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Immune Regulatory Effects of Enteromorphaclathrata Polysaccharides on Nd Attenuated Vaccine in a Chicken Model Infected with Reticuloendotheliosis Virus

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ABSTRACT

Reticuloendotheliosis virus (REV) infection has frequently affected the poultry industry in recent years. The infection with REV weakens the immune responses of the infected poultry. It is reported that *Enteromorphaclathrata* polysaccharides are capable of regulating immune function. In order to investigate the immuno regulatory effects of *Enteromorphaclathrata* polysaccharides (EPS) on the response of REV-infected broilers to a live attenuated Newcastle disease (ND) vaccine. Broilers were intraperitoneally injected with REV at one day of age, subcutaneously infected with EPS at 2 days of age, and vaccinated by nasal drip with a live attenuated ND (Lasota strain) vaccine at 5 days of age. Immune organ index, secretory immunoglobulinA (SIgA), peripheral blood heterophil to lymphocyte ratios (H/L ratio), peripheral blood lymphocyte transformation rates, and interferon-gamma (IFN-γ) and interleukin-2 (IL-2) levels were measured at 3, 7, 14, 21, 28, 35, 42, and 56 days of age. The results showed that EPS increased the immune organ index, and the secretion of small intestine secretory immunoglobulin A, serum ND antibody titers, blood H/L ratio, peripheral blood lymphocyte transformation rates, and IL-2 and IFN-γ levels. These results indicate that EPS are able to enhance the immune responses of chickens both to REV infection and to ND vaccination. Therefore, *Enteromorphaclathrata* polysaccharides can be considered as an immune regulator in the future.

INTRODUCTION

The reticuloendotheliosis virus (REV) is a common immunosuppressive and oncogenic virus (Cui *et al,* 2006), which may infect several domestic and wild bird species. The infection with REV can cause organ damage, and weaken the immune system of poultry, resulting in poor immune responses to vaccines and immune failure. In addition, the REV increases the sensitivity of poultry to secondary infections. These mixed infections increase mortality and considerably reduce production performance, resulting insignificant damage and losses to the breeding and food-processing industries.

Today, antibiotics are widely used in the Chinese poultry industry. However, antibiotics may cause drug resistance and antibiotic residue problems, which not only restrict the exports of animal products, but also threaten human life. Therefore, natural biological agents that have low toxicity and do not result drug residue in animal products have been researched and developed as alternative to the use of antibiotics.

Enteromorpha, commonly known as sea lettuce, are aquatic green algae of the family Ulvaceae. As it is a natural seaweed, Enteromorpha is a low-cost green plant, and has high production potential. In addition, high volumes of Enteromorpha can cause ecological damage to the

marine environment. Polysaccharides derived from Enteromorpha have a wide range of pharmacological properties (Jianteng *et al*. 2014), including antitumoral activity, reduction of blood lipids and glucose, as well as antioxidant, antibacterial, antiviral, and immuno regulatory functions (Wang *et al*. 2012; Shao *et al*. 2014; Teng *et al*. 2013; Shihong *et al*. 2010; Mezghani *et al*. 2013; Lü *et al*. 2014; Wei *et al*. 2014). Therefore, *Enteromorphaclathrata* polysaccharides have a higher potential application value compared with antibiotics. However, there are no studies on the immunoregulatory effects of *Enteromorphaclathrata* polysaccharides on the response of REV-infected chickens to ND vaccine.

This study aimed at investigating the immuno-regulatory effects of *Enteromorphaclathrata* poly-saccharides on the response of REV-infected chickens to an attenuated live ND vaccine. The REV infected chicken model was established, the immune organ index, secretory immunoglobulin A (SIgA), peripheral blood H/L ratios, peripheral blood lymphocyte transformation rates, and iInterferon-gamma (IFN-γ) and interleukin-2 (IL-2) levels were measured to evaluate immuno regulatory effects of EPS, which laid the foundation for the further development of *Enteromorphaclathrata* polysaccharides as a new immune adjuvant and vaccine immune enhancer in the poultry industry.

MATERIALS AND METHODS

Reagents

Monosaccharide standards were purchased from Showa Denko (Japan). Trifluoroacetic acid (TFA), 1-phenyl-3-methyl-5-pyrazolone (PMP), concanaval in A (ConA), lymphocyte separation medium, and interleukin 2 (IL-2) and interferon gamma(IFN-γ) ELISA kits were purchased from Sigma (USA). Methanol and acetonitrile were bought from the Fisher (USA). Chicken secretory IgA (sIgA) ELISA kit was purchased from Shanghai Meixuan Biotechnology Company (China). The antigen, positive serum, and negative serum used for the hemagglutination (HI) test were purchased from Qingdao Yibang Biology Engineering Company (China). The Lasota strain ND live vaccine was purchased from Shandong Qilu Animal Health Care Products Company (China). RPMI-1640 medium and fetal bovine serum were purchased from Invitrogen (USA).

Virus strains

The REV (SNV-ori-p4 strain) was donated by Professor Cui, Z (College of Veterinary Medicine, Shandong Agricultural University). The tissue culture infectious dose (TCID50) of REV-SNV titer was calculated by the Reed-Muench method, using indirect immunofluorescence to identify REV-positive virus cultures in DF-1 chicken fibroblasts (Liang *et al*. 2013), the TCID50 was 10-4.58/0.1 mL.

Isolation of *Enteromorphaclathrata* poly-saccharides

Fresh Enteromorpha were collected from Qingdao (China), and the polysaccharides were obtained by hot water extraction and ethanol precipitation after cleaning, incubation in an oven at 37°C, and pulverization in a Ultra-Micro Pulverizer. The details of this method are described as follows (Fuchao *et al*. 2010). Flesh Enteromorpha were precisely weighed, and then deionized water was added in the proportion of 1:100 (quality ratio). The mixture was stirred for 3 hours under 100°C. The solution was filtered using filter paper while still hot, the residue was washed three times with deionized water, 95% ethanol was three times to the filtrate, which was stored at 4°C overnight, and then centrifuged (3,000 rpm for 20 min). After drying in oven at 37°C, the precipitate was washed with ether for fat removal. The precipitate was completely dissolved in deionized water (1 g:50 mL), 0.1% trypsin was added and the precipitate was digested for 2 h at 37°C to remove proteins using Sevage reagent, and the supernatant was removed after stirring (3,000 rpm for 10 min) at room temperature. The Sevage solvent was added three times to the supernatant. Anhydrous ethanol was added to the supernatant fluid at 1:3 (volume ratio), precipitated overnight at 4°C, and then centrifuged at 3,000 rpm for 20 min. After centrifuging (3,000 rpm for 20 min), the precipitate consisted of raw Enteromorpha polysaccharides. Enteromorpha polysaccharides were purified in C18 solid-phase extraction column to determine polysaccharide yield (Zuo *et al*. 2013). Finally, PMP precolumn derivatization ultra-high performance liquid chromatography-tandem mass spectrometry was used to determine the composition and yield of the *Enteromorphaclathrata* polysaccharides.

Experimental birds

This experiment was approved by the Committee on the Ethics of Animal of Shandong (Permit No.: 20143782). In total, 175 one-d-old broiler chicks were randomly divided into five groups. Groups I and group II were intraperitoneally injected at 1 d of age with 0.1 mL of REV at $10^{4.58}$ $TCID_{50}$. Groups I and III were subcutaneously injected at 2d of age with 0.1 mL of

Enteromorphaclathrata polysaccharides at 5 mg/L for three days. Group IV was intraperitoneally injected at 1 d of age with 0.1 mL physiological saline solution. Birds of Groups I to IV were vaccinated with an attenuated live NDV LaSota strain vaccine at 5d of age by nasal drip. Group V was set as the blank control. Five chicks per group were randomly sacrificed after feed and water fasting for 6 h on days 3, 7, 14, 21, 28, 35, 42, and 56 for the measurement of the evaluated indices.

Immune organ index

Five chicks per group were randomly selected to determine their weight and immune organ indexes. Birds were euthanized and dissected in a UV radiation-sterilized isolation chamber. The spleen, thymus, and bursa of Fabricius were surgically excised and weighed after the removal of surface moisture with a sterile filter paper. Immune organ index was expressed as their weight organs relative to live bodyweight (McCarty *et al*. 2004).

Detection of small intestine secretory immunoglobulin A (SIgA)

A 10-cm segment of the small intestine between the duodenum and the jejunum was sectioned, rinsed with sterile saline until clean. TwomL PBS (pH 7.4), containing0.1% bovine serum albumin and protease inhibitors were added, and the solution was homogenized. The homogenate was incubated at room temperature for 4 h, centrifuged for 10 min at 4,000 rpm, and the supernatant was collected to determine sIgA levels, using a chicken secretory IgA (sIgA) ELISA kit.

Serum ND-HI antibody assay

Cardiac blood (1.5 mL/bird) was collected and into a 1.5 mL Eppendorf tube under sterile conditions, stored at 37ºC for 2 h, and then the supernatant was collected. ND antibody titers were determined by hemagglutination inhibition (HI) using two-fold dilution series of the sera. The titers were expressed as log2 values of the highest dilution showing the complete inhibition of agglutination.

Blood heterophil to lymphocyte ratio detection

Approximately 1 mL blood was aseptically collected from the heart of each bird and placed in EDTA-Na anticoagulant tubes. Blood H/L ratios were determined using an automatic hematology analyzer (model XT 1800i, SYSMEX, Japan).

Peripheral blood lymphocyte transformation assay

A volume of 2 mL blood/bird was aseptically collected from the heart and placed into EDTA-Na anticoagulant tubes. Blood was diluted with 2 mL of serum-free RPMI-1640, gently shaken, and then 4 mL of lymphocyte separation medium were added. The solution was centrifuged at 2000 rpm for 20 min. The resulting fluid was clearly separated into four layers. The second layer was a yellowish-white membrane, consisting of lymphocytes, which was removed and suspended in RPMI-1640 culture medium, centrifuged at 2000 rpm for 20 min, after which the supernatant was discarded. This procedure was repeated twice. The precipitated cells were then suspended in RPMI-1640 culture medium containing 10% fetal bovine serum. Cell density was adjusted to 2×10^6/mL using RPMI-1640 media, and incubated in 96-well culture plates (0.1mL/well, and four wells/sample). Con A (30 mg/mL) was added to three of the four wells. The plates were incubated at 37ºC in humid atmosphere of 5% CO_2 until a monolayer of lymphocytes grew. Subsequently, 30 mL of MTT stain (5 mg/mL) were added to each well, and the plates were re-incubated for 4 h. Absorbance was read at 490 nm, and the values were used to calculate lymphocyte transformation rates (LTR) (Guo *et al*. 2014).

Detection of IL-2 and IFN-γ in the serum

A volume of 1.5 mL aseptic cardio blood was pipetted into Eppendorf tubes and allowed to clot at 37ºC for 1h. The serum was then separated by centrifugation (4,000 rpm for 15 min) and stored at –20ºC for IFN-γ and IL-2 detection. The levels of IL-2 and IFN-γ were determined using a chicken IL-2 ELISA kit (Life Span BioSciences, Inc., USA) and an IFN-γ ELISA kit (Aviva Systems Biology, Inc., USA).

Statistical analysis

All data were analyzed by SPSS Statistics 19.0 software and expressed as mean±SD. One-way analysis of variance was applied to perform multiple comparison analysis. P values of <0.05 were used to define statistical significance.

RESULTS

The isolation of *Enteromorphaclathrata* polysaccharides

The yield rate of *Enteromorphaclathrata* polysaccharides (EPS) was 23.5%, and their purity rate was 69.2%. PMP precolumn derivatization in ultra-high

performance liquid chromatography in tandem mass spectrometry was used to obtain EPS composition and yield, as shown in Table 1. The purified EPS consisted of six compounds, out of which the most abundant were the monosaccharidesxylose (32.3%) and rhamnose (28.7%).

Table 1 – Composition and monosaccharide contents of in EPS

Compound	Molar percentage (%)
Xylose	32.3
Rhamnose	28.7
Glucosamine	3.4
Glucuronic acid	17.3
Mannitol	8.6
Galactose	9.7

Immune organ index

The immune organ indexes obtained in the experimental birds on different days of evaluation are shown in Figure 1. The immune organ index of all five groups peaked on day 21 post-injection and then decreased. The immune organ index of group III was significantly higher compared with other four groups (p<0.05). Groups I and V presented similar immune organ indexes (p>0.05). The immune organ index of group II was significantlylower compared with those of groups I and III (p<0.01).

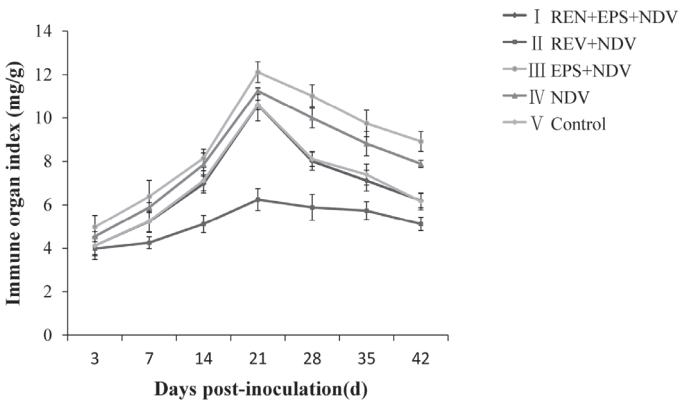

Figure 1 – Immune organ index changes according to experimental group and days post-inoculation.(REV: reticuloendotheliosis virus, EPS: *Enteromorphaclathrata* polysaccharides, NDV: ND vaccine).

Small intestine secretory immunoglobulin A (sIgA) titers

As shown in Figure 2, the small intestine sIgA titers of groups I, III, and IV peaked on day 21 post-injection, then gradually decreased, whereas in groups II and V, the peak was reached on day 7 post-injection and then began to decrease. In addition, sIgA secretion in group III was significant higher than that of other four groups (p<0.01), and in group II, it was lower compared with other four groups (p<0.05) at any time point.

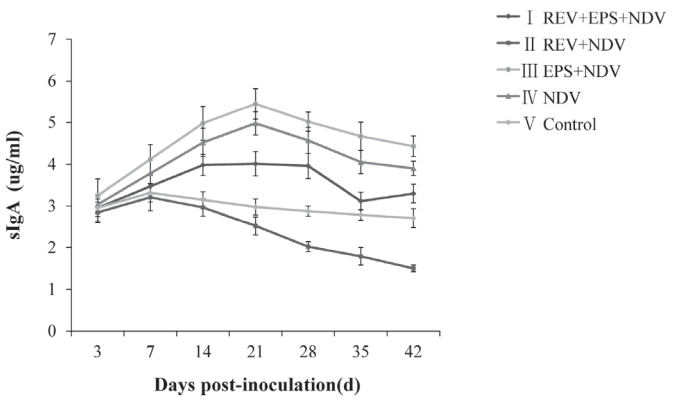

Figure 2 – The changes of sIgA in small intestine according to experimental group and days post-inoculation. (REV: reticuloendotheliosis virus, EPS: *Enteromorphaclathrata* polysaccharides, NDV: ND vaccine).

Serum ND antibody titers

The changes in serum ND antibody titers are presented in Figure 3. The ND antibody titer in group V was zero, as expected. The ND antibody titers of the infected groups (I, II, III, IV) reached a maximum value on day 21. The ND antibody titers of Group III were significantly higher than those of other groups (p<0.01) and remained high for until 42 days. The ND antibody titers of Group II were significantly lower than those of Groups I, III and IV (p<0.05). No significant difference was observed between Groups I and IV (p>0.05).

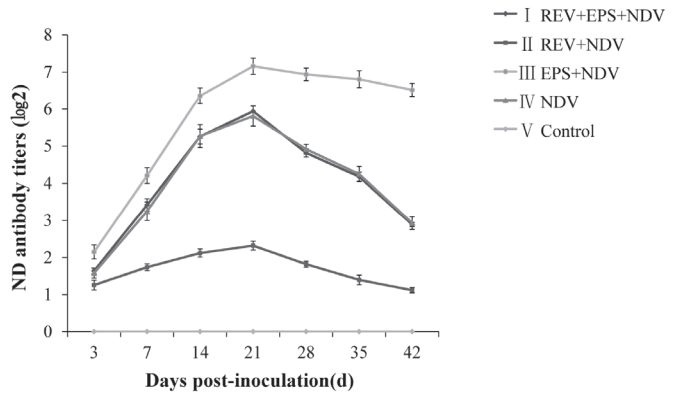

Figure 3 – Changes of serum ND antibody titers according to experimental group and days post-inoculation. (REV: reticuloendotheliosis virus, EPS: *Enteromorphaclathrata* polysaccharides, NDV: ND vaccine).

Blood heterophil to lymphocyte ratio

The blood H/L ratios of all groups are listed in Table 2. The H/L ratio of groups I, III and IV reached a maximum value on day 21. The H/L ratios of Group III were significantly higher than those of other groups (p<0.05) at all time points. No significant difference was observed between Groups I and IV (p>0.05). The H/L ratios of Group II were significantly lower than those of Groups I, III and IV (p<0.01) at all time points.

Table 2 – Effect of EPS on the blood H/L ratio (%), according to experimental group and days post-inoculation.

Group	Days post-inoculation						
	3	7	14	21	28	35	42
I	86.7±1.1	88.8±2.3	90.9±3.1	91.0±1.8	90.1±2.2	90.8±2.4	89.9±1.5
II	83.2±2.5*	80.4±1.8*	74.1±2.9*	72.0±2.1*	76.2±3.1*	75.9±1.8*	76.0±2.3*
III	87.5±1.9	90.8±3.0	94.7±2.7	95.1±2.2	94.7±2.3	94.2±2.1	93.9±2.0
IV	86.9±2.1	89.2±2.2	91.2±1.9	91.5±2.1	90.3±1.7	89.9±2.5	89.7±1.9
V	85.7±3.3	87.2±2.7	84.1±2.1	86.2±1.9	86.8±2.1	86.7±3.6	86.3±2.2
p-value	<0.05	<0.05	<0.05	<0.05	<0.05	<0.05	<0.05

*indicates significant difference in the results of group II compared with the other groups at $p<0.05$.

Changes in peripheral blood lymphocyte transformation rates

The blood lymphocyte transformation rates of all experimental groups obtained on the different days post-injection are listed in Table 3. The lymphocyte transformation rates of groups I, III, and IV reached maximum values on day 28 post-injection. The lymphocyte transformation rates of Group III were significantly higher than those of other groups ($p<0.05$) at all time points. Significant differences were observed between Groups I and IV ($p<0.05$). The lymphocyte transformation rates of Group I were lower that Group IV at all time points. The lymphocyte transformation rates of Group II was significantly lower than that of other groups ($p<0.05$) and reached peak value on day 7 post-injection.

Table 3 – Effect of EPS on blood lymphocyte transformation ratio in chickens (%), according to experimental group and days post-inoculation.

Group	Days post-inoculation						
	3	7	14	21	28	35	42
I	20.18±0.86	20.52±1.11	19.02±0.75	19.57±0.82	20.95±0.65	20.2±0.52	19.7±0.47
II	19.28±0.51*	19.92±0.78*	14.52±0.23*	10.3±0.57*	9.8±0.59*	10.5±0.43*	10.42±0.55*
III	22.96±0.76	24.02±0.43	24.98±0.42	25.75±0.45	25.95±0.54	25.9±0.68	24.76±0.71
IV	22.18±0.52	22.95±0.85	22.98±0.54	23.01±0.62	23.27±0.73	23.25±0.72	22.19±0.36
V	21.95±1.2	22.74±0.7	21.02±0.92	21.13±0.87	21.98±0.75	21.79±0.76	20.23±0.98
p-value	<0.05	<0.05	<0.05	<0.05	<0.05	<0.05	<0.05

*indicates significant difference in the results of group II compared with the other groups at $p<0.05$.

Peripheral blood IL-2 levels

The IL-2 levels in the peripheral blood of all groups obtained on the different days post-injection are presented in Figure 4. The IL-2 levels of group I, III, and IV gradually increased. In group II, IL-2 values peaked on day 21 post-inoculation and then decreased, while in group III, peak values observed on day 14 and then a decrease until day 21. The IL-2 levels of Group III were significantly higher than those of other groups ($p<0.01$) at all time points. The IL-2 levels of Groups I and IV were similar until day 21 post-inoculation ($p>0.05$), but were significantly different thereafter ($p<0.05$). In comparison with other groups, the IL-2 levels of Group II were significantly lower ($p<0.05$) at all time points.

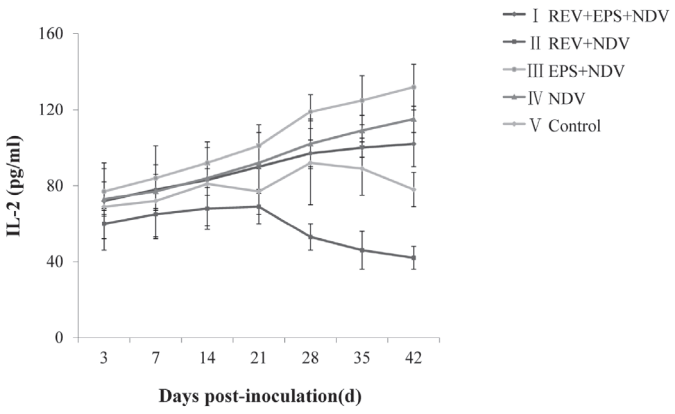

Figure 4 – Changes in IL-2 levels in the peripheral blood of chickens, according to experimental group and days post-inoculation. (REV: reticuloendotheliosis virus, EPS: *Enteromorphaclathrata* polysaccharides, NDV: ND vaccine).

Changes of IFN-γ levels in the peripheral blood

The IFN-γ level changes in the peripheral blood of the experimental groups on the different days post-injection are shown in Figure 5. The IFN-γ levels of Groups I, III and IV were gradually increased. In Group II, peak IFN-γ values were observed on day 21 post-inoculation, then sharply decreased to levels lower than those of the control group. The IFN-γ levels of

Group III were significantly higher than those of other groups except Group II at all time points (p<0.05). The IFN-γ levels were not significantly different between group I and IV before day 14 post-inoculation (p>0.05), showed significant difference after day 14 (p<0.05) and no significant differences was observed on day 42 (p>0.05). In comparison with group I, III and IV, the IL-2 level of Group V was significantly lower (p<0.05).

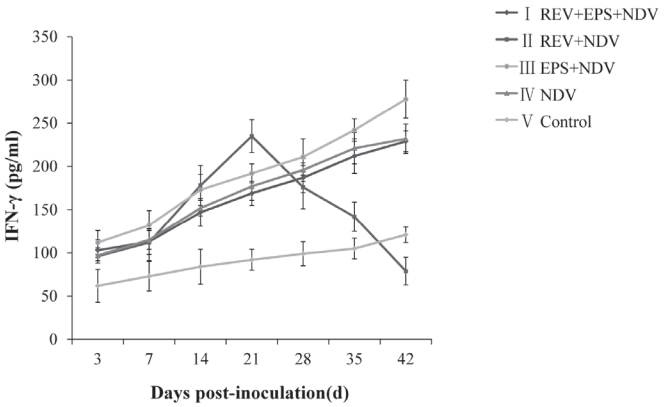

Figure 5 – Changes in IFN-γ levels in the peripheral blood of chickens, according to experimental group and days post-inoculation. (REV: reticuloendotheliosis virus, EPS: *Enteromorphaclathrata* polysaccharides, NDV: ND vaccine)

DISCUSSION

Enteromorpha, which is widely distributed along the coast of China, is a type of edible marine algae used as a traditional Chinese medicine and as food additive. It shows a huge development potential. On the other hand, due to its massive growth capacity, it may damage the marine ecosystem, causing environmental problems and economic losses. The polysaccharides are the main active components of *Enteromorphaclathrata*. In our study, hot water extraction and ethanol precipitation method was used to extract *Enteromorphaclathrata* polysaccharides. The study on the extraction of *Enteromorphaclathrata* polysaccharides and the analysis its components laid the foundation for the research on their functions.

The thymus, bursa of Fabricius, and spleen are the main immune organs of birds. These organs are the main sites for immune cells formation, differentiation, and production of antibodies. The developmental status of the immune organs directly influences the immune response levels of broilers (Montzouris *et al*. 2007). In the present study, the weight of the immune organs (spleen, thymus, and bursa of Fabricius) relative to the live body weight was used immune organ index. The results showed that the immune organ index of group III was significantly higher than that of other groups (p<0.01), indicating *Enteromorphaclathrata* polysaccharides are able to stimulate the development of immune organs.

Small intestine SIgA titers are used as an indication of humoral immune status of chickens (Zhang *et al*. 2009). In our study, the results showed that the small intestine SIgA titer of group III was significantly higher than that of other groups (p<0.01), demonstrating that *Enteromorphaclathrata* polysaccharides are able to stimulate the production of sIgA induced by vaccination. The significantly lower small intestine SIgA titers of group II compared with that of other groups (p<0.01) shows the immunosuppressive effects of REV, which were partially counteracted by the addition of *Enteromorphaclathrata* polysaccharides, as demonstrated by the higher SIgA titers of Group I.

Changes in serum antibody titers can accurately and directly reflect humoral immunity (Chattopadhyay *et al*. 2009). The serum antibody titer against ND of Group III was significantly higher than that of other groups (p<0.01), also indicating that EPScan enhance antibody titers stimulated by vaccine and extend the antibody peak period. Again, the antibody titer of Group II was significantly lower than that of Group I (p<0.05), demonstrating the immunosuppression caused by the REV.

The blood H/L ratio and lymphocyte transformation rates indicate cellular immunity (Li *et al*. 2007). In our study, blood H/L ratios and lymphocyte transformation rates of Group III were significantly higher that of other groups (p<0.05), which shows that *Enteromorphaclathrata* polysaccharides can enhance H/L ratios and lymphocyte transformation rates in the peripheral blood.

Interleukin 2 (L-2) is a glycoprotein released from activated T cells or CD4+ T cells. It promotes T cell proliferation and cytokine secretion, enhances the activity of T, NK, and LAK cells, and stimulates B lymphocyte proliferation and antibody secretion. IL-2 is an indirect measure of the cellular and humoral immune status of chickens (Barmeyer *et al*. 2003). The IL-2 level of Group III was significantly higher than that of other groups (p<0.01), also indicating that *Enteromorphaclathrata* polysaccharides enhance the vaccinal response of chickens.

IFN-γ is an another important immune-regulating factor in poultry, upregulating MHCI class II antigen processing, and exhibits broad antiviral activity, enhancing cellular and humoral immunity (Ottenhoff *et al*. 1995). In our study, IFN-γ level of Group III was significantly higher than that of other groups (p<0.01), demonstrating that *Enteromorphaclathrata*

polysaccharides are able to strengthen vaccinal response by increasing the production of IFN-γ in chickens.

CONCLUSION

The purified *Enteromorphaclathrata* polysaccharides contains six components, out of which xylose and rhamnose are the most abundant. *Enteromorphaclathrata* polysaccharides can not only strengthen the immune responses of chicken infected with reticuloendotheliosis virus, but also significantly enhance the response to an ND vaccine. Therefore, *Enteromorphaclathrata* polysaccharides can be used as an immune regulator.

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REFERENCE

Barmeyer C, Horak I, Zeitz M, et al. The interleukin-2 deficient mouse model. Pathobiology 2002/2003;70(3):139-142.

Chattopadhyay G, Chen Q, Colina J, et al. Intact bacteria inhibit the induction of humoral immune responses to bacterial-derived and heterologous soluble T cell-dependent antigens. Journal Immunology 2009;182(4):2011-2019.

Cui Z, Meng S, Jiang S, Wei J. Serological surveys of chicken anemia virus, avian reticuloendotheliosis virus and avian reovirus infections in white meat-type chickens in China. Acta Veterinaria et Zootechnica Sinica 2006;37:152-157.

Fuchao X, Zhixin, Naihao Y. Extraction, purification and analyzing of Polysaccharides from Enteromorpha. Science Technology and Engineering 2010;10(10):2413-2433.

Guo F, Xue C, Wu C, Zhao X, Qu T, He X, et al. Effects of polysaccharide on chicks co-infected with Bordetella avium and Avian leucosis virus. Carbohydrate Polymers 2014;109:71-76.

Jianteng W, Dong P, Yongfeng L, Yi L, Long DD. Research progress on polysaccharides from Enteromorpha. Marine Sciences 2014;38(1):91-94.

Li CH, Santoso S, Lo DD. Quantitative analysis of T cell homeostatic proliferation Cellular Immunology 2007;250(1-2),45-54.

Liang M, Zhao Q, Liu G, Yang H, Zuo X, Cui G, et al. Pathogenicity of Bordetella avium under immunosuppression induced by reticuloendotheliosis virus in specific-pathogen-free chickens. Microbial Pathogenesis 2013;54:40-45.

Lü H,Gao Y, Shan H, Lin Y. Preparation and antibacterial activity studies of degraded polysaccharide selenide from Enteromorpha prolifera. Carbohydrate Polymers 2014;107(7):98-102.

McCarty N, Shinohara ML, Lu L, Cantor H. Detailed analysis of gene expression during development of T cell lineages in the thymus. Proceedings of the National Academy of Sciences of the United States of America 2004;101(25):9339-9344.

Mezghani S,Bourguiba I, Hfaiedh I, Amri M Antioxidant potential of Ulva rigida extracts: protection of HeLa cells against H_2O_2 cytotoxicity. The Biological Bulletin 2013;225(1):1-7.

Montzouris KC, Tsirtsikos P, Kalamara E, Nitsch S, Schatzmayr G, Fegeros K. Evaluation of the efficacy of a probiotic containing Lactobacillus, Bidobacterium, Biterococcus, and Pediococcus strains in promoting broiler performance and modulating cecal microflora composition and metabolic activities. Poultry Science 2007;86:309-317.

Ottenhoff TH, Mutis T. Role of cytoxic cells in the protctive immunity against and immunopathology of intracellular infections. European Journal of Clinical Investigation 1995;25:2530-2536.

Shao P, Pei Y, Fang Z, Sun P. Effects of partial desulfation on antioxidant and inhibition of DLD cancer cell of*Ulva fasciata* polysaccharide. International Journal of Biological Macromolecules 2014;65(4):307-313.

Shihong A, Yan C, Ying L. Study on reducing blood glucose effects of polysaccharides from Enteromorpha prolifera extracted by base. Medical Inovation of China 2010;7(20):181-182.

Teng Z, Qian L, Zhou Y. Hypolipidemic activity of polysaccharides from Enteromorpha prolifera. International Journal of Biological Macromolecules 2013;62(11):254-256.

Wang J. Research progress on antitumor effects mechanism of polysaccharides from Enteromorpha. Practical Journal of Medicine & Pharmacy 2012;29(7);655-656.

Wei J, Wang S, Liu G, Pei D, Liu Y, Liu Y, et al. Polysaccharides from Enteromorpha prolifera enhance the immunity of normal mice. International Journal of Biological Macromolecules 2014;64:1-5

Zhang XH, Wang DY, Hu YL, Sun JL. Immunoligic enhancement of Astragalus polusaccharide on the humoral immunity of chicken. Chinese Journal of Veterinary Science 2009;29(3):312-314.

Zuo X, Cui L,Yang S, Liang M, Zhong S, Zhu R. The synergistic effect of Taishanpinepollen polysaccharide and Lactobacillus on enhancing the immunity of subunit vaccine of Bordetella avium OMP. Chinese Journal of Veterinary Science 2013;33(11):1662-1667