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Effects of polysaccharide on chicks co-infected with *Bordetella avium* and *Avian leukosis* virus

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ABSTRACT

Chicks' co-infection with immunosuppressive virus and bacteria seriously threaten the development of the poultry industry. In this study, a model was established in which chicks were injected with either subgroup B ALV (ALV-B) + *Bordetella avium* (*B. avium*), or ALV-B + *B. avium* + Taishan *Pinus massoniana* pollen polysaccharide (TPPPS), or *B. avium* only, or *B. avium* + TPPPS. The data showed that the group injected with ALV-B and *B. avium* exhibited significant inhibition of the immune function and therefore increased pathogenicity compared with the group injected with *B. avium*-only. Application of TPPPS effectively alleviated immunosuppression, and body weights increased sharply in the TPPPS groups compared with non-TPPPS groups. To some extent, TPPPS may reduce the proliferation of ALV-B. These results suggest that *Pinus* pollen polysaccharides are beneficial treating co-infections with immunosuppressive virus and bacteria and therefore have potential for development into safe and effective immunoregulator.

1. Introduction

Immunosuppression is a state of permanent or temporary immunity dysfunction that can cause an organism to become more sensitive to pathogens due to the damage to the immune system. *Avian leukosis* virus (ALV), which is considered one of the most common immunosuppressive viruses in chickens (Shi, Li, Chen, & Guan, 2012), is a disease with both vertical propagation and horizontal propagation, although vertical propagation remains the main mode of infection. The earlier ALV infects chickens, the more severe the resulting pathogenicity (Wang, Wang, Chen, Liu, & Cheng, 2011). Chickens infected with ALV are more susceptible to secondary infections. The phenomenon of co-infection in the poultry industry is fairly common. In particular, co-infection with *Bordetella avium* and ALV has been increasingly observed in recent years (Loving et al., 2010; Tan et al., 2011). *B. avium* is a highly infectious, upper respiratory infection that results in high morbidity and low mortality. Affected chickens present acute death of young birds, ophthalmia of adult birds, death of embryos, and low hatchability (10–40%) (Zhu, Zhang, & Tang, 1991). *B. avium* is a highly contagious, vertically and horizontally propagating disease (Raffel, Register, Marks, & Temple, 2002). Most studies of infectious models have focused on singular infections; however, co-infections are very common in practice. The symptomatic and pathological changes of co-infection are atypical, often resulting in immunization failure and severe economic losses. Therefore, the prevention and treatment of co-infections with immunosuppression viruses and bacteria should be given extensive attention.

Antibiotics are frequently abused in the poultry industry, which often leads to maladjustment of the normal flora and the outbreak of diseases. This phenomenon is especially serious in young chicks. Moreover, products from diseased poultry indirectly harm the health of consumers. Research on new and safe immunoregulators, especially plant polysaccharides, which have diverse biological activities, has become a popular topic (Xue et al., 2009). Previous studies have demonstrated that plant polysaccharides can improve specific or nonspecific immunological functions in several ways (Jiang et al., 2003). Polysaccharides from *Pinus massoniana* pollen, in particular, exhibit various biological and physiological activities including antihyperlipidemic, antitumor, antioxidant, anticoagulant and immunological activities (Meng, 2007).

Pinus pollen polysaccharide belongs to water-soluble polysaccharides and has extremely high hydrophilicity and viscidity

Abbreviations: TPPPS, Taishan *Pinus massoniana* pollen polysaccharide; ALV-B, subgroup B Avian leukosis virus; *B. avium*, *Bordetella avium*; IL-2, interleukin-2; IFN-γ, interferon-γ; LTR, lymphocyte transformation rate; p27, group-specific antigen; ConA, concanavalin A; TFA, trifluoroacetic acid; PMP, 1-phenyl-3-methyl-5-pyrazolone.

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(Wei et al., 2011). Research shows that Taishan *P. massoniana* pollen polysaccharide (TPPPS), which is extracted from *P. massoniana* pollen collected from Mount Tai (Chinese name: Taishan), can enhance immunologic function and production performance of animal subjected (Wei et al., 2011). What is more, it can significantly strengthen immune response and enhance the effects of the subunit vaccine (Cui et al., 2013; Zhao et al., 2013). Mixing TPPPS with vaccine could protect antigen and prolong the release of antigen (Wei et al., 2011). Therefore, TPPPS has the potential to be developed into plant-derived medicine or a vaccine adjuvant. However, it was not clear about the composition and content of monosaccharide in TPPPS. What is more, no report about its role in regulating co-infections with immunosuppression viruses and bacteria on chicks had been published. In this study, we detected the composition and content of monosaccharide in TPPPS by precolumn derivatization ultra-high performance liquid chromatography–tandem quadrupole mass spectrometry (Xu et al., 2003). TPPPS has a variety of outstanding biological activities (Wei et al., 2011; Zhao et al., 2013). To evaluate the immunoregulation effects of TPPPS on chicks, we designed the following experiment to create an immunoregulation model for the prevention and treatment of co-infection of subgroup B Avian leukosis virus (ALV-B) and *B. avium*. First, we created an ALV-induced immunosuppression model co-infected with *B. avium* and then evaluated the immunoregulatory effects of TPPPS by measuring the immune indices and antigen levels of the chicks. We also evaluated the immunoregulatory effects of TPPPS on the condition of single infection with *B. avium*.

2. Materials and methods

2.1. Materials

2.1.1. Reagents

Monosaccharide standards and trifluoroacetic acid (TFA) were purchased from Sigma (USA). RPMI-1640 and fetal bovine serum were purchased from Gibco (USA). Concanavalin A (ConA; Sigma, USA). Lymphocyte separation medium was purchased from Cedarlane (Canada). *B. avium* standard positive serum was preserved by the microorganism research laboratory of Shandong Agricultural University.

2.1.2. Extraction, purification and isolation of polysaccharide

Taishan *P. massoniana* pollen was collected in Taishan region, and TPPPS was obtained through hot water extraction and ethanol precipitation (Wei et al., 2011), and the details of this method were described as the following.

Taishan *P. massoniana* pollen was sieved by a 260-mesh sieve, and break-walled by Ultra-Micro Pulverizer. Wall-broken *P. massoniana* pollen was packed with filter paper and placed in a Soxhlet extractor, its fat extracted with ethyl ether. Pollen without fat was then mixed with deionized water in the proportion of 1:20 (volume ratio), and then 0.5% of pepsin was added. After reaction at 48 °C for 1.5 h and extraction at 80 °C for 6 h, polysaccharide was fully dissolved out. *P. massoniana* pollen suspension was precipitated overnight at 4 °C, sediment eliminated. Supernatant fluid was filtered through filter net, centrifuged (10,000 rpm/min, 10 min), and then concentrated under reduced pressure with rotary evaporator to prevent destruction of polysaccharide structure. Protein was removed from the concentrated solution using Sevage reagent twice. Finally, polysaccharide was precipitated with a quadruple volume of absolute ethyl alcohol and freeze-dried, and TPPPS was obtained. We used the anthrone–sulphoacid method (Miao, 2009) with a glucose standard curve to determine the yield and purity of the TPPPS.

We detected the composition and content of monosaccharide in TPPPS by precolumn derivatization ultra-high performance liquid chromatography–tandem quadrupole mass spectrometry. TPPPS was cleaned with C_{18} SPE cartridge, and then hydrolysised with TFA at 110 °C. The products were derivated with 50 μL 0.2 mol/L 1-phenyl-3-methyl-5-pyrazolone (PMP) for 30 min. The obtained monosaccharides were separated at a flow rate of 0.5 mL/min on a Waters ACQUITY UPLC BEH C_{18} column (2.1 mm i. d. × 50 mm, 1.7 μm) at 37 °C, by using acetonitrile and buffered salt solution (0.5 mmol/L ammonium acetate and 0.05% acetic acid) as the mobile phase in the gradient elution. The analysis of target compounds was performed in multiple reaction monitoring (MRM) mode via positive electrospray ionization, and quantified by the internal standard method.

2.1.3. Virus strains, bacterial strains

B. avium strain P_8 isolated from chicks of high virulence was selected from available *B. avium* strains preserved in our laboratory. The LD_{50} of P_8 in 1-day-old chicks was 1.458×10^4 CFU/mL (Hu, Zhu, Liu, & Bi, 2007). ALV-B (SDAU09C2) was generously provided by Prof. S. Sun (College of Veterinary Medicine, Shandong Agricultural University of China). The $TCID_{50}$ of the stock was calculated by the Reed–Muench method using an ALV-p27 antigen ELISA kit to identify ALV-positive virus cultures in DF-1 cells (Wu et al., 2011); the $TCID_{50}$ of ALV-B was $10^{-3.8}$/0.1 mL.

2.1.4. Experimental chicks

This experiment was approved by the Committee on the Ethics of Animal of Shandong (Permit No.: 20127716). One-day-old males of specific pathogen-free (SPF) Hy-line brown breed were purchased from SPAFS. All chicks were raised in isolation under the same conditions (i.e., in individual isolators with filtered air of positive pressure; drinking water and feeds were autoclaved before use).

One hundred fifty SPF chicks were randomly divided into five groups. Group I was injected intraperitoneally at the age of 1 d with ALV-B of 10^4 $TCID_{50}$/0.1 mL and then injected intraperitoneally with *B. avium* of 1.458×10^4 CFU/0.1 mL 3 d later. Group II was injected intraperitoneally at the age of 4 d with *B. avium* of 1.458×10^4 CFU/0.1 mL. Group III was simultaneously injected intraperitoneally at the age of 1 d with ALV-B of 10^4 $TCID_{50}$/0.1 mL and injected cervical subcutaneously with TPPPS of 400 mg/kg (once per day for 3 d), and then injected intraperitoneally with *B. avium* of 1.458×10^4 CFU/0.1 mL 3 d later. Group IV was injected cervical subcutaneously at the age of 1 d with TPPPS of 400 mg/kg (once per day for 3 d) and injected then intraperitoneally with *B. avium* of 1.458×10^4 CFU/0.1 mL 3 d later. The injection dose of viral and bacterial media given to each chick was 0.1 mL. Group V equivalent physiological saline was injected with the above doses as the blank control. Five chicks from each group were randomly sampled on days 7, 14, 21, 28, 35, 42, and 56 (without feed and water, before weighing for 4–6 h), and aseptic blood samples were collected from the heart to detect the relevant indices.

2.2. Index detection

2.2.1. Weight and immune organ indices

Five chicks were sampled randomly from each group to determine their weight and immune organ indexes. The chicks were then euthanized and dissected in a UV radiation-sterilized isolation chamber. The spleen, thymus, and bursa were excised surgically and weighed. The index of immune organs was expressed as the weight of immune organs relative to the live body.

2.2.2. Blood lymphocyte ratio

Approximately 1 mL of aseptic blood was collected from the heart in EDTA anticoagulant tubes. Blood lymphocyte ratios were detected by the PE-6800VET automatic blood cell analyzer.

2.2.3. Peripheral blood T-lymphocyte transformation

Lymphocytes were separated from peripheral blood (Xie, Jiang, Quan, & Wang, 2011), counted, and then compounded into a liquid. The cell density of this liquid was adjusted to 1×10^6 cells/mL by RPMI1640 medium containing 1% fetal bovine serum. The liquid was seeded into 96-well plates (Corning Costar) at 100 mL/hole. Each sample was seeded into six wells. Con A was added to three of the well (final concentration: 25 μg/mL); the negative control well was arranged simultaneously. The plates were incubated at 37 °C in a humid atmosphere of 5% CO_2 until the lymphocytes grew into a monolayer. Afterwards, 20 μL of MTT (5 μg/mL) was added into each well, and the plates were re-incubated for 3–4 h. The plates were then centrifuged at 1000 r/min for 10 min at room temperature. The supernatant was removed carefully, and 150 μL of DMSO was added into each well to dissolve the formazan crystals. The plates were shaken for 5 min to dissolve the crystals completely. The absorbance of the solution in each of the wells was detected after 15 min using the microplate reader at 490 nm (A_{490}). The lymphocyte transformation rate (LTR) was calculated as follows: LTR = (A_{490} value of ConA-stimulated − A_{490} value of non-stimulated cultures)/A_{490} value of control group × 100%.

2.2.4. Serum antibody titer

Serum antibody agglutination titers of *B. avium* were detected by a microagglutination test using the method of Zhu (Zhu et al., 1991). *B. avium* diagnostic antigen was prepared by our team at 4 °C. The serum antibody titer was expressed as mean log 2.

2.2.5. Interferon-gamma (IFN-γ) and interleukin-2 (IL-2)

Blood samples (1.0 mL/chicken) were drawn into Eppendorf tubes and allowed to clot at 37 °C for 2 h. Serum was separated by centrifugation and stored at −20 °C for IFN-γ and IL-2 detection. The concentrations of IFN-γ and IL-2 were detected using the chicken IFN-γ ELISA kit and IL-2 ELISA kit.

2.2.6. Viremia

The plasma was separated from blood under sterile conditions and incubated in 48-well culture plates with a monolayer of DF-1 cells (Feng, Li, Wu, Cao, & Liao, 2011). The plates were incubated for 7 d, after which the cell culture medium was obtained. Cell culture medium stored at −20 °C was tested for the presence of a group-specific antigen (p27). The presence of p27 on the plasma was determined using ALV-p27 antigen ELISA kit.

2.3. Statistical analysis

Data are expressed as mean ± SD. Duncan's multiple comparison analysis was performed using SPSS 17.0. *P* values of <0.05 were used to define statistical significance.

3. Results and discussion

3.1. Extraction of TPPPS

The yield rate of TPPPS was 0.83%, and the purity of the gained TPPPS was 79.4%.

Discrepancies in TPPPS extractions can cause differences in TPPPS contents, used doses and pharmacological effects (Xing & Li, 2009). Optimized hot water extraction and ethanol precipitation method were adopted to extract polysaccharide from Taishan *P. massoniana* pollen. In the process of extraction, protein and lipid were eliminated to the maximum. Therefore, the TPPPS obtained in this assay had high purity and with high reference value. Owing to the special structures of polysaccharides, they do not contain the functional groups that can absorb ultraviolet and fluorescence. Therefore, derivatization not only increases the measurement sensitivity significantly, but also makes the isolate of monosaccharide easier.

Table 1
Composition and content of monosaccharides in TPPPS.

Peak no.	Compound	Molar percentage (%)
1	Mannose	10.9
2	Ribose	8.6
3	Glucuronic acid	2.1
4	Galacturonic acid	26.8
5	Glucose	20.9
6	Galactose	18.9
7	Xylose	11.2
8	Arabinose	0.6

The results of optimized precolumn derivatization ultra-high performance liquid chromatography–tandem quadrupole mass spectrometry showed that TPPPS consist of eight monosaccharides, as shown in Table 1. We can see that the most abundant monosaccharides in TPPPS are galacturonic acid and glucose. The sensitivity of this new method is so high that it can be used for the exact determination of low-monosaccharide.

3.2. Immunoregulatory evaluation by body weight gain and relative weight of immune organs

The thymus, bursa Fabricii, and spleen are the main immune organs of birds. These organs are the main sites for immune cell formation, differentiation, and antibody formation. The developmental status of immune organs directly influences immune response levels as well as the ability of organisms to resist pathogenic microorganisms. Body weights and immune organs can be used to reflect the immune status of birds (Kim, Brown, & Pantin-Jackwood, 2003).

Growth retardation was observed throughout the experimentation period and became more severe from 28 d onwards in Group I. The average body weight in Group I was only 45% of that in Group II and about 35–40% of those in the TPPPS groups on day 35. Growth retardation was significantly adjusted in the TPPPS groups (III and IV), but no significant differences between these groups and the control group were observed. Body weights in the TPPPS groups, especially Group IV, increased more sharply than that in the control group ($P < 0.05$). Spleen, thymus, and bursa atrophy were observed in Groups I and II on relative days. Chicks in Group I showed more severe immune organ atrophy than chicks in Group II ($P < 0.05$) in the overall level. While they had been adjusted significantly in Groups III and IV (Group IV increased most sharply). Comparison of the change trends showed that the spleen weight first increased and then decreased in Groups I and III on day 28, whereas other groups showed increasing weights throughout the experimental period. The thymus weight in Group I decreased throughout the experiment; by contrast, thymus weights significantly increased in other groups. The bursa weight also generally increased throughout the experimental in all groups except Group II, in which the thymus weight first increased and then decreased from day 35 onwards. We found that the effects of ALV-B-induced immunosuppression exhibited as arrested development of immune organs, immunosuppression and *B. avium* infection exhibited as stunted increases in body weight. The body weights and immune organs of chicks in the infected groups increased continuously and steadily after treatment with TPPPS.

3.3. Immunoregulatory evaluation by lymphocyte ratio

The lymphocyte ratio is defined as the percentage of lymphocytes relative to the total number of leucocytes. Changes in the blood lymphocyte ratio can reflect humoral immunity conditions (Peltola, Mertsola, & Ruuskanen, 2006).

Overall lymphocyte ratios in Group I were lowest compared with all other groups. The lymphocyte ratio of Group II was slightly higher than that of the control group on days 7–28 and significantly higher on days 14–21. Both TPPPS groups showed significantly increased lymphocyte ratios. Group III showed lymphocyte ratios slightly higher than that of the control group. Group IV showed significantly higher lymphocyte ratios than the control group on days 14–42 ($P < 0.05$). Comparison of the change trends showed that overall lymphocyte ratios in TPPPS groups were higher than those in non-TPPPS groups. Differences were significant between the TPPPS groups and Group I ($P < 0.05$) (Table 2). TPPPS can promote the proliferation of lymphocytes and alleviate the harmful influence induced by ALV-B. It can also modulate immune functions by increasing lymphocyte ratios, thereby improving the immune response capacity of chicks.

3.4. Changes in peripheral blood lymphocyte transformation rates

T lymphocytes can transform into lymphoblasts stimulated by mitogen. Mitogen is the nonspecific stimulus of T lymphocyte proliferation. The LTR is the most direct indicator of the state of cellular immunity (Li, Santoso, & Lo, 2007).

The overall LTR in Group I was lowest compared with all other groups. The LTR of Group II was slightly higher than that of the control group on days 14–28 but showed no significant difference ($P > 0.05$). The LTRs of both TPPPS groups increased significantly. No significant difference was observed between Group III and the control group ($P > 0.05$). Group IV showed a significantly higher LTR compared with the control group on days 14–28 ($P < 0.05$). Comparison of the change trends showed that the overall LTRs of the TPPPS groups were higher than those of the non-TPPPS groups. Differences were significant between the TPPPS group and Group I from day 14 onwards (Table 3). TPPPS can elevate the state of cellular immunity and enhance the power of the body's resistance to antigens, which might be related to the immune-enhancement mechanism of the immunoregulation.

3.5. Changes in B. avium serum antibody titer

Antibodies are not the only products of immune responses but also the most effective modulatory factors of humoral immunity. Changes in serum antibody titers can accurately and directly reflect the state of humoral immunity (Zhang, Wang, Hu, & Sun, 2009).

Serum antibody agglutination titers of *B. avium* are presented in Fig. 1. Similar change trends in *B. avium* antibody titers, which first increased and then decreased slightly, was observed in all of the experimental groups. The *B. avium* antibody titer in the control group was zero. The quantity of antibody in the infected groups (I, II, III, IV) reached a maximum value on day 35. The antibody titer of Group I was significantly lower than that of Group II ($P < 0.05$). Both TPPPS groups showed significantly increased titers. Comparison of the titers of the infected groups indicated overall *B. avium* serum antibody titers were higher in the TPPPS groups than in non-TPPPS groups on relative days. No significant difference was observed between Groups II and III ($P > 0.05$). The serum antibody titer in Group IV was significantly higher than those of other groups. By detecting the serum antibody titer of *B. avium*, we showed that TPPPS can stimulate B lymphocytes to convert into plasmocytes that secrete specific antibodies. It can also modulate immune functions by increasing antibody titers and prolonging the antibody peak period, thereby improving the immune response capacity of chicks.

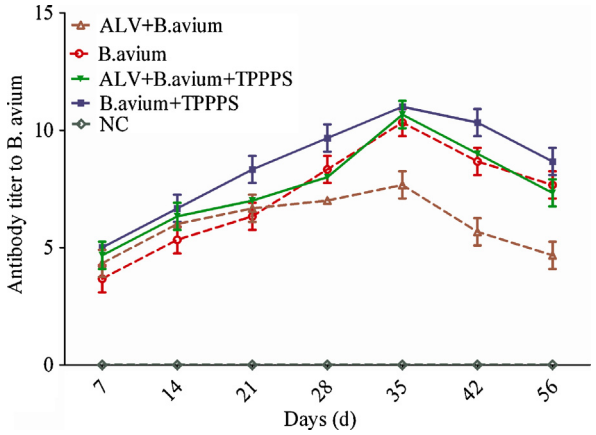

Fig. 1. Antibody titer to *B. avium* in the different experimental groups (log 2). Groups of chicks were co-infected with Avian leukosis virus (ALV-B) and *B. avium*; *B. avium*, Taishan pine pollen polysaccharides (TPPPS), ALV-B and *B. avium*; TPPPS and *B. avium*, or physiological saline. Serum was collected on days 7, 14, 21, 28, 35, 42, 56, and the antibody titers were determined by micro-agglutination test. Data are represented as mean ± SD at each time point.

3.6. Changes in IFN-γ and IL-2

IL-2, a glycoprotein released from activated T cells or CD4+ 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 (Jin, Hirano, & Murakami, 2008). IL-2 reflects the state of cellular immunity of chickens indirectly. Similar changes in IL-2 concentration were observed between Groups I and II, as shown in Fig. 2. IL-2 concentrations first increased and then decreased. Peak values for Groups I and II were observed on days 21 and 28, respectively. Overall, however, IL-2 concentrations in the former were significantly lower than those of the latter ($P < 0.05$). The same findings were observed in Groups III and IV. IL-2 concentrations in these groups rose throughout the entire experimental period. Comparison of change trends showed that the IL-2 concentrations in the TPPPS groups were higher than those in the non-TPPPS groups on relative days.

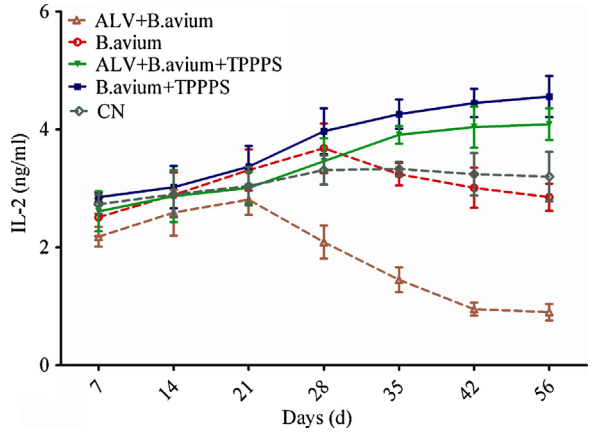

Fig. 2. Changes in IL-2 levels in the peripheral blood. Groups of chicks were co-infected with Avian leukosis virus (ALV-B) and *B. avium*; *B. avium*, Taishan pine pollen polysaccharides (TPPPS), ALV-B and *B. avium*; TPPPS and *B. avium*, or physiological saline. Serum was collected on days 7, 14, 21, 28, 35, 42, 56, and the concentration of IL-2 was determined by ELISA. Data are represented as mean ± SD at each time point.

Table 2
The effect of TPPPS on blood lymphocyte ratio in chickens (%).

Groups		Age of chicks (days) and lymphocyte ratio						
		7	14	21	28	35	42	56
I	ALV + *B. avium*	81.2 ± 2.0^{a}	73.6 ± 5.2^{b}	70.5 ± 1.8^{b}	74.6 ± 2.2^{a}	75 ± 1.2^{a}	76.9 ± 2.0^{a}	73.9 ± 2.7^{a}
II	*B. avium*	90.5 ± 1.0^{bc}	913 ± 4.2^{a}	89.9 ± 4.7^{a}	88.9 ± 3.5^{c}	85.1 ± 3.2^{ac}	83.1 ± 2.0^{c}	85.2 ± 1.2^{b}
III	ALV + *B. avium* + TPPPS	86.4 ± 1.6^{b}	88.2 ± 1.1^{ac}	90.2 ± 2.1^{a}	89.9 ± 1.8^{c}	93.7 ± 2.5^{c}	87.2 ± 3.1^{b}	89.8 ± 1.1^{c}
IV	*B. avium* + TPPPS	91.7 ± 1.1^{c}	95 ± 1.8^{a}	95.0 ± 2.4^{a}	91.8 ± 5.0^{c}	90.6 ± 1.2^{ac}	88.5 ± 1.2^{b}	92.5 ± 2.2^{d}
V	Control	87.9 ± 5.1b^{bc}	83.8 ± 3.1^{c}	81.2 ± 1.9^{c}	86.6 ± 2.4^{c}	87.8 ± 2.6^{ac}	86.5a ± 1.7^{c}	89.4 ± 2.8^{c}

Notes: The values in the table were means ± SD. The data in the same columns were compared, different small letters at the upper-right corner indicates significant difference ($P < 0.05$).

Table 3
The effect of TPPPS on lymphocyte transformation rates (%).

Groups		Age of chicks (days) and lymphocyte transformation rates						
		7	14	21	28	35	42	56
I	ALV + *B. avium*	19.55 ± 1.0^{a}	13.27 ± 0.1^{a}	9.31 ± 2.0^{a}	9.03 ± 1.2^{a}	10.11 ± 0.7^{a}	10.24 ± 0.6^{b}	10.53 ± 3.3^{a}
II	*B. avium*	20.11 ± 3.1^{a}	21.43 ± 1.9^{c}	21.85 ± 1.0^{c}	22.68 ± 0.3^{c}	19.88 ± 0.1^{b}	18.76 ± 0.5^{a}	17.84 ± 1.0^{b}
III	ALV + *B. avium* + TPPPS	19.77 ± 1.2^{a}	17.83 ± 0.1^{b}	17.80 ± 0.1^{b}	19.75 ± 1.1^{b}	22.31 ± 2.2^{c}	20.58 ± 0.04^{c}	19.36 ± 1.1^{c}
IV	*B. avium* + TPPPS	21.64 ± 0.3^{b}	22.55 ± 2.7^{d}	21.89 ± 2.5^{d}	23.74 ± 2.1^{d}	23.66 ± 1.3^{c}	23.68 ± 3.0^{d}	22.54 ± 1.5^{d}
V	Control	22.86 ± 1.1^{b}	20.95 ± 0.8c	19.65 ± 0.1^{bc}	21.47 ± 1.2^{bc}	21.31 ± 1.7^{c}	20.55 ± 0.44^{c}	18.85 ± 2.3^{bc}

Notes: The values in the table were means ± SD. The data in the same columns were compared, different small letters at the upper-right corner indicates significant difference ($P < 0.05$).

Avian IFN-γ has been used as an indicator of cell-mediated immunity in infected hosts. IFN-γ has a variety of pharmacological activities, such as antitumor and antiviruses (Sun, Xu, Xu, & Li, 2009). Similar changes in IFN-γ concentration were observed between Groups I and II, as shown in Fig. 3. IFN-γ concentrations in these groups first increased first and then decreased. Concentrations in Groups I and II were highest on days 21 and 28, respectively. Similar phenomena were observed between Groups III and IV. In these groups, IFN-γ concentrations increased throughout the experimental period. IFN-γ concentrations of the first two groups were significantly higher than that of the last one. Furthermore, Group III showed the highest IFN-γ concentration from day 28 onwards.

Our research showed that TPPPS can eliminate reductions in T lymphocyte transformation induced by ALV-B. Activated T lymphocytes secrete IL-2 and IFN-γ, remarkably promoting the cellular immunity, which might be related to the immune-enhancement mechanism of the immunoregulation.

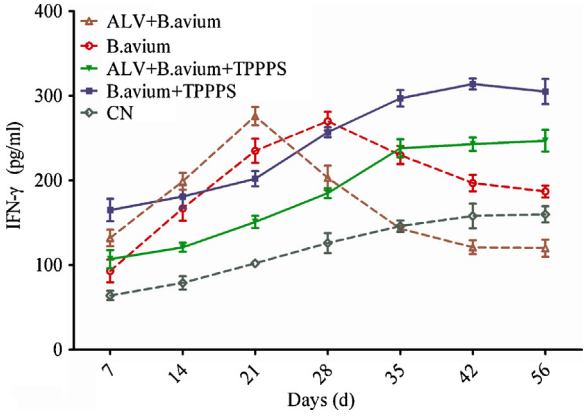

Fig. 3. Changes in IFN-γ levels in the peripheral blood. Groups of chicks were co-infected with Avian leukosis virus (ALV-B) and *B. avium*; *B. avium*, Taishan pine pollen polysaccharides (TPPPS), ALV-B and *B. avium*; TPPPS and *B. avium*, or physiological saline. Serum was collected on days 7, 14, 21, 28, 35, 42, 56, and the concentration of IFN-γ was determined by ELISA. Data are represented as mean ± SD at each time point.

3.7. Dynamic changes in viremia

Dynamic changes in viremia were observed, as shown in Table 4. Chicks in Group I developed viremia at 7 d old. Viremia developed in all chicks at 28 d old but was delayed by about two weeks in the TPPPS group. The application of TPPPS decreased positive rates of viremia. Thus, we believe that TPPPS can reduce the proliferation of ALV-B to some extent.

Co-infection of *B. avium* and immunity-inhibiting viruses (Liang et al., 2013) has created favorable conditions for the outbreak of poultry epidemic diseases. However, co-infection with ALV and *B. avium* has been observed to occur with increasing frequency year after year. Such infection causes serious economic losses in the poultry industry. This study showed that bacteria were more pathogenic and disease courses were longer under the immunosuppression induced by ALV-B. The immunosuppressive effects of ALV-B were more serious than those reported in a previous research (Wu et al., 2011). Thus, we infer that ALV-B and *B. avium* have coordinating roles during co-infection. During vaccine development, Wang injected inactivated *B. avium* into 1-day-old chicks (Wang & Zhu, 2001) but the outcomes were not ideal. Today, no effective commercial vaccines for the prevention of ALV infection are available and the prevention and treatment of such diseases have become more challenging. Antibiotic residues and food safety problems further hinder the development of the poultry industry in China. Thus, the development of more efficient immunity intensifiers and production of secure animal products are main concerns in the poultry industry.

Research on *Pinus* pollen polysaccharides began fairly recently (Wei et al., 2010), and the application of these polysaccharides to the poultry industry as immunoregulators has seldom been reported. Polysaccharides help adjust the immunity of organisms (Jin, Huang, Zhao, & Shang, 2012), enhance antiviral effects (Xie et al., 2012), stimulate serum antibody levels, and improve the proliferation of lymphocytes. Wei et al. (2010) reported that TPPPS can elevate the immune indices of normal and immunosuppressed mice. In this study, we found that application of TPPPS not only improved the resistance of chicks to *B. avium* and ALV-B, but also attenuated the immunosuppression induced by ALV-B. TPPPS can adjust specific and nonspecific immunological functions without toxicity and side-effects. *Pinus* pollen polysaccharide

Table 4
The effect of TPPPS on dynamics of viremia in chickens.

Groups		Age of chicks (days) and dynamics of viremia						
		7	14	21	28	35	42	56
I	ALV + *B. avium*	3/20	8/20[a]	13/17[a]	14/14	10/10[a]	7/7	4/4
II	*B. avium*	0/20	0/20	0/18	0/14[a]	0/11	0/8	0/5
III	*B. avium* + ALV + TPPPS	0/20	3/20	7/18	9/14[a]	10/11[a]	8/8	5/5
IV	*B. avium* + TPPPS	0/20	0/20	0/18	0/15	0/12	0/9	0/6
V	Control	0/20[b]	0/20[b]	0/18	0/15	0/12	0/9	0/6

[a] Experimental chicken die because of pathogeny infection.
[b] Experimental chicken die accidental.

thus appears to have high clinical value and great application potential.

4. Conclusion

TPPPS consists of eight monosaccharides, and the most abundant monosaccharides in TPPPS are galacturonic acid and glucose. ALV-B can reduce the immune function of chicks significantly, causing them to become more susceptible to and even exacerbating the pathogenicity of *B. avium*. TPPPS can improve the functions of humoral and cellular immunity that are damaged by ALV-B, reduce adverse effects on the growth of chicks, and improve the development of immune organs by enhancing host immunity.

The mechanism of interaction between ALV-B and *B. avium* remains unclear, and the mechanism by which TPPPS attenuates the effects of co-infection with ALV-B and *B. avium* requires further investigation.

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