Probiotics and Antimicrobial Proteins
https://doi.org/10.1007/s12602-023-10205-z

RESEARCH

Pediococcus pentosaceus MIANGUAN Enhances the Immune Response to Vaccination in Mice

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Accepted: 10 December 2023

Abstract

Increasing evidence shows that some probiotics can improve vaccine responses as adjuvants. This study aimed to evaluate the effect of *Pediococcus pentosaceus* MIANGUAN (PPM) on SARS-CoV-2 vaccine-elicited immune response in mice. Six-week-old female ICR mice were primed and boosted with SARS-CoV-2 vaccine intramuscularly at weeks 0 and 4, respectively. Mice were gavaged with PPM (5×10^9 CFU/mouse) or PBS (control) for 3 days immediately after boosting vaccination. Compared to the control, oral PPM administration resulted in significantly higher levels of RBD-specific IgG binding antibodies (> 2.3-fold) and RBD-specific IgG1 binding antibodies (> 4-fold) in the serum. Additionally, PPM-treated mice had higher titers of RBD-specific IgG binding antibodies (> 2.29-fold) and neutralization antibodies (> 1.6-fold) in the lung compared to the control mice. The transcriptional analyses showed that the B cell receptor (BCR) signaling pathway was upregulated in both splenocytes and BAL cells in the PPM group vs. the control group. In addition, the number of IFN-γ-producing splenocytes (mainly in CD4 + T cells as determined by flow cytometry) in response to restimulation of RBD peptides was significantly increased in the PPM group. RNA sequencing showed that the genes associated with T cell activation and maturation and MHC class II pathway (*CD4*, *H2-DMa*, *H2-DMb1*, *H2-Oa*, *Ctss*) were upregulated, suggesting that oral administration of PPM may enhance CD4 + T cell responses through MHC class II pathway. Furthermore, PPM administration could downregulate the expression level of proinflammatory genes. To conclude, oral administration of PPM could boost SARS-CoV-2 vaccine efficacy through enhancing the specific humoral and cellular immunity response and decrease the expression of inflammation pathways.

Keywords COVID-19 · *Pediococcus pentosaceus* · Probiotics · Immune modulation · Vaccine · Adjuvant

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Introduction

The global spread of coronavirus disease 2019 (COVID-19) caused by severe acute respiratory syndrome coronavirus 2 (SARS-CoV-2) has profoundly affected public health and economics. From December 2019 to February 2023, there were more than 757 million confirmed cases and the disease claimed more than 6.8 million lives globally (WHO Coronavirus (COVID-19) Dashboard; https://covid19.who.int). To control the COVID-19 pandemic and decrease morbidity and mortality, COVID-19 vaccination has been conducted worldwide. To date, there are various types of COVID-19 vaccines available including inactivated vaccines, mRNA vaccines, and adenoviral-based vaccines [1–4]. Although these vaccines have prevented the COVID-19 pandemic to certain extent, their effectiveness may only last for a few months [5]. Therefore, it is necessary to find alternative

Published online: 02 January 2024

strategies to prolong memory immune responses to vaccination. Previous studies have shown that vaccine effectiveness and immunogenicity were influenced by various factors such as age, sex, genetics, environment, adjuvant, and microbiota [6]. Increasing evidence from animal models and clinical studies shows that the composition and function of gut microbiota can modulate host immune responses to the vaccines [7–9], which prompts the investigators to pursue optimizing vaccine efficacy by modulating the gut microbiota [10]. Probiotics are defined as live microorganisms that are beneficial to health [11]. Studies have shown that certain probiotics have positive effects on the host by changing the gut or skin microbiota [10]. Some specific probiotics defined as "immunobiotic" can serve as vaccine adjuvants to enhance the vaccine's efficacy through modulating gut microbiota and participating in the interaction of mucosal, humoral, and cell-mediated immune responses [12, 13]. Investigators are currently pinning their hopes on probiotics as an inexpensive way to boost vaccine effectiveness and prolong the duration of protection [14]. Many animal and human studies have demonstrated the feasibility of this approach in enhancing the effectiveness of several vaccines [15], including those for influenza [16, 17] and rotavirus [18, 19]. Since the COVID-19 vaccine could only induce relatively short-term anti-SARS-COV-2 immunity, it is reasonable to believe that the use of probiotics may be beneficial in terms of boosting immune response to the vaccine. We recently showed that administration of *Lactobacillus plantarum* GUANKE (LPG) not only promoted the specific neutralization antibodies > 2-fold in sera and > 8-fold in bronchoalveolar lavage, but also increased IFN-γ-producing CD4 + T cells in spleen [20]. Some of the probiotic functions are strain-specific, and different probiotics may have similar effects but through different mechanisms. Therefore, it is necessary to find more efficient probiotic candidates to design a consortium of probiotics that may work synergistically in enhancing the immune response to the SARS-CoV-2 vaccine.

Pediococcus pentosaceus is a gram-positive, nonmotile, and coccus-shaped lactic acid bacteria (LAB) with facultative anaerobic features. *P. pentosaceus* is considered a probiotic, as certain strains of *P. pentosaceus* are known to possess probiotic properties, such as antimicrobial [21, 22], anticarcinogenic [23, 24], anti-inflammatory [25, 26], lipid-lowering [27, 28], detoxification [29, 30], and modulation of immune function [31–34]. Although several studies have reported the ability of *P. pentosaceus* to modulate immune response, its role as a vaccine adjuvant and how it works in this matter remain to be studied. In this study, we demonstrated that oral administration of *P. pentosaceus* MIANGUAN (PPM) could promote SARS-CoV-2-specific humoral and cell-mediated immune responses through enhancing BCR and MHC II signaling pathways. Furthermore, PPM could decrease the expression of the genes related to inflammatory pathways.

Materials and Methods

Strain and Culture Condition

PPM was isolated from healthy human feces and stored in glycerol preservation solution at − 80 ℃. PPM was grown in de man, Rogosa, Sharpe (MRS) medium (Oxoid, Thermo Fisher Biochemicals Ltd., Beijing, China) on a 37 ℃ incubator shaker to reach $OD_{600nm} = 1.0$. PPM was then washed twice with sterile phosphate-buffered saline (PBS) and resuspended in PBS to a concentration of 2.5×10^{10} CFU/mL before gavage in mice. *Lactobacillus plantarum* GUANKE [20] and other bacteria were isolated from stool specimens of healthy human and identified by 16S rRNA gene sequencing. The same culture method and condition were used as for PPM.

Ethics Statement and Animals

The specific pathogen-free (SPF) female ICR mice (6–8 weeks old) were obtained from the Beijing Vital River Laboratory Animal Technology Co., Ltd. [laboratory animal permit no. SYXK (Jing) 2022–0029], and housed in the Chinese Center for Disease Control and Prevention Laboratory Animal Center. In the SPF environment, a 12-h light/dark cycle was maintained, mice were assigned to cages with a maximum occupancy of five, and they were provided unrestricted access to both food and water. All animal-related procedures were conducted by following the protocol approved by the Institutional Animal Care and Use Committee (IACUC) in Chinese Center for Disease Control and Prevention Laboratory Animal Center (Approval code: 2022–023).

In Vitro Screening of Candidate Strains that Potentially Enhance SARS-CoV-2 Specific Cellular Responses Using ELISpot

In this study, we first conducted the screening experiments to select candidate strains. ICR mice were primed and boosted with 100 μg of SARS-CoV-2 vaccine (DNA-S, the DNA sequences encoding SARS-CoV-2 spike (S) protein (YP_009724390.1) were cloned into the pcDNA3.1 vector to construct pcDNA3.1-S plasmid [20]) via intramuscular route at 0 and 2 weeks, respectively. On day 7 after boosting, the mice were sacrificed and splenocytes were isolated using Red Blood Cell Lysis Buffer (Cat#R1010, Solarbio) (Fig. 1A). Then, the splenocytes were incubated with candidate strains (MOI = 20) at 37 °C in a 5% CO_2 incubator.

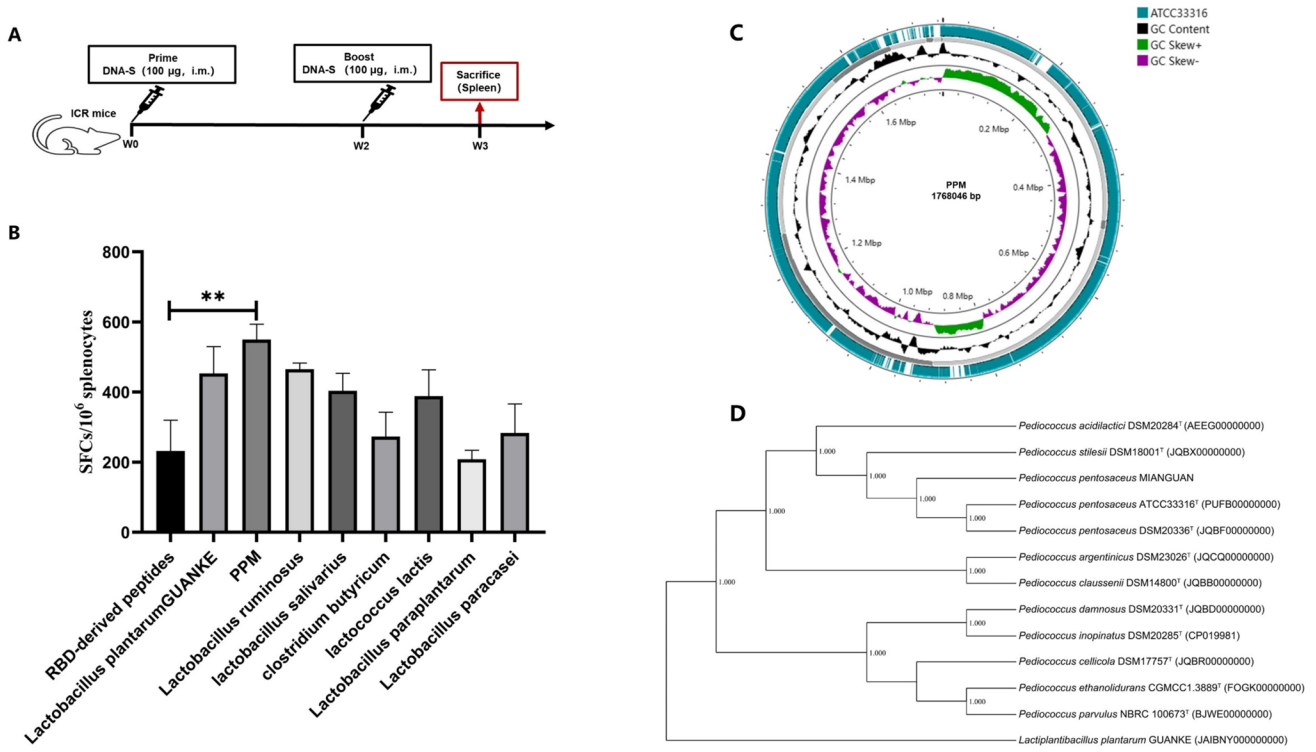

Fig. 1 PPM enhanced SARS-CoV-2 vaccine-elicited cellular immunity in cell model in vitro. **A** Experimental schedule. ICR mice were primed and boosted with SARS-CoV-2 vaccines (DNA-S) intramuscularly at 0 and 2 weeks separately. The spleens were collected and prepared as single cell suspension at day 7 after priming, followed by measuring IFN-γ-producing splenocytes using ELISpot assay. **B** The number of IFN-γ-producing splenocytes in different groups after treatment by different bacteria. **C** Comparison of *Pediococcus pentosaceus* MIANGUAN with *Pediococcus pentosaceus* ATCC33316. **D** The position of *Pediococcus pentosaceus* MIANGUAN (PPM) relative to the representative strains, neighbor-joining phylogenomic tree based on 741 core genes of genomic sequences. The data are shown as mean ± SD and conducted three independent experiments. $^{**}P < 0.01$

After 3 h, the splenocytes were collected by centrifugation. For assessment of RBD-specific T cell responses of the splenocytes, the IFN-γ-producing cells were measured by mouse IFN-γ ELISpot assay set (Cat#51–9,000,209, BD Bioscience) [20]. The RBD peptides (13 peptides, 15-mer with 11 overlapped amino acids) were synthesized in China Peptides Co., Ltd. and covered the entire RBD sequence, which provided distinct antigenic peptide fragments and enabled activation of multiple RBD-specific T cell subpopulations and thus can be used in ELISpot to measure the RBD-specific T cell immune response. The spot forming cells (SFC) on Millipores were counted using Biospot plate reader (iSpot Spectrum, AID, Germany).

Mouse Immunization and Treatment with Immunobiotics In Vivo

The objective of this study focused on investigating the impact of oral administration of PPM on the immune reaction elicited by the SARS-CoV-2 vaccine in immunized mice, and the experimental procedure is shown in Fig. 2A. A total of 12 ICR mice were initially subjected to priming through intramuscular administration of 5×10^{10} vp of AdC68-Delta-S. At week 4 after priming, all mice were given a broad-spectrum antibiotic cocktail for 3 days to deplete gut microbiota. Ampicillin (1 g/L, INALCO, American), gentamicin sulfate (0.5 g/L, INALCO, American), and vancomycin hydrochloride (0.5 g/L, INALCO, American) were administered in the drinking water. Amphotericin B (0.1 g/L, INALCO, American) and metronidazole (8 g/L, Solarbio, China) were administered in 200 μL dosage by oral gavage once daily [35]. Fecal samples were suspended in PBS and plated on Brain–Heart Infusion (BHI) plate with 5% sheep blood and then cultured under anaerobic and aerobic conditions to verify microbiota depletion. After treatment with antibiotics, all mice were boosted with 5×10^{10} vp of AdC68-Delta-S via intramuscular routes. Then, mice were randomly divided into two groups with one group receiving oral administration of 200 μL of PPM (5×10^9 CFU) and the other group receiving oral administration of PBS (control). This procedure was done once daily for 3 days. Mice were sacrificed on day 7 post administration of PPM or PBS and tissue samples (serum, bronchoalveolar lavage (BAL) and spleen)

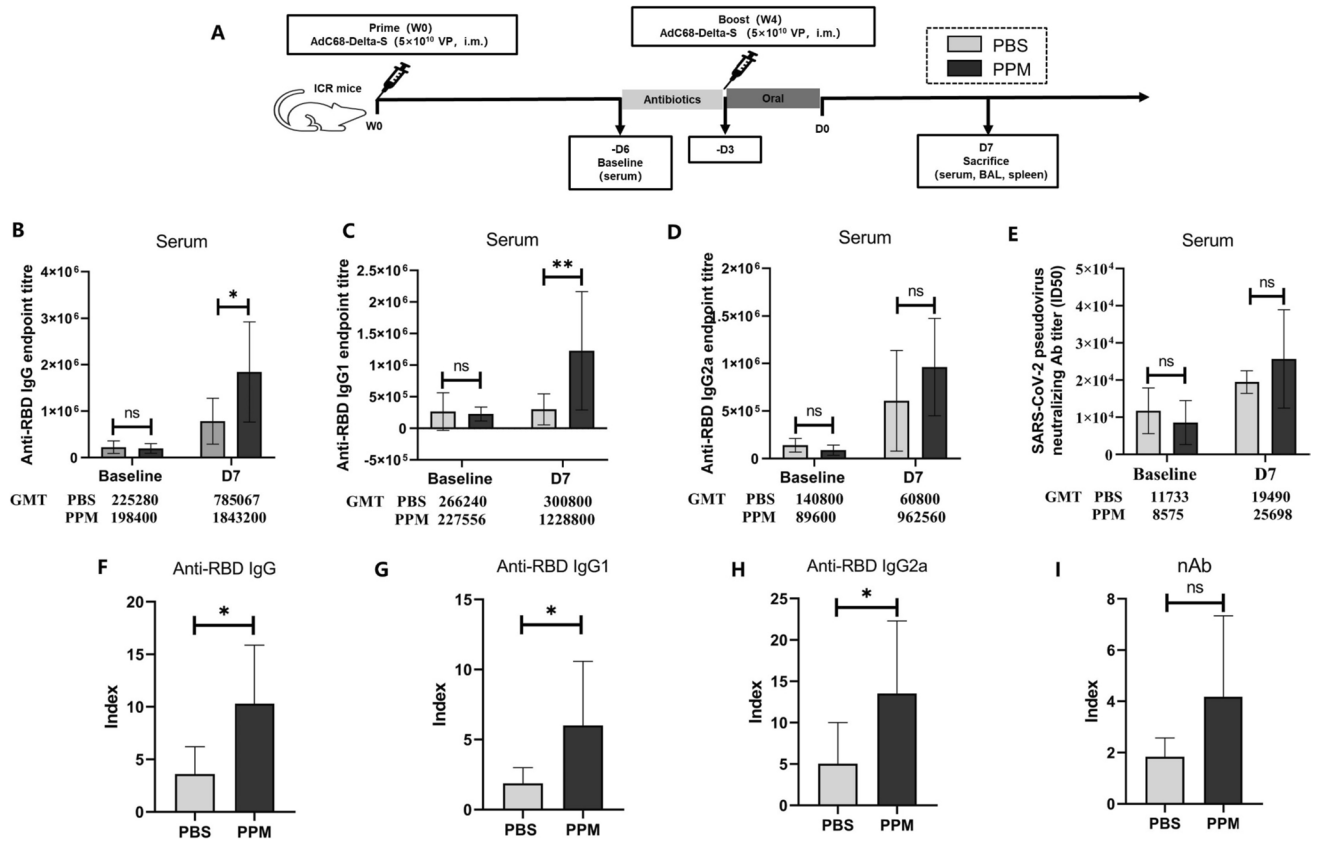

Fig. 2 Oral PPM administration immediately after immunization enhanced serum humoral responses in mice. **A** The outline of animal experiment. Twelve 6-week-old female ICR mice were randomly allocated into PBS or PPM groups, 6 mice/group; all mice were primed and boosted with SARS-CoV-2 vaccine followed by the oral administration of PBS or PPM for 3 days. Serum RBD-specific IgG (**B**), IgG1 (**C**), and IgG2a (**D**) and neutralizing antibodies (**E**) were assessed using ELISA and the pseudovirus neutralization assay. D: day; GMT: geometric mean titer. The data are shown as means ± standard deviation from three independent experiments. Differences of antibody titers between the PBS and PPM groups were analyzed by using the Mann–Whitney test. $^{*}P < 0.05$, $^{**}P < 0.01$

were collected for subsequent antibody titer analysis and transcriptome sequencing [20].

Measurement of RBD-binding Antibodies Using ELISA

RBD-binding antibody (IgG, IgG1, IgG2a) titers in serum and BAL were measured using ELISA. Serum and BAL were collected before antibiotic treatment (set as the baseline) and day 7 post administration of PPM or PBS for RBD-binding antibody measurement. Firstly, 96-well ELISA plates were coated with 100 μL of SARS-CoV-2 RBD protein (Cat#BDAB0017, Biodragon, Beijing, China) which was diluted with coating buffer (Cat#SFQ012-250, Beijing 4A Biotech Co., Ltd., Beijing, China) at a final concentration of 1 μg/mL at 4 ℃ overnight. Afterward, the plates underwent three times of washing with 220 μL PBST (PBS with 0.5% Tween-20), following which they were subsequently blocked at room temperature for a duration of 2 h with 200 μL of PBST encompassing 0.5% nonfat milk. The plates were washed three times; 100 μL of twofold serially diluted serum and BAL samples was added and incubated for 3 h at RT. To measure the titers of RBD-binding immunoglobulins (IgG, IgG1, or IgG2a), a diluted solution (1:5000) of horseradish peroxidase (HRP)-conjugated goat anti-mouse immunoglobulins IgG (ab6789, abcam, Shanghai, China), IgG1 (ab97240, abcam, Shanghai, China), and IgG2a (ab97245, abcam, Shanghai, China) was added to ELISA plates (100 μL/ well) separately. The incubation was performed for 1 h at RT. After washing with 220 μL PBST, 100 μL of mixed TMB substrate (A:B = 1:1) (TMB Substrate Reagent Set, Cat#555,214, BD Bioscience) was added to each well and allowed for color-forming reaction for 5 min at RT. Finally, the reaction was terminated by adding 50 μL termination solution (Cat#SFQ009-500, Beijing 4A Biotech Co., Ltd., Beijing, China), followed by reading at OD_{450nm} with a Synergy Microplate Reader (Bio-Tek, Winooski, VT, USA). The ELISA endpoint titers were defined as the highest serum or BAL dilution that produced an absorbance twofold over the background value.

Pseudovirus Neutralization Assay

To assess neutralizing activity of serum and BAL against SARS-CoV-2, 50 µL serially diluted heat-inactivated samples were prepared with RPMI-1640 medium (Cat#11,875,093, Gibco) in 96-well plates and mixed with an equal volume of diluted FNV-SARS-CoV-2-S (Cat#BDAAA0026, Biodragon, Beijing, China), and the virus control wells were added with 50 µL RPM1640 instead of diluted samples. After incubation at 37 ℃ for 1 h, 100 µL of hACE2-293 T cells (104 cells/well) was added into the plates and then incubated for 48 h at 37 ℃ in an incubator containing 5% CO_2. After washing with 100 µL of PBS, 50 µL of luciferase cell culture lysis reagent (Cat# E1531, Promega, Madison, WI, USA) was added into each well and incubated for 30 min at RT on a horizontal shaker to fully lyse the cells. Finally, the Luciferase Assay System (Cat#E1483, Promega, Madison, WI, USA) was utilized to measure the activity of luciferase on a luminometer (Promega GloMax® 96). The titers of neutralizing antibodies, denoted as the 50% inhibitory doses (ID50), were determined by identifying the highest dilution causing a 50% decrease in relative light units (RLUs) compared to the control wells infected with the virus. This calculation was performed by subtracting the background signal (RLUs obtained from wells without the virus).

Flow Cytometric Analysis

Spleens were collected on day 7 after oral PPM or PBS administration and splenocytes were isolated using Red Blood Cell Lysis Buffer (Cat#R1010, Solarbio). The splenocytes were activated with Brefeldin A (Cat#423,303, Biolegend), followed by incubation at 37 ℃ with 5% CO_2 for 6 h. The splenocytes were stained with 0.25 µg TruStain FcX™ (anti-mouse CD16/32) (Cat#101,319, Biolegend) in 100 µL staining buffer (Cat#420,201, Biolegend) to block Fc receptor. After incubation at 4 ℃ for 10 min, the following antibodies were added to the cells: FITC anti-mouse CD3 antibody (Cat#100,203, Biolegend), PerCP/Cyanine5.5 anti-mouse CD4 antibody (Cat#100,433, Biolegend), and APC anti-mouse CD8a antibody (Cat#162,305, Biolegend). The samples were incubated in the dark at 4 ℃ for 15 min. After washing with the staining buffer, the cells were fixed and permeabilized with 150 µL Cyto-Fast™ Fix Perm Solution (Cat#426,803, Biolegend) for 20 min at RT, followed by two times of washing with 500 µL 1 × Cyto-Fast™ Perm wash buffer (Cat#426,803, Biolegend). Next, the cells were suspended in 100 µL 1 × Cyto-Fast™ Perm wash buffer, and 1 µL PE anti-mouse IFN-γ antibody (Cat#505,808, Biolegend) was added and incubated for 20 min in the dark at RT. After washing twice with Intracellular Staining Perm wash buffer (Cat#421,002, Biolegend), the samples were suspended in 500 µL cell staining buffer and analyzed by using a BD FACSCalibur. The data analysis was performed using FlowJo software (v10.8.1).

RNA Sequencing

The spleen and BAL cells collected on day 7 after oral PPM administration (Fig. 2A) were used for transcriptome analysis by Beijing Genomics Institute Co., Ltd. (Beijing, China). The extraction of total RNA from the samples by employing Trizol was performed following the guidelines provided by the manufacturer. To assess the concentration and integrity of the RNA, an Agilent 2100 bioanalyzer was utilized, and the qualified samples were used to construct a data library, followed by sequencing on DNBAEQ by Beijing Genomics Institute Co., Ltd. The raw data were filtered with SOAPnuke (v1.5.6) [36] to remove adapter-containing reads and low-quality reads and the reads containing more than 5% of unknown N-base. Bowtie2 (v2.3.4.3) [37] and HISAT2 (v2.1.0) [38] were used to separately align the clean data with the reference gene and genome. The matched data were calculated and normalized to FPKM by RSEM (v1.3.1) [39]. Differentially expressed genes (DEGs) were measured using DESeq2 (v1.4.5) [40] with a threshold at $|\log2FC| \geq 0$, Q value ≤ 0.05. For further functional classification of DEGs, KEGG (https://www.kegg.jp/) enrichment analysis was conducted using Phyper (https://en.wikipedia.org/wiki/Hypergeometric_distribution) in R software. Furthermore, the gene set enrichment analysis (GSEA) was also conducted. Hierarchical cluster analysis of DGEs was performed by heatmap (v1.0.8).

Statistical Analysis

GraphPad Prism 8.0.1 (GraphPad Inc., San Diego, CA, USA) was used for statistical analysis. All data were obtained from three independent experiments and expressed as the means ± standard deviation (SD). Significant differences between the two groups were evaluated by the Mann–Whitney U tests for samples that were not normally distributed, and the two-tailed unpaired Student t-test was used for data with normal distributions. To evaluate the significant difference among three or more groups, the one-way ANOVA and Tukey's post hoc test were used for normally distributed data, and the Kruskal–Wallis was used for nonnormally distributed data analysis. P value < 0.05 was considered statistically significant.

Results

PPM Enhances SARS-CoV-2 Vaccine-elicited Cellular Immunity in Cell-based Model

We previously showed that oral administration of *Lactobacillus plantarum* GUANKE (LPG) could effectively promote

humoral response against COVID-19 in mice and significantly increase the number of specific IFN-γ-producing splenocytes in response to re-stimulation of RBD-derived peptides [20]. In the present study, we intended to find more effective immunobiotics to enhance SARA-CoV-2 vaccine-elicited immune response for prevention of COVID-19. We first screened candidate strains using the in vitro cell model and found that PPM treatment was able to increase the number of IFN-γ-producing splenocytes compared with LPG treatment (Fig. 1A, B), suggesting that PPM may be more efficient in enhancing SARA-CoV-2 vaccine-elicited cellular immunity than LPG. We obtained the draft genome of PPM, which was 1,768,046 bp in size and possessed a mean G + C content of 37.24% (Fig. 1C). Phylogenetic tree highlights the position of PPM relative to the representative strains (Fig. 1D).

Oral PPM Administration Significantly Boosts Humoral Response Against SARS-CoV-2 in Mice

To evaluate the effect of PPM on humoral immune response to SARS-CoV-2 vaccine, ICR mice were primed and boosted with SARS-CoV-2 vaccines at weeks 0 and 4 followed by oral administration of PBS or PPM as shown in the flowchart (Fig. 2A). The serum RBD-binding antibody titers of IgG, IgG1, and IgG2a were measured on day 7 post-administration of PPM. The results showed that the serum geometric mean titers (GMT) of IgG, IgG1, and IgG2a in the PBS and PPM groups were not significantly different at the baseline (before antibiotic treatment). On day 7 after oral PPM administration, the titers of RBD-specific IgG increased to GMT 785,067 and 1,843,200 in the PBS and PPM groups, respectively ($P = 0.0345$; Fig. 2B). Similarly, fourfold higher RBD-specific IgG1 titers were observed in the PPM (GMT = 1,228,800) versus the PBS group (GMT = 300,800) ($P = 0.0038$; Fig. 2C). The RBD-specific IgG2a titers were also 15.8-fold higher in PPM (GMT = 962,560) than the PBS group (GMT = 60,800) (Fig. 2D). The neutralizing antibody (nAb) titers were only insignificantly higher (1.3-fold) in the PPM than the PBS group (Fig. 2E). Furthermore, we analyzed the antibody index, which is the ratio of antibody titers at indicated time points to their counterparts at baseline, and is often used to normalize the baseline values. The results showed that the antibody indexes of IgG, IgG1, and IgG2a, but not nAb, were significantly larger in the PPM than the PBS group (Fig. 2F–I).

We further assessed RBD-specific binding antibody and nAb titers in BAL collected on day 7 after oral PPM administration. RBD-specific IgG titers in the PPM group (GMT 8800) were significantly higher by 2.29-fold than those in the PBS group (GMT = 3840) ($P = 0.0254$; Fig. 3A). However, there was no significant difference in the titers of specific IgG1 and IgG2a in BAL between the PBS and PPM groups (Fig. 3B, C). The nAb titers in the PPM group (GMT = 282) were 1.6-fold higher than those in the PBS group (GMT = 180) ($P = 0.0397$; Fig. 3D).

Together, these results indicate that oral PPM administration enhanced effective humoral immune response in SARS-CoV-2 vaccinated mice.

Oral PPM Administration Promotes Humoral Response Through Enhancing the B Cell Receptor Signaling Pathway

To explore how PPM affects the immune system, we collected spleen and BAL cells to perform transcriptome analysis after 7 days of oral PPM administration. We identified 1070 differentially expressed genes (DEGs) in the spleens

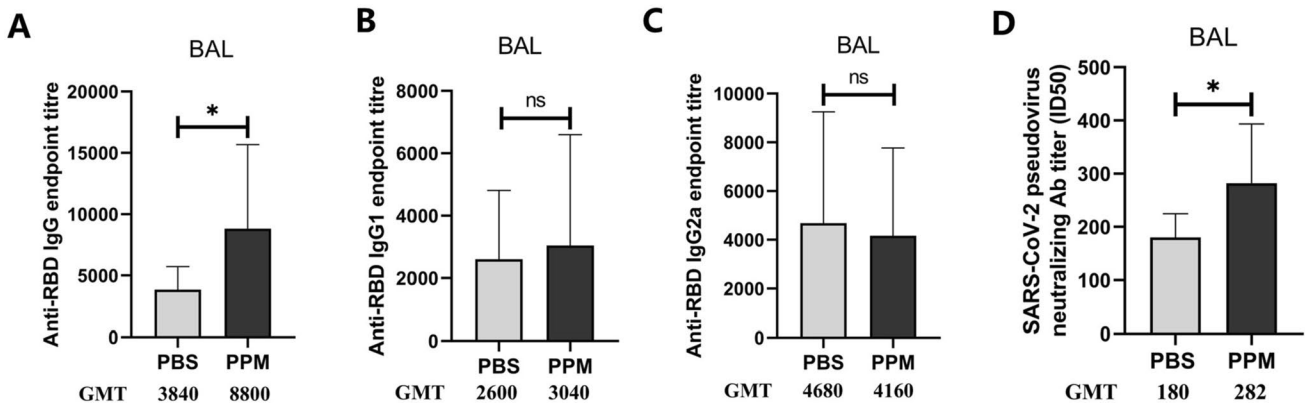

Fig. 3 Oral PPM administration immediately after immunization improved BAL antibody levels in mice. BAL RBD-specific IgG (**A**), IgG1 (**B**), and IgG2a (**C**) and neutralizing antibody (**D**) levels were assessed at day 7 post-oral PBS or PPM administration by ELISA and the pseudovirus neutralization assay. BAL: bronchoalveolar lavage; GMT: geometric mean titer. The data are showed as means ± SD from three independent experiments, and the Mann–Whitney tests were performed to analyze differences between the PBS and PPM groups. *$P < 0.05$

of the PBS and PPM groups including 653 upregulated and 417 downregulated genes, whereas 2103 DEGs were discovered in BAL cells of the PBS and PPM groups including 1183 upregulated and 920 downregulated genes (Fig. 4A). To identify the effects of these DGEs on the KEGG pathways, DEGs were subjected to gene set enrichment analysis. Interestingly, the B cell receptor signaling pathway was enriched in both spleen (Fig. 4B) and BAL cells (Fig. 4D), suggesting that oral administration of PPM can activate B cell receptor signaling pathway in mice, which is consistent with the observed enhancement of SARS-CoV-2 humoral responses induced by PPM administration. We further conducted a hierarchical cluster analysis of DGEs. As shown in Fig. 4C, E, a number of significant genes in

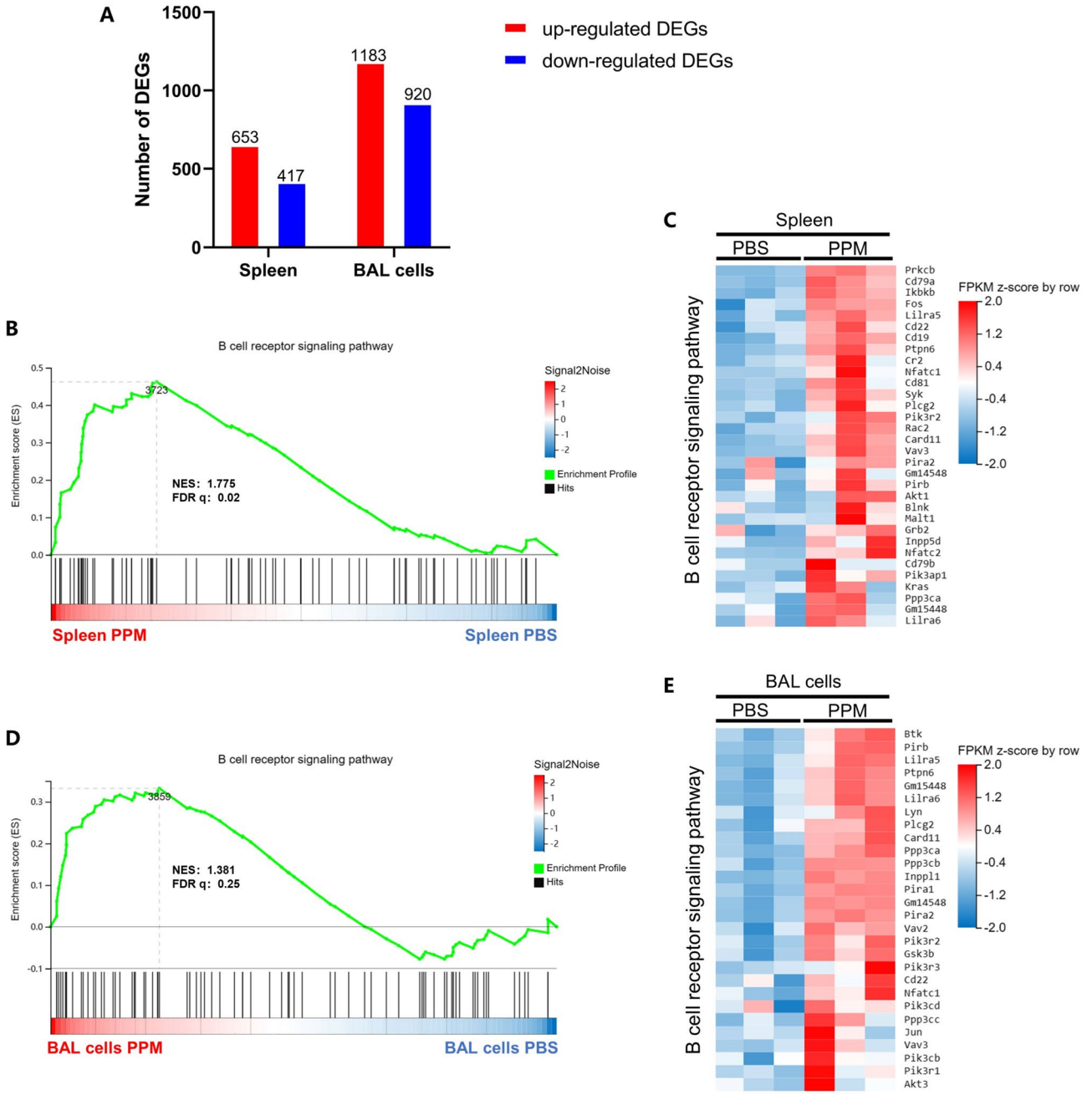

Fig. 4 Oral PPM administration stimulated the B cell receptor signaling pathway in the mouse spleen and BAL cells. **A** The number of differentially expressed genes (DEGs) in the spleen and BAL cells between the PPM and PBS groups. The gene set enrichment analysis (GSEA) showed the activation of B cell receptor signaling pathway in both the spleen (**B**) and BAL cells (**D**) in the PPM group compared with the PBS group. The clustering heatmap depicts the FPKM values of genes with dominant roles in the B cell receptor signaling pathway in the spleen (**C**) and BAL cells (**E**). NES, normalized enrichment score; FDR-q, false discovery rate *q* value

the B cell receptor signaling pathway were upregulated in the spleen and BAL cells of the PPM group compared with the PBS group. Specifically, the expression of critical genes related to the activation of B cells such as *CD19* [41, 42] was upregulated in the spleen of the PPM group. In addition, the genes associated with signal transduction of the B cell receptor signaling pathway such as *CD79a/CD79b*, *Syk* [43], and *Blnk* [44] were upregulated in the PPM group. The gene expression of *Akt1* [45], which is critical for B cell maturation and survival, was also higher in the PPM group (Fig. 4C). In BAL cells, several key genes related to B cell activation and maturation such as *Akt3* and *Lyn* [46] were upregulated in the PPM group (Fig. 4E). Collectively, the critical genes for B cell activation, maturation, and proliferation in the B cell receptor signaling pathway were upregulated in both the spleen and BAL cells of the PPM group, which may explain the increased SARS-CoV-2-specific humoral responses observed in the PPM group.

Oral PPM Administration Promotes CD4 + T Cell Response Which May Help Fight Against COVID-19

We next investigated if oral PPM administration could affect the cellular response to the vaccine. The number of IFN-γ-producing splenocytes in response to restimulation of RBD-derived peptide pools on day 7 post oral PPM administration was detected by ELISpot. The results showed that IFN-γ-producing cells in response to RBD were dramatically increased in the PPM group compared with the PBS groups (Fig. 5A), indicating that oral PPM can promote RBD-specific T cell response. To further explore the mechanism of how T cells responded to PPM treatment, we performed RNA-seq and observed the upregulation of key genes related to T cell activation and proliferation (Fig. 5B). CD28 is a receptor for co-stimulatory signal which binds CD80 and CD86 leading to activation of naïve T cells [47]. CD2 also has the function of T cell activation [48]. Upregulation of *Cd28* and *Cd2* in the PPM group indicates that oral PPM administration could enhance T cell response. Furthermore, the expression of critical genes including *Cd4*, *H2-Oa*, *H2-DMb*, *H2-DMb1*, and *Ctss* involved in the MHC-II pathway was upregulated (Fig. 5C). Thus, we speculate that the PPM administration may mainly induce CD4 + T cells to produce IFN-γ in response to specific RBD stimulation in the splenocytes as shown in the ELISpot assay, leading to improvement of the cellular immunity of SARS-CoV-2 vaccine. To verify our hypothesis, we performed flow cytometric analysis and found that the percentage of CD4 + IFN-γ + T cells in PPM mice was significantly increased (Fig. 5D) while CD8 + IFN-γ + T cells had no significant difference (Fig. 5E), suggesting that the more IFN-γ-producing cells induced by PPM was dominated by CD4 + T cell subset. Together, these results suggest that oral PPM administration may enhance CD4 + T cell response through the MHC II pathway, which may be responsible for protection against COVID-19.

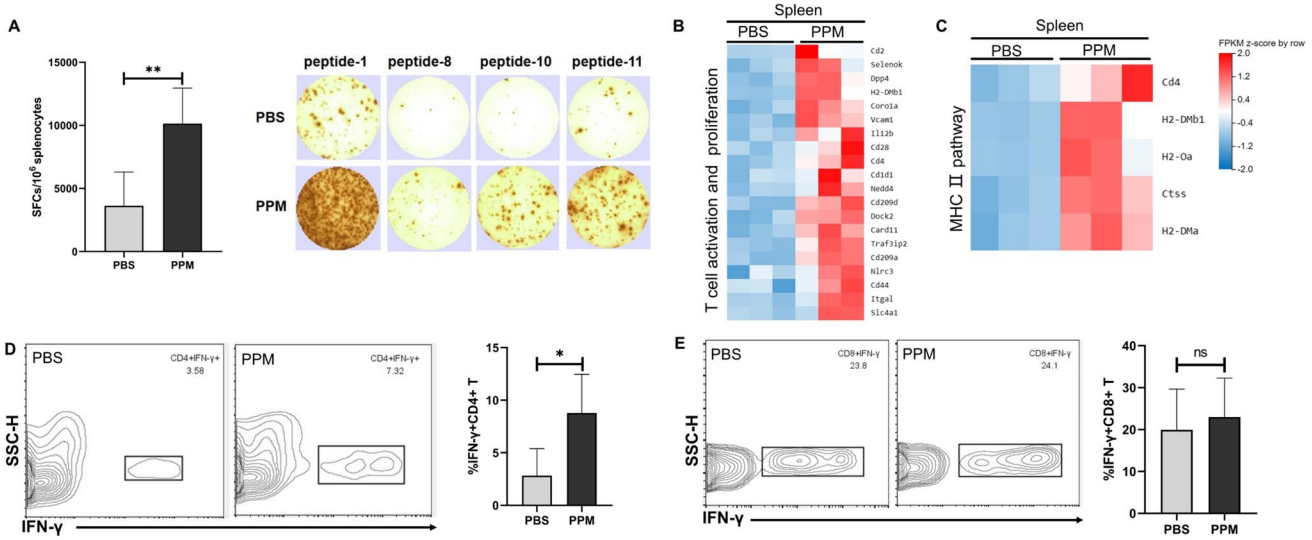

Fig. 5 Oral PPM administration promoted the number of IFN-γ-producing CD4 + T cells in the spleen in response to specific RBD peptides through the activation of the MHC II pathway. **A** The number of IFN-γ-producing splenocytes was accessed by ELISpot; the numbers of IFN-γ secreting cells (left) and the representative spot plots (right) are shown. The heatmap shows the FPKM values of the genes associated with T cell activation and proliferation (**B**) and the MHC II pathway (**C**) in the spleen. **D** Representative FACS plots (left) and frequencies of IFN-γ + cells (right) among CD4 + T cells isolated from the spleen. **E** Representative FACS plots (left) and frequencies of IFN-γ + cells (right) among CD8 + T cells isolated from the spleen of mice. Bar figures represent means ± SD of three independent experiments. Statistical differences were analyzed by Mann–Whitney tests. $^{*}P < 0.05$, $^{**}P < 0.01$

Oral PPM Administration Immediately After SARS-CoV-2 Vaccination May Downregulate Inflammation Pathway

To further investigate the immune modulation of oral PPM administration in SARS-CoV-2-vaccinated mice, KEGG enrichment analysis for the transcriptome data of spleen and BAL cells was conducted. The bubble diagram displayed the top 20 KEGG pathways downregulated in the PPM group relative to the PBS groups. The results showed that several inflammatory pathways including TNF, MAPK, and NF-kappa B were downregulated in BAL cells (Fig. 6A), and a downregulated MAPK signaling pathway was also observed in spleen cells (Fig. 6E), suggesting that oral administration of PPM could regulate the systemic inflammatory response in SARS-CoV-2-vaccinated mice. We further analyzed the change of DEGs in these inflammatory-associated pathways between the PPM and PBS groups, and the results of hierarchical clustering analyses indicated that several key genes such as *Fas*, *Met*, *Myd88*, and *Tnf* in the TNF, MAPK, and NF-kappa B signaling pathways were downregulated in the PPM group (Fig. 6B, C, D, F). Hsps are implicated in proinflammatory responses [49]. The coding genes of Hsps including *Hspa1a*, *Hspa1b*, *Hspa2*, *Hspa8*, *Hspa11*, and *Hspb1* in the MAPK signaling pathway were also downregulated in splenocytes of PPM-treated mice compared with PBS-treated mice (Fig. 6F). Furthermore, the expression of genes for chemokines *Cxcl5* and *Cxcl10* in the TNF signaling pathway was significantly lower in BAL cells of PPM mice than that in PBS mice (Fig. 6D).

Discussion

COVID-19 caused by SARS-CoV-2 has resulted in more than 6 million deaths worldwide, and an efficient vaccine is considered helpful to prevent further morbidity and mortality [50–52]. It has been reported that the effectiveness and durability of vaccines are associated with many factors including age, sex, and microbiota [6], and increasing animal and clinical trials have indicated that certain immunobiotics can promote the efficacy of vaccines [14, 53]. In our earlier study, we found that a special strain *Lactobacillus plantarum* GUANKE (LPG) could boost humoral and cellular response to SARS-CoV-2 vaccine in mice [20]. We intended to find more immunobiotics with better efficacy or synergetic

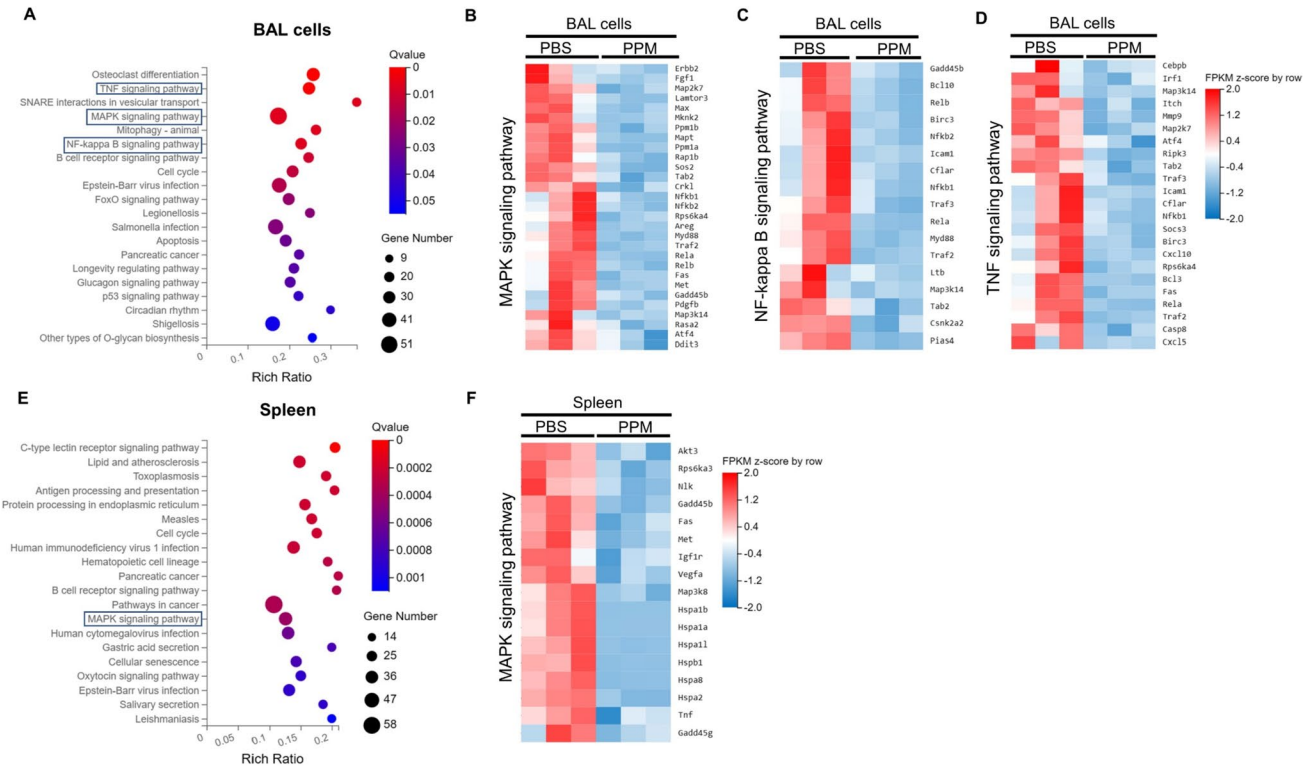

Fig. 6 Transcriptomic data indicated the potential anti-inflammatory function of PPM. The bubble diagram shows the top 20 KEGG pathways activated in the BAL cells (**A**) and spleen (**E**) upon the oral PPM administration compared with the PBS group. The FPKM values of genes involved in the TNF (**B**), MAPK (**C**), and NF-kappa B (**D**) signaling pathways in BAL cells isolated from the PPM and PBS groups are presented as the clustering heatmaps. **F** The clustering heatmap shows the FPKM values of genes involved in the MAPK signaling pathway in the spleens of the PPM and PBS groups

effect in modulating the immune response to SARS-CoV-2. In this study, we demonstrated that PPM could boost specific humoral and CD4 + T cell-mediated response through the BCR signaling and MHC II pathways, respectively, in SARS-CoV-2-vaccinated mice.

The humoral response of the host defense is critical in response to vaccination and natural infections. There is increasing evidence supporting that immunobiotics can enhance the production of vaccine-induced specific binding antibodies. A randomized, double-blind, placebo-controlled study showed that oral BB-12® and *L. casei* 431® increased influenza-specific IgG, IgG1, and IgG3 levels 4 weeks after vaccination [54]. The cholera-specific IgG levels were increased at day 7 after the oral cholera vaccine administration with *B. lactis* Bl-04 or *L. acidophilus* La-14 compared with cholera vaccine alone [55]. Consistent with other studies, we observed a ~ 2.3-fold and ~ 2.29-fold increase of RBD-specific IgG binding antibody in serum and lung, respectively, in the PPM group. Among all antibodies, nAb within the airways is considered to play a critical role in protecting susceptible individuals from SARA-CoV-2 infection [56, 57]. Passive transfer of nAb also can prevent severe COVID-19 in animal models [58, 59]. Thus, we assessed the effect of PPM on nAb levels and observed a ~ 1.6-fold increase of nAb in the lung of the PPM group. Transcriptome analysis indicated that several genes in the BCR signaling pathway including *CD19*, *CD79a*/*CD79b*, *SyK*, *Blnk*, and *Akt1* were upregulated in the PPM group. CD19 is involved in B cell proliferation, differentiation, activation, and antibody production [41]. CD79a/CD79b, SyK [43], and Blnk [44] play a key role in transmitting signals in the BCR signaling pathway. Akt1 is involved in B cell maturation [45], and it is required for germinal center formation and maintenance [60]. Together, these studies support PPM administration being used to boost the humoral response by upregulating the BCR signaling pathway.

The cellular immune response is also a significant component of immune defense. Passive transfer of adoptive CD4 + T cells has been shown to protect the host against SARS-CoV-1 and MERS-CoV infections [61]. Increasing evidence indicates that T cells play an important role in conferring protection against SARS-CoV-2 [62]. It has been reported that individuals with durable and stronger memory T cell responses have mild COVID-19 symptoms or asymptomatic infections [63]. Thus, enhancing T cell responses may contribute to combating COVID-19. Several studies have demonstrated that certain immunobiotics can enhance T cell-mediated immune response in vaccination. We also previously found a prolonged specific T cell response in the immunobiotic-treated group compared with the PBS-treated group [20]. *Lactobacillus rhamnosus* GG can enhance HRV-specific T cell responses to produce more IFN-γ [64], which is consistent with our results that oral PPM administration enhanced IFN-γ-producing CD4 + T cells in response to specific RBD re-stimulation. Furthermore, our transcriptome analysis also supported the PPM-boosted T cell response. CD28 and CD2 are significant receptors for T cell activation, and their coding genes are upregulated in the PPM group [47, 48]. IFN-γ plays a significant role in antivirus response. A study showed that low levels of IFN-γ secretion across all stimuli/viruses were related to more morbidity and mortality from respiratory virus infections [65]. PPM can stimulate IFN-γ-producing CD4 + T cells to enhance protection against COVID-19. Furthermore, we also observed upregulation of genes associated with the MHC II pathway such as *Cd4*, *H2-Oa*, *H2-DMb*, *H2-DMb1*, and *Ctss*. The primary function of MHC II is to present the processed exogenous antigens to CD4 + T cells [66, 67], indicating that PPM may enhance CD4 + T cell response through upregulating the MHC-II pathway.

An increasing body of evidence has revealed that the inflamm-aging, characterized as increased chronic and systemic sterile inflammatory state, may be a determinant of vaccine responsiveness and thus reducing the baseline inflammation might be an attractive strategy to boost vaccine responses[68, 69]. Recent data suggests that the low-level chronic inflammatory status may hamper immune responses to vaccination, thus, novel strategies aiming at reducing background inflammation may offer opportunity to improve vaccine responses in the elderly [70]. Our transcriptome analysis results showed that PPM administration immediately after vaccination significantly downregulated the TNF, NF-kappa B, and MAPK signaling pathways. This result suggests that PPM might be a potential new strategy as immunomodulators for older subjects who have an impaired immune response or develop severe inflammation after SARS-Cov-2 vaccination. In addition, activation of NF-kappa B pathways can upregulate inducible enzymes, alarmins, chemokines, and inflammatory cytokines resulting in cytokine storm, which is a major contributor to the high mortality rate observed in COVID-19 patients [71]. Furthermore, activation of the MAPK pathway can be triggered by viral infection and then potentially exacerbates the infection [72]. In this study, both NF-kappa B and MAPK pathways were downregulated in the PPM group indicating that PPM may exhibit the anti-inflammatory function.

In summary, in this study, we demonstrated that administration of *Pediococcus pentosaceus* MIANGUAN enhanced SARS-CoV-2 vaccine-induced humoral and cellular immune responses by upregulating both the BCR and MHC II pathways. We further found that oral administration of PPM downregulated the critical proinflammatory genes involved in the TNF, MAPK, and NF-kappa B signaling pathways, highlighting the potential anti-inflammatory function of PPM. These results suggest that PPM may enhance vaccine

effectiveness by boosting the specific humoral and cellular immunity response and downregulate the expression of inflammation pathways. A limitation of this study is that due to lack of adequate hACE-2 knockout mice and availability of P3 biosafety-required animal room, we did not conduct a challenge experiment to further validate the potentiating effect of PPM on SARS-CoV-2 vaccine's protection against future infection. While this needs to be considered in the future studies, more importantly, the translational value (applicability to humans) of the results in this animal study should be verified in the clinical trials.

Supplementary Information The online version contains supplementary material available at https://doi.org/10.1007/s12602-023-10205-z.

Acknowledgements We thank the member of JGX and ZHR laboratory for advice and assistance during the study. We also thank Dr. Jianqing Xu and Dr. Kangli Cao from Zhongshan Hospital, Institutes of Biomedical Sciences, Shanghai Medical College, Fudan University for SARS-CoV-2 vaccine (DNA-S and AdC68-Delta-S).

Author Contribution JGX and ZHR conceived and designed the experiments. YLC conducted animal experiments and prepared the paper. ZJC constructed screening system of immunobiotics in vitro. SML, ZHW, and CYM assisted in animal study and ELISA. GZ isolated the PPM. MSC and JY analyzed data. All authors contributed to the article and approved the submitted version.

Funding This work was supported by National Key R and D Program of China (2023YFC0871200) and Research Units of Discovery of Unknown Bacteria and Function (2018RU010) to J.G.X.

Data Availability The original contributions presented in the study are publicly available. This data can be found in the NCBI SRA database (accession numbers: PRJNA953511).

Declarations

Ethics Approval The protocol for animal study was reviewed and approved by the Laboratory Animal Welfare and Ethics Committee of the National Institute for Communicable Disease Control and Prevention, Chinese Center for Disease Prevention and Control.

Competing Interests The authors declare no competing interests.

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