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Effects of Taishan Pinus massoniana pollen polysaccharide on the subunit vaccine of *Proteus mirabilis* in birds

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ABSTRACT

Three adjuvants, namely, Taishan Pinus massoniana pollen polysaccharide (TPPPS), white mineral oil (WO) and propolis (PP), were added to the outer membrane protein (OMP) of *Proteus mirabilis* (*P. mirabilis*) and their effects were compared. Three hundred 1-day-old chicks were randomly divided into five groups (I–V), with 60 chicks per group, and injected subcutaneously with WO-OMP vaccine (I), PP-OMP vaccine (II), TPPPS-OMP vaccine (III), OMP-only vaccine (IV) and physiological saline (V) at 3, 7 and 12 days old. On days 3, 7, 14, 21, 28, 35, 42 and 49 after the first vaccination, the antibody titers, interleukin-2 levels (IL-2) and T-lymphocyte proliferation rates in the peripheral blood as well as the secreting-type IgA levels (SIgA) in the duodenum were measured. On day 7 after the third vaccination, the chicks were challenged with *P. mirabilis* strain Q1 and the protective effects of each group were observed. The highest protective rate was observed in group III. Moreover, the antibody titers as well as IL-2, SIgA and T-lymphocyte proliferation rates in this group significantly increased and were significantly higher than those in the other groups at most time points. The results indicate that TPPPS could significantly enhance the effects of the subunit vaccine of *P. mirabilis*; induced stronger humoral, cellular and mucosal immunity as compared with WO and PP; and should be developed as a vaccine adjuvant.

1. Introduction

Vaccines based on the outer membrane protein (OMP) have been showed to possess excellent immunogenicity and stimulate not only humoral immunity but also cellular immunity [1]. They exclude prime bacterial virulence factors and have a better immunogenicity compared with those based on whole cells. We have previously demonstrated that subunit vaccines based on the OMPs of *Bordetella avium* and *Proteus mirabilis* (*P. mirabilis*) in chickens are safe and could stimulate immune responses [2,3]. However, subunit vaccines achieve a robust immune response against weakly immunogenic targets by inclusion of an adjuvant, which augments the immune response to a co-delivered antigen [4].

Adjuvants are nonspecific immunoenhancers used to strengthen the immune response of the body or modulate immune response types [5]. They may serve as a depot for an antigen to extend its retention time in the body, as a means to target the antigen to immune cells, or as a stimulator of the immune response [6]. Traditional vaccine adjuvants, such as white mineral oil (WO) and propolis (PP), can induce humoral immunity [7,8]. PP, as the primary choice of many modern vaccine adjuvants, presents a number of biological and pharmacological properties, such as antitumor, anti-inflammatory, antioxidant, antibacterial, antiviral and immunomodulatory activities, among others, and does not exhibit antigenicity, irritability and toxicity [9]. However, this classic oil adjuvant is used in only a few vaccines because of its severe side effects [10].

A number of botanical polysaccharides have been proven to possess immune-enhancement effects [11,12]. Due to their diversity, abundance and effectiveness, potential use of botanical polysaccharides as new adjuvants for medical and veterinary use has aroused the interests of many researchers in recent years [13]. Pine pollen is a kind of omnipotent nutritional pollen, gloriously known as the 'King of Pollen' [14]. We have previously demonstrated that oral administration of cell wall-broken pine pollen can significantly increase immune functions in both chickens and rabbits and that the hypodermic action of Taishan Pinus massoniana pollen polysaccharide (TPPPS) significantly increases immune responses to vaccines against rabbit hemorrhagic disease in rabbits [15].

This study compared the effects of adding TPPPS, PP and WO to the subunit vaccine of *P. mirabilis*. Its objectives were to determine

Abbreviations: TPPPS, Taishan Pinus massoniana pollen polysaccharide; WO, white mineral oil; PP, propolis; OMP, outer membrane protein; *P.mirabilis*, *Proteus mirabilis*; IL-2, the interleukin-2; SIgA, the secreting-type IgA; SDS-PAGE, sodium dodecyl sulfate polyacrylamide gel electrophoresis; ConA, concanavalin A.

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the effects of TPPPS on the subunit vaccine of *P. mirabilis*, identify the main superiorities of TPPPS as compared with conventional adjuvants and provide theoretical evidence for its development as a new adjuvant.

2. Materials and methods

2.1. Preparation of adjuvants

TPPPS (net content = 72.20%) was provided by our laboratory and prepared according to a certain of proportion based on our previous experiment [16]. It was diluted to 100 mg/ml (net content) with deionized water, sterilized by filtrating and stored at 4 °C. PP and WO were prepared as previously described [17], sterilized and then stored at 4 °C.

2.2. Preparation of the subunit vaccines

The bacterial strain used for this study, *P. mirabilis* strain Q1 of chickens, was identified and preserved previously by the Animal Biotechnology and Disease Control and Prevention Laboratory of Shandong Agriculture University. The LD_{50} of strain Q1 was determined to be 1.545×10^8 CFU/ml in the preliminary test.

The OMP of *P. mirabilis* was extracted according to the method reported by Wooldridge and Williams [18], sterilized and stored at −20 °C. The protein content of samples was calculated using the method of Bradford [19].

The molecular weight of the extracted OMP was detected by sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE). A standard curve was drawn based on a logarithm of the protein molecular weight marker and the electrophoretic migration rate to calculate the molecular weight of the protein samples.

TPPPS was diluted and mixed with the OMP of *P. mirabilis* at the ratio of 1:1, reaching the final concentration of 200 mg/ml. The solution was immingled enough using a vortex mixer (TPPPS-OMP vaccine) and stored at 4 °C after the routine control was determined to be up to standard [20]. The PP-OMP and WO-OMP vaccines were simultaneously prepared following the same method and stored at 4 °C.

2.3. Animals

One-day-old specific-pathogen-free (SPF) male chicks were purchased from Jinan Spafas Poultry Co. Ltd. and housed in wire cages (150 cm × 100 cm × 50 cm) in air-conditioned rooms at 37 °C for 24-h light cycle at the beginning of the pretrial period. The temperature was gradually reduced to room temperature and the photoperiod was adjusted to a 12-h light cycle per day, and these settings were kept constant in the following days. The chicks were fed with a commercial starter diet provided by Taian Liuhe Lelin Feed Co. Ltd. All procedures related to the animals and their care conformed to internationally accepted principles as found in the Guidelines for Keeping Experimental Animals issued by the Government of China.

2.4. Immunization

Three hundred 1-day-old chicks were randomly divided into five groups (n = 60 per group). Groups I–V were injected subcutaneously with 0.2 ml of WO-OMP vaccine, PP-OMP vaccine, TPPPS-OMP vaccine, OMP-only vaccine and physiological saline, respectively, at 3, 7 and 12 days old. The final concentrations of OMP were 500 μg/ml at the first vaccination, 1 mg/ml at the second vaccination and 2 mg/ml at the third vaccination. Seven days after the third vaccination, 30 chicks from each group were challenged with 100 LD_{50} *P. mirabilis* strain Q1 by oral administration.

On days 3, 7, 14, 21, 28, 35, 42 and 49 after the first vaccination, the peripheral blood and duodenum were sampled for analysis. The antibody titers were detected using indirect enzyme-linked immunosorbent assay (ELISA), T-lymphocyte proliferation rates were detected with flow cytometry and secreting-type IgA (SIgA) and interleukin-2 (IL-2) were measured using ELISA kits (Shanghai Hengyuan Bio-technology Co. Ltd.). The pathogenesis and survival status of chicks were clinically examined daily for 14 successive days after challenge. The mortality, morbidity and protective rates in each group were calculated as follows:

Mortality (%) = number of dead chicks within 14 days post-challenge/number of sample × 100%.
Morbidity (%) = number of dead chicks and chicks with clinical symptoms on day 14/number of sample × 100%.
Protective rate (%) = number of chicks without clinical symptoms on day 14/number of sample × 100%.

2.5. Serum antibody titers detection

One milliliter of blood sample was collected from five chicks per group. Sera were collected and antibody titers were detected with indirect ELISA method [21].

2.6. Serum IL-2 detection

IL-2 levels in peripheral blood were detected using a chicken IL-2 ELISA kit.

2.7. T-lymphocyte proliferation assay

One milliliter of aseptic blood was collected from experimental SPF chicks and transferred immediately into aseptic capped tubes containing EDTA Na_2. Lymphocytes were separated and diluted to 1×10^6/ml in RPMI-1640 medium with 10% fetal bovine serum after cell viability was assessed by trypan blue exclusion [22]. The solution was added to 96-well culture plates at 100 μl per well. ConA (0.025 mg/ml in RPMI-1640 medium) was then added, resulting in the final concentration of 10 μg/ml after culture plates were incubated for approximately 2 h. The negative control wells were arranged at the same time [23].

After 48 h of cultivation, the solution of 96-well culture plates with ConA and that without ConA (control) were blended into corresponding suspensions by blowing. The suspensions were moved into experimental and control centrifuge tubes and then washed twice with PBS through centrifugation and supernatant elimination. Finally, lymphocytes were suspended in 1 ml of PBS and counted. Fifty micrograms of PI dye was added to each tube with 1×10^6 cells. Next, these tubes were placed in the dark for 30 min and washed with PBS once again. Upon irradiation with laser generated by flow cytometry at the wavelength of 488 nm, lymphocyte percentages at the S phase in both experimental and control tubes were measured. Percentages at the S phase (SPF) of the tubes containing ConA represented the degree of T-lymphocyte proliferation [24]. SPF was calculated as follows:

$$SPF = \frac{S}{(G_0 + G_1) + S + (G_2 + M)} \times 100\%$$

2.8. Duodenum SIgA detection

Five centimeters of duodenum were harvested from three chicks per group. The enteric cavity was washed with 5.0 ml of ice-cold 1% BSA-PBST containing 0.1 mg/ml pancreatic trypsin inhibitor 10 times, and the supernatant of the rinse solution was stored at–20 °C after the latter was centrifuged at 10,000 rpm for

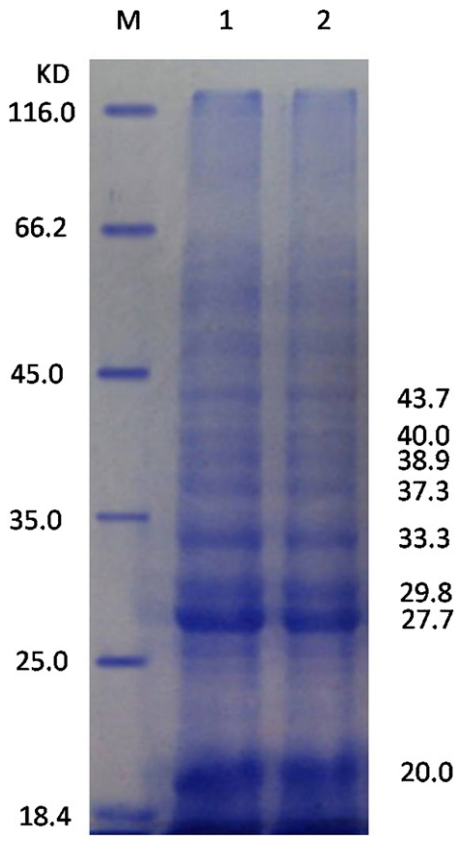

Fig. 1. SDS-PAGE of the outer membrane protein (OMP) of *P. mirabilis* strain Q1: M, Marker; 1, 2, the parallel OMP samples of strain Q1. Strain Q1 was cultured at 37 °C for 18 h. OMP was extracted according to the method of Wooldridge and eight straps were observed.

5 min. The duodenum SIgA was then detected according to the protocol described for the chicken SIgA ELISA kit.

2.9. Statistical analysis

Data were expressed as mean ± SD, and Duncan's multiple-range test was performed to analyze the differences among groups using SPSS 17.0. χ^2-Test was used to analyze differences in morbidity, mortality and protective rates. $P < 0.05$ was considered statistically significant.

3. Results

3.1. Preparation of the subunit vaccines

Eight straps were observed by SDS-PAGE from the OMP of *P. mirabilis* strain Q1 (Fig. 1). The results slightly differ from the conclusion of Zhu [25], which can be attributed to the variations in the culture conditions of bacteria, preparation of OMP and strains used. The content of OMP was 5.43 mg/ml.

The safety of the subunit vaccines was determined as follows:

i. After centrifugation at 3000 rpm for 15 min and not layering, the subunit vaccine demonstrated good stability.
ii. The subunit vaccine was inoculated on nutrient broth, blood agar, SDS agar and anaerobic beef liver broth, and no bacterium grew.
iii. SPF chicks were injected with the subunit vaccine, and clinical symptoms did not appear in the experimental and blank control groups after a certain period.

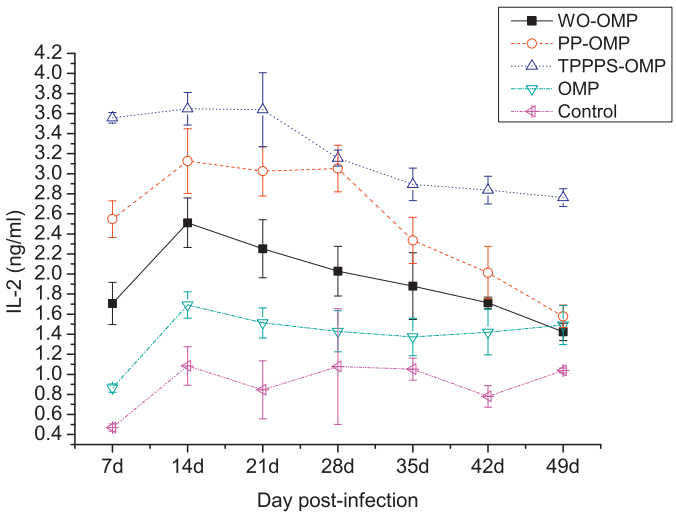

Fig. 2. Changes in IL-2 levels in the peripheral blood. Chicks were immunized with WO-OMP vaccine, PP-OMP vaccine, TPPPS-OMP vaccine, OMP-only vaccine and physiological saline at 3, 7 and 12 days old. On days 7, 14, 21, 28, 35, 42 and 49 after the first vaccination, sera were collected for IL-2 detection. Data are represented as mean ± SD at each point.

3.2. Changes in antibody titers

The antibody titers in each group are illustrated in Table 1. On day 3 after the first vaccination, the antibody titers in the immune groups (I–IV) increased and were significantly higher than those in the blank control group (V) ($P < 0.05$). On days 7–49, the antibody titers in groups I–III were significantly higher than those in groups IV and V ($P < 0.05$). On day 21, the antibody titers in group II and group III reached their peak value and were significantly higher than those in group I ($P < 0.05$), but the antibody titers in group III were higher ($P > 0.05$). On day 35, the antibody titers in group I reached their peak value but were indistinctive compared with those in groups II and III ($P > 0.05$).

3.3. Changes in IL-2

The IL-2 contents in each group are illustrated in Fig. 2. On days 7–21 and 35–49 after the first vaccination, the IL-2 contents in group III were significantly higher than those in the other groups ($P < 0.05$). The IL-2 contents in groups II were significantly higher than those in group I, but those in group I were significantly higher than those in groups IV and V on days 7–35 ($P < 0.05$). On day 14, the IL-2 contents in each group reached their peak value.

3.4. Changes in T-lymphocyte proliferation

The lymphocyte proliferation rates in each group are illustrated in Table 2. On days 7–49 after the first vaccination, the T-lymphocyte proliferation rates in groups II and III were significantly higher than those in group I ($P < 0.05$), with those in group III being significantly higher than those in group II ($P < 0.05$) except on day 42. On day 14, the T-lymphocyte proliferation rates in group I reached their peak value; on day 28, those in groups II and III reached theirs.

3.5. Changes in SIgA

The SIgA contents in each group are illustrated in Fig. 3. On days 21–42, the SIgA contents in group III were significantly higher than those in the other four groups ($P < 0.05$). On days 7–21, the SIgA contents in groups I–III were significantly higher than those in groups IV and V ($P < 0.05$). The SIgA contents in groups II and

Table 1
The changes of serum antibody titers in experimental chicks (mean ± SD, $n = 5$, $\log_2$).

Group	Day post-vaccination							
	3d	7d	14d	21d	28d	35d	42d	49d
I	3.33 ± 0.58^a	5.00 ± 0.00^a	4.67 ± 0.58^a	5.00 ± 0.00^a	6.33 ± 0.16^a	7.33 ± 0.58^a	5.00 ± 0.00^a	5.00 ± 0.00^a
II	3.00 ± 0.00^a	5.00 ± 0.00^a	5.00 ± 0.00^a	7.00 ± 0.00^b	6.00 ± 0.00^a	6.67 ± 0.58^a	5.67 ± 0.53^a	5.33 ± 0.58^a
III	3.33 ± 0.58^a	5.33 ± 0.58^a	4.67 ± 0.58^a	7.67 ± 0.58^b	6.67 ± 0.58^a	6.67 ± 0.58^a	5.67 ± 0.58^a	5.33 ± 0.16^a
IV	3.00 ± 0.00^a	3.00 ± 0.00^b	2.33 ± 0.58^b	3.00 ± 0.00^c	3.00 ± 0.00^b	2.67 ± 0.58^b	2.67 ± 0.58^b	2.33 ± 0.58^b
V	0	0	0	0	0	0	0	0

a–c: the data with different letters in a column show significant difference ($P < 0.05$).

Table 2
The changes of T lymphocyte proliferation in experimental chicks (mean ± SD, $n = 3$, %).

Group	Day post-vaccination						
	7d	14d	21d	28d	35d	42d	49d
I	22.19 ± 0.10^a	26.48 ± 0.15^a	24.25 ± 0.25^a	23.11 ± 0.10^a	17.79 ± 0.15^a	18.71 ± 0.31^a	20.24 ± 0.25^a
II	30.22 ± 0.17^b	29.88 ± 0.18^b	30.30 ± 0.31^b	38.27 ± 0.26^b	30.36 ± 0.42^b	29.30 ± 0.25^b	29.16 ± 0.12^b
III	33.94 ± 0.10^c	30.37 ± 0.32^c	28.84 ± 0.20^c	38.86 ± 0.15^c	33.31 ± 0.31^c	29.93 ± 0.85^b	31.15 ± 0.15^c
IV	19.21 ± 0.10^d	16.22 ± 0.20^d	17.81 ± 0.15^d	21.86 ± 0.19^d	15.46 ± 0.15^d	14.34 ± 0.30^c	11.64 ± 0.21^d
V	5.20 ± 0.21^e	6.20 ± 0.15^e	7.67 ± 0.20^e	9.21 ± 0.20^e	6.45 ± 0.20^e	7.92 ± 0.12^d	10.92 ± 0.10^e

a–e: the data with different letters in a column show significant difference ($P < 0.05$).

III were significantly higher than those in group I on days 14 and 28 ($P < 0.05$). The SIgA contents in groups I–III reached their peak value on day 21.

3.6. Protective effects of the subunit vaccines

The protective effects of the subunit vaccine in each group are illustrated in Fig. 4. The morbidity and mortality in group III were the lowest, and they were significantly lower than those in group IV ($P < 0.05$). The protective rate in group III was the highest, and it was significantly higher than that in group IV ($P < 0.05$).

4. Discussion

In this study, the extracted OMP of *P. mirabilis* removed many irrelevant components to immunity, overcame the main defects of attenuated live as well as inactivated vaccines and exhibited good safety and immunogenicity (Fig. 1). Meanwhile, TPPPS as an adjuvant served as a natural botan-immunoenhancer and had outstanding effects on drug residue resolution and disease control. In recent years, with its industrialization, exploitation and utilization, TPPPS has demonstrated great application prospects as a high-performance adjuvant.

The antibody titer is an indicator reflecting the state of humoral immunity of chickens [26]. The results showed that the subunit vaccines containing TPPPS, WO and PP could induce high antibody titers. The antibody titers in groups II and III reached their peak value earlier and those in group III were higher or significantly higher than those in the other groups during the whole test period (Table 1). These results confirmed that the effects of the TPPPS-OMP vaccine are better than those of the WO-OMP and PP-OMP vaccines in promoting humoral immunity.

IL-2, as an important cytokine in the body, is secreted by active T cells or CD^4+ T cells, mainly promotes T-lymphocyte proliferation and cytokine secretion, enhances the cytoactivity of Tc, NK and LAK cells and stimulates B lymphocyte proliferation and antibody secretion [27]. It indirectly reflects the state of cellular immunity

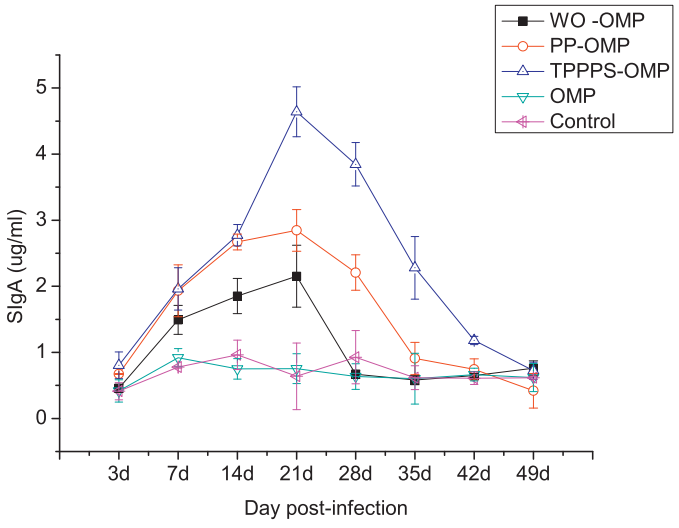

Fig. 3. Changes in SIgA levels in the duodenum. Chicks were immunized with WO-OMP vaccine, PP-OMP vaccine, TPPPS-OMP vaccine, OMP-only vaccine and physiological saline at 3, 7 and 12 days old. On days 3, 7, 14, 21, 28, 35, 42 and 49 after the first vaccination, duodenums were harvested for SIgA detection. Data are represented as mean ± SD at each point.

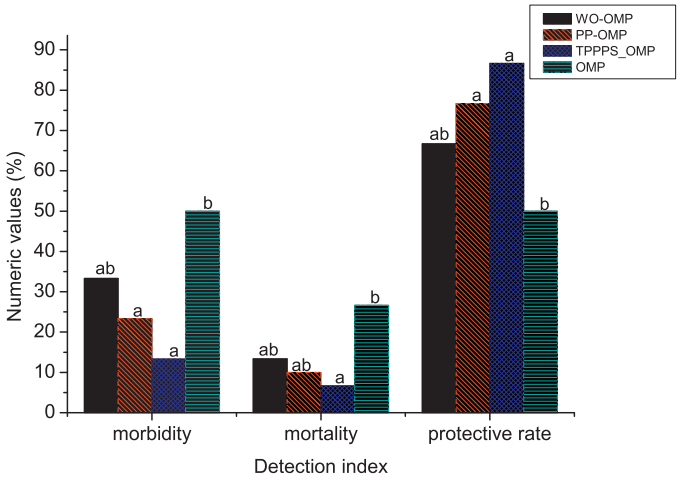

Fig. 4. The mortality, morbidity and protective rate in the test. On day 7 after the third vaccination, 30 chicks of each group were challenged with 100 LD_{50} *P. mirabilis* by oral administration. The pathogenesis and survival status of chicks were clinically examined daily for 14 successive days after challenge. a and b: bars without the same letter differ significantly ($P < 0.05$).

of chickens. Our experiment showed that the IL-2 contents in groups I–III significantly increased but that those in group III were significantly higher than those in the other groups at most time points (Fig. 2). These results indicate that the TPPPS-OMP vaccine can remarkably promote cellular immunity, which may be relevant to the immune-enhancement mechanism of the adjuvant.

Static lymphocyte stimulated by mitogen can translate to lymphocytoblast, and the method of detecting lymphocyte transformation function using the properties of lymphocyte is called the lymphocyte proliferation test. Lymphocyte proliferation is the most direct indicator of the state of cellular immunity [28]. In the present study, ConA as a T-cell mitogen was used to promote the proliferation of T lymphocytes. The results showed that the T-lymphocyte proliferation rates in groups I–III significantly increased; however, the T-lymphocyte proliferation rates in group III were higher than those in the other groups and those in group I were the lowest among these three experimental groups (Table 2). These results are similar to the results obtained from the IL-2 assay, thus further confirming that the TPPPS-OMP vaccine can elevate the state of cellular immunity and enhance the power of the body's resistance to antigens.

The mucosal immune system, which is distributed on the gut mucosal surface directly or indirectly communicating with external animal organisms, is a critical component of the body's defense against pathogenic organisms, especially those responsible for enteric infections [29]. It produces a marked effect by secreting SIgA and IgM, and SIgA prevents pathogenic organisms from penetrating the upper cortex by stopping them from residing on the mucosal surface [30]. The results showed that the SIgA contents in group III increased significantly and that, although they also increased, the effects of those in groups I and II were worse compared with the former (Fig. 3). These results indicate that the TPPPS-OMP vaccine performed better in inducing mucosal immunity.

P. mirabilis is widely distributed in nature, such as polluted water, soil or stool of humans or animals and infects through the gut [31]. Therefore, in the present study, the chicks were challenged by oral administration in our study, which is similar to the natural infection of *P. mirabilis* in chickens. The protective effects of the subunit vaccines in groups I–III showed that the protective rates in group III were the highest, followed by those in group II and that those in group I were the lowest (Fig. 4). These results indicate that group III exhibited the best immune protection.

In conclusion, this study has demonstrated that TPPPS could enhance the effects of the subunit vaccine, such as by stimulating the body to develop enough immunity against pathogenic microorganisms and inducing better humoral, cellular and mucosal immunity compared with WO and PP. In addition, TPPPS is a low-cost immune enhancer. Therefore, of the three adjuvants tested, TPPPS has the greatest potential as a vaccine adjuvant, which also lays the foundation for the development of new-generation vaccine adjuvants.

Acknowledgments

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