 100 ng/μL for polymerase chain reaction (PCR) to detect the targeted genes. PCR was performed using a commercial protocol amplification kit (TransGen, China), with the 50 μL reaction mixture containing 100 ng DNA extract, 1x TransStart FastPfu buffer, 20 pmol primers, 2.5 μM dNTP, and 2.5 units of TransStart FastPfu DNA polymerase. A sequence library was generated by purifying, quantifying, and homogenizing the PCR product (Maughan *et al*., 2012). The finished library was checked using a library quality test and sequenced using the Illumina PE250. The original 16S rDNA sequencing data underwent splicing, followed by mass filtering of the spliced sequences and removal of chimeras to obtain high-quality tag sequences. The sequences were clustered at a 97% similarity level, and a threshold of 0.005% of all sequences was used to filter operational taxonomic units. The bacterial community was annotated using the RDP Classifier software based on information from the Silva database (Maidak *et al*., 1996; Quast *et al*., 2013).

All data are expressed as the mean ± standard deviation (SD), and SPSS 19.0 was used for data analysis. Differences between groups were compared using one-way ANOVA, and the Duncan method was used for multiple comparisons. A *P*-value of less than 0.05 was considered statistically significant.

Results

The body weights and spleen and thymus indices were substantially lower in the CTX group than in the BC group. Following treatment with *B. papyrifera* leaf extract, the body weights and spleen and thymus indices in the BPL, BPM, and BPH groups were substantially higher than in the CTX group (Table 1). These results indicate that *B. papyrifera* leaf extract can substantially improve the body weight and the spleen and thymus index of cyclophosphamide-induced immunosuppressed mice.

Table 1 Body and organ weights (mean ± standard deviation) of cyclophosphamide-induced immunosuppressed mice administered *Broussonetia papyrifera* leaf water extract

	BC	CTX	BPL	BPM	BPH	*P*-value
Body weight (g)	$26.65^{a} \pm 2.17$	$17.15^{b} \pm 1.41$	$24.06^{c} \pm 0.84$	$27.34^{a} \pm 1.56$	$23.55^{c} \pm 1.22$	<0.001
Spleen (mg/g)	$3.78^{a} \pm 0.61$	$1.87^{b} \pm 0.21$	$2.96^{c} \pm 0.18$	$3.68^{a} \pm 0.29$	$3.10^{c} \pm 0.23$	<0.001
Thymus (mg/g)	$2.39^{a} \pm 0.20$	$1.35^{b} \pm 0.30$	$1.91^{c} \pm 0.12$	$2.24^{a} \pm 0.18$	$2.01^{c} \pm 0.18$	<0.001

BC: blank control group; CTX: cyclophosphamide-induced immunosuppressed model group; BPL: low dose *B. papyrifera* treatment group; BPM: medium dose *B. papyrifera* treatment group; BPH: high dose *B. papyrifera* treatment group

^{abc} Matching superscript letters indicate non-significant differences ($P > 0.05$), whereas different superscript letters indicate significant differences ($P < 0.05$)

Cyclophosphamide can cause severe immunosuppression in humans and animals, and the results demonstrated that the serum IgM and IgG levels in the CTX group were substantially lower than in the BC group, indicating that immunosuppressed mice were successfully produced. *Broussonetia papyrifera* leaf extract was used to treat the CTX mice, and the results showed that the serum IgM and IgG levels in the BPL, BPM, and BPH groups were substantially higher than in the CTX group (Table 2).

Table 2 IgM and IgG contents (mean ± standard deviation) of cyclophosphamide-induced immunosuppressed mice administered *Broussonetia papyrifera* leaf water extract

Indicators	BC	CTX	BPL	BPM	BPH	*P*-value
IgM (μg/mL)	$0.34^{a} \pm 0.063$	$0.15^{b} \pm 0.034$	$0.29^{a} \pm 0.062$	$0.49^{c} \pm 0.068$	$0.35^{a} \pm 0.081$	<0.001
IgG (μg/mL)	$7.82^{a} \pm 1.04$	$1.96^{b} \pm 0.38$	$4.93^{c} \pm 0.36$	$7.01^{a} \pm 1.17$	$5.79^{c} \pm 0.96$	<0.001

BC: blank control group; CTX: cyclophosphamide-induced immunosuppressed model group; BPL: low dose *B. papyrifera* treatment group; BPM: medium dose *B. papyrifera* treatment group; BPH: high dose *B. papyrifera* treatment group

^{abc} Matching superscript letters indicate non-significant differences ($P > 0.05$), whereas different superscript letters indicate significant differences ($P < 0.05$)

WBC mainly consist of lymphocytes (Lymph#), monocytes (Mon#), and neutrophils (Gran#), and their counts are often used as an important haematological indicator of immunity. The counts of WBC, Lymph#, Mon#, and Gran# in the CTX group were substantially lower than in the BC group, whereas the counts of WBC, Lymph#, Mon#, and Gran# in the BPM and BPH groups were substantially higher than in the CTX group, and the counts of WBC, Lymph#, and Gran# in the BPL group were substantially higher than in the CTX group (Table 3).

Table 3 White blood cell (WBC) counts (mean ± standard deviation) of cyclophosphamide-induced immunosuppressed mice administered *Broussonetia papyrifera* leaf water extract

	BC	CTX	BPL	BPM	BPH	*P*-value
WBC (× 10^9 cells/L)	11.33^{a} ± 1.75	4.67^{b} ± 1.31	7.70^{c} ± 026	10.37^{a} ± 0.71	7.80^{c} ± 1.05	<0.001
Lymphocytes (× 10^9 cells/L)	9.07^{a} ± 1.03	3.83^{b} ± 1.06	6.00^{c} ± 0.85	7.20^{ac} ± 0.95	5.83^{c} ± 1.23	<0.01
Monocytes (× 10^9 cells/L)	0.67^{a} ± 0.15	0.20^{b} ± 0.10	0.30^{bc} ± 0.10	0.67^{a} ± 0.12	0.47^{c} ± 0.058	<0.01
Neutrophils (× 10^9 cells/L)	1.6^{a} ± 0.52	0.63^{b} ± 0.21	1.30^{a} ± 0.26	1.60^{a} ± 0.10	1.17^{c} ± 0.25	<0.05

BC: blank control group; CTX: cyclophosphamide-induced immunosuppressed model group; BPL: low dose *B. papyrifera* treatment group; BPM: medium dose *B. papyrifera* treatment group; BPH: high dose *B. papyrifera* treatment group
^{abc} Matching superscript letters indicate non-significant differences (P >0.05), while different superscript letters indicate significant differences (P <0.05).

The bacterial community was similar between the treatment groups at the order level, with all five groups containing Bacteroidales, Clostridiales, Lactobacillales, Desulfovibrionales, Coriobacteriales, Verrucomicrobiales, and Erysipelotrichales (Figure 1). However, the relative abundance of Bacteroidales in the CTX group (27.61%) was lower than in the BC (41.31%) and BPM (33.32%) groups, and the relative abundance of Clostridiales in the CTX group (37.94%) was higher than in the BC (26.37%) and BPM (31.36%) groups. Lactobacillales was higher in the CTX group (28.50%) than in the BC (20.44%) and BPM (26.27%) groups. These data indicate that Bacteroidales, Clostridiales, and Lactobacillales were the most abundant orders in all groups, and that *B. papyrifera* leaf extract administration can increase the relative abundance of Bacteroidales and decrease the relative abundance of Clostridiales and Lactobacillales at the order level.

Regarding genera, *Lactobacillus, Bacteroidales, Lachnospiraceae*_(NK4A136 group, unclassified, uncultured and UCG-006), *Roseburia, Rikenella, Desulfovibrio, Alistipes, Odoribacter*, and *Bacteroides* were identified as the nine most prevalent genera in the samples. After analysing the relative abundance of the bacterial community, the *Bacteroidales*_S24-7 group norank, *Rikenella*, *Desulfovibrio*, *Alistipes*, *Odoribacter*, *Bacteroides*, *Lachnospiraceae* UCG-006, *Akkermansia, Enterorhabdus, Blautia*, and *Romboutsia* were found to be lower in the CTX group than in the BC group, and the *Bacteroidales*_S24-7 group norank, *Desulfovibrio*, *Akkermansia, Enterorhabdus, Blautia*, and *Romboutsia* were found to be higher in the BPM group than in the CTX group. The relative abundance of *Lactobacillus*, *Lachnospiraceae*_(NK4A136 group, *uncultured*, and unclassified), *Roseburia*, *Lachnoclostridium*, *Anaerotruncus*, and *Ruminiclostridium 9* were higher in the CTX group than in the BC group, and *Lactobacillus*, *Lachnospiraceae*_(NK4A136 group and uncultured), *Roseburia*, *Lachnoclostridium*, and *Anaerotruncus* were lower in the BPM group than in the CTX group. These results demonstrate that *B. papyrifera* leaf extract administration can improve the structure of the gut bacterial community in cyclophosphamide-induced immunosuppressed mice.

The overall bacterial community was substantially affected by *B. papyrifera* leaf extract administration (Figure 2). Coriobacteriales, Enterorhabdus, Porphyromonadaceae, and Odoribacter, which were most abundant in the BC group, and Epsilonproteobacteria, Helicobacter, Candidatus Arthromitus, Campylobacterales, and Clostridiales, which were most abundant in the CTX group, and Prevotellaceae, Eisenbergiella, Peptostreptococcaceae, Eubacterium, Romboutsia, Turicibacter, and Clostridiales, which were most abundant in the BPM group, were the dominant microflora that contributed to the differences between the groups.

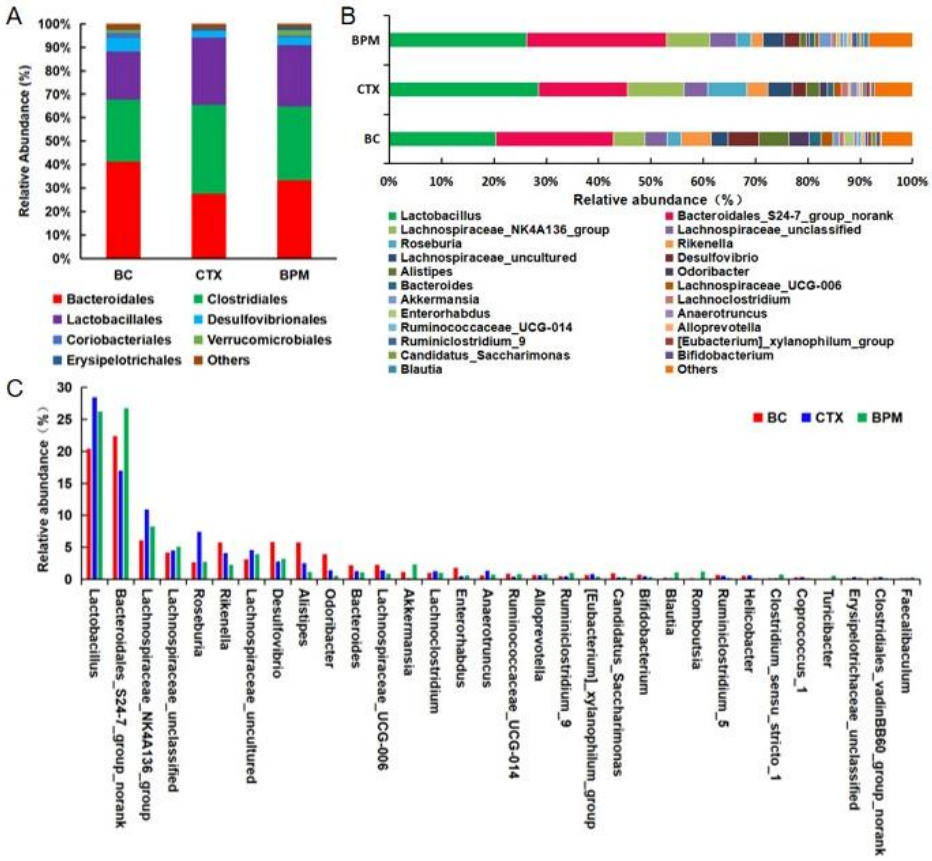

Figure 1 Relative abundance of caecal faecal bacteria at the order (A), genus (B), and species (C) levels in the blank control group (BC), cyclophosphamide-induced immunosuppressed model group (CTX), and medium dose *Broussonetia papyrifera* treatment group (BPM)

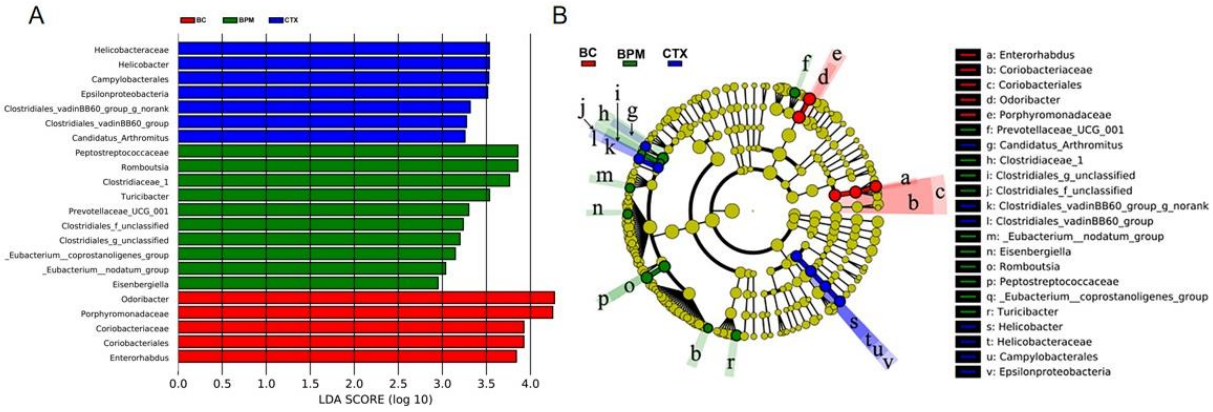

Figure 2 Comparison of microbial variation in the caecal faeces between the blank control (BC), cyclophosphamide-induced immunosuppressed model (CTX), and medium dose *Broussonetia papyrifera* treatment (BPM) groups using the LEfSe tool: (A) Linear discriminant analysis (LDA) scores; (B) Cladogram

Discussion and Conclusions

Traditionally, *B. papyrifera* leaves have been known as herbs for clearing heat, cooling the blood, and removing dampness, and are they are recorded in *Mingyi Bielu* as being used to treat the diseases of enteritis and dysentery, as well as severe nosebleeds. Furthermore, *B. papyrifera* has been shown to effectively enhance the immunity of carp and mice (Chen *et al*., 2020; Xu *et al*., 2023). The

composition and homeostasis of the gut microbiota also influences the human and animal immune system, especially in cases of immunosuppressive diseases. Recent research has produced evidence that herbal medicine can regulate and control immunological function in immunosuppressed humans and animals, and, in particular, has shown a positive therapeutic effect in patients with systemic lupus erythematosus and intestinal dysbacteriosis (Dhanisha *et al*., 2020; Chen *et al*., 2021; Wei *et al*., 2021). This study therefore aimed to evaluate the immunopotentiation potential of *B. papyrifera* leaf extract, as well as its effects on the regulation of gut microorganisms. The effects of administering the extract on immune cells, immunoglobulins, and the gut microbiota were also investigated to explore the mechanisms of immunoenhancement. The results suggest that *B. papyrifera* leaf extract is a promising immunomodulating and microbial community regulating agent.

Cyclophosphamide can cause severe immunosuppression in humans and animals, resulting in the inhibition of haematological immune-related factors, such as immune cells and immunoglobulins (Legrand *et al*., 2013; Qi *et al*., 2018). To verify the success of the immunosuppressive model, the WBC count in the blood and the IgG and IgM antibody levels in the serum were determined. The results showed that the counts of WBC, Lymph#, Mon#, Gran#, IgG, and IgM in the CTX mice were substantially lower than in the BC mice, indicating that immunosuppressed mice were successfully produced.

The homeostasis of the gut microbiome is involved in a number of life activities, including the immune response, nutrient uptake, and biological rhythm (Leser *et al*., 2009; Pickard *et al*., 2017; Kuang *et al*., 2019;; Bishehsari *et al*., 2020; Matenchuk *et al*., 2020). Furthermore, gut microorganisms are closely related to disease onset and development (Gomaa, 2020; Lee *et al*., 2021). The pathogenic factor of immune diseases such as lupus erythematosus, Behçet's disease, and Hashimoto's thyroiditis can destroy the homeostasis of gut microbiome (Ye *et al*., 2018; Virili *et al*., 2018; Chen *et al*., 2021). The homeostasis of the gut microbiota therefore has a strong correlation with immunity.

In the current study, the relative abundance of some microbes, including *Bacteroidales*_S24-7 group norank, *Rikenella*, *Desulfovibrio*, *Alistipes*, *Odoribacter*, *Bacteroides*, *Lachnospiraceae*_UCG-006, *Akkermansia, Enterorhabdus, Blautia*, and *Romboutsia* were lower, and *Lactobacillus*, *Lachnospiraceae*_ NK4A136 group, *Lachnospiraceae*_unclassified, *Roseburia*, *Lachnospiraceae_uncultured*, *Lachnoclostridium*, *Anaerotruncus*, and *Ruminiclostridium 9* were higher in the CTX group, demonstrating that cyclophosphamide disrupted the homeostasis of the gut microbiota. Maintaining the homeostasis of the gut microbiome is particularly important for human and animal health. Fortunately, several studies have shown that herbs can effectively regulate and control the balance of the human and animal gut microbiota. Wang *et al.* (2019) found that essential oil, limonene, linalool, and citral improved the prevalence of *Lactobacillus* in the caeca and colon of mice, and polysaccharides from *Auricularia auricula* restored the composition of the gut microbiome to close to normal levels by decreasing the ratio of *Firmicutes/Bacteroidetes* in cyclophosphamide-induced immunosuppressed mice (Kong *et al*., 2020).

Our study similarly found that *B. papyrifera* leaf extract had positive effects on immunity, increasing the WBC, Lymph#, Mon#, Gran#, IgG, and IgM counts in immunosuppressed mice. In mice treated with *B. papyrifera* leaf extract, the relative abundance of *Bacteroidales* S24-7 group norank, *Desulfovibrio*, *Akkermansia, Enterorhabdus, Blautia*, and *Romboutsia* were higher, and the relative abundances of *Lactobacillus*, *Lachnospiraceae* NK4A136 group, *Roseburia*, *Lachnospiraceae uncultured*, *Lachnoclostridium*, and *Anaerotruncus* were lower. These results demonstrate that the *B. papyrifera* leaf extract can improve the structure of the caecum's bacterial community in cyclophosphamide-induced immunosuppressed mice.

In this study, *B. papyrifera* leaf extract administration had immune-protective effects in cyclophosphamide-induced immunosuppressed mice by substantially increasing the counts of WBC, Lymph#, Mon#, Gran#, IgG, and IgM. Furthermore, the results suggest that this extract can help regulate the homeostasis of the gut microecological system in immunosuppressed mice. These findings suggest that *B. papyrifera* leaf extract can be used as an immunomodulator of the gut microbiota, with the potential to promote animal health.

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Author Contributions

Conceptualization, P.J.; methodology, P.J. and G.P.X.; validation, C.B.S.; formal analysis, P.J.; investigation, G.P.X.; resources, P.J.; data curation, P.J. and G.P.X.; writing—original draft preparation, P.J.; writing—review and editing, P.J.; supervision, C.B.S. and Y.F.H.; funding acquisition, R. H. Z. and P.J., All authors have read and agreed to the published version of the manuscript.

Conflict of Interest Declaration

The authors declare that they have no conflicts of interest relevant to the content of this paper.

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