Kafkas Universitesi Veteriner Fakultesi Dergisi
Journal Home-Page: http://vetdergikafkas.org
E-ISSN: 1309-2251

Kafkas Univ Vet Fak Derg
30 (3): 335-340, 2024
DOI: 10.9775/kvfd.2023.31166

Research Article

Effect of Dietary Inclusion of Alfalfa Polysaccharide on Performance and Immune System Efficacy of Broiler Chickens

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How to cite this article?
Dong Y, Wang L, Zhang M, Zhang K, Zhang S: Effect of dietary inclusion of alfalfa polysaccharide on performance and immune system efficacy of broiler chickens. *Kafkas Univ Vet Fak Derg*, 30 (3): 335-340, 2024.
DOI: 10.9775/kvfd.2023.31166

Article ID: KVFD-2023-31166
Received: 14.11.2023
Accepted: 27.03.2024
Published Online: 03.04.2024

Abstract

This study was conducted to investigate the effect of Alfalfa polysaccharide (APS) on the growth performance and immune responses of broilers. A total of 400 1-day-old Avian broiler chicks were randomly divided into four treatment groups and administered intranasal with different levels of APS (0, 2.5, 5.0 or 10.0 mg/dose) on day 1 and day 14. Growth performance, phagocytic activity, T lymphocyte transformation, and serum antibody titers against the Newcastle disease virus (NDV) vaccine were examined. During the study period of 42 days, no significant differences were observed in food intake among treatment groups (P>0.05). The APS administered groups displayed a low FCR, especially the 2.5 mg groups (P<0.05). Broilers with 2.5 mg APS displayed a much higher phagocytic activity than the control group (P<0.05). At the same time, APS enhanced the T lymphocyte transformation in response to phytohaemagglutinin (PHA). The antibody titer to NDV was not influenced by APS on day 14, while it improved on day 28 and day 42, especially in the 2.5 mg group (P<0.05). And the above data suggest that APS could improve innate and cellular immune responses in broilers.

Keywords: Alfalfa polysaccharide, Broilers, Diseases, Growth, Immunity

Introduction

Polysaccharides are biomolecules existing in higher plants, animal cell membranes, and microbial cell walls [1]. They are important components of all living organisms, serving as both energy source for cell metabolism and structural element of the cell. They are involved in several physiological and pathological processes of the body [2]. Studies have proved that polysaccharides have anti-inflammatory and anti-infective properties, prevent gastrointestinal inflammation, promote the development of immune organs, and enhance the body's immune system [3,4]. However, although polysaccharides have a wide range of functions, they are difficult to produce synthetically on a large scale due to their complex structure, difficult synthesis process, and high production costs. Based on that, naturally extracted plant polysaccharides appear particularly convenient.

Plants polysaccharides can be extracted from a wide range of plant-based sources and have low cost, low toxicity, and lesser side effects, thus overcoming the shortcoming of synthetic polysaccharides. This has attracted the attention of many scholars [3]. So far, a variety of polysaccharides have been isolated from plants. Research has suggested that plant polysaccharides have obvious effects on growth promotion, macrophage phagocytosis, cellular immunity, and humoral immunity of livestock and poultry in a certain dose range [5]. The effectiveness of plant polysaccharides as an immuno-enhancer is influenced by various factors, such as their components and structure, the dosage used, the route of administration, and the species of animal being treated. It has been found that a variety of plant polysaccharides with immune activity, such as Astragalus polysaccharide, can significantly enhance the phagocytic function of macrophages, increase the spleen weight of mice, and have bactericidal, antiviral and anti-infective effects [6,7]; another polysaccharide lentinan can promote the oxidative stress around lung cancer cell tumor [8].

Alfalfa is an important source of legume feed for livestock. It is called the king of forage. It has the effects of clearing heat, detoxicating, and curing jaundice, urinary calculus, and other diseases [9,10]. The alfalfa polysaccharide (APS) is extracted from the stems and leaves of alfalfa. The

extraction process is simple and feasible, and the cost is low. It is suitable for large-scale production and feed supplementation [11]. However, the effect of additive APS in the diet of broiler remains unclear.

In the present research, different doses of APS were administered to 1-day- old broilers to study its effect on growth performance and immune function, including macrophage phagocytosis activity, peripheral lymphocyte transformation and antibody titer. These findings can provide a theoretical reference for the application of APS as an immune-enhancer in the poultry industry.

Material and Methods

Ethical Statement

Experimentation with animals was approved by the Experimental Animal Management Methods of Xinxiang Medical University (Approval number: 201206078) and followed Henan Authority's Experimental Animal Regulations.

Alfalfa Polysaccharide

The APS used in this study was purchased from Shanghai Moqi Biology Co.Ltd. (Shanghai, China). The purity of APS was 95%. The molecular weight of APS was determined by LC-MS and ranged from 8 to 160.000 daltons.

Experimental Animals and Management

A total of 400 1-day-old Avian broiler female chicks (Dayong Broiler Breeding Corp., Henan, China) were randomly assigned into four groups, with 10 replicate pens per group and 10 birds per pen for a 42-day feeding trial. Different dosage of APS (0, 2.5 mg, 5 mg, 10 mg) was dissolved in 50 μL PBS and administered intranasally to broilers on day 1 and day 14.

Maize-soybean-based basal diets without antibiotics were formulated to meet the nutrient requirements as recommended by the National Research Council (1994) during the starter (days 1-21) and finisher (days 22-42) periods. The ingredient compositions of the basal diet are shown in *Table 1*. Body weight gain (BWG) was recorded on day 1 and day 42, and feed intake (FI) was measured over the period of 42 days to calculate the feed conversion ratio (FCR).

All the birds were housed in electrically heated cages and had free access to clean water and feed. Under the lighting program, the broilers were provided light for 23 h for the first 2 weeks, and 20 h afterwards. The birds were kept at a temperature of 33-34°C for the first week. Then, the temperature was decreased by 2-3°C per week till 24°C was attained.

Macrophages Phagocytic Activity Assay

Phagocytic ability was determined by carbon clearance, as proposed by previous study elsewhere [12]. Briefly, two broilers from each cage on day 14 and 28 were randomly selected and injected colloidal carbon (drawing ink, Pelikan; 1 mL/kg BW) intravenously. Two (T1) and ten (T2) minutes after the injection, 200 μL blood was collected from the wing and mixed with 2 mL sodium citrate, which was then centrifuged at 530 g for 5 min. The supernatant was used to measure the optical density (OD) at 600 nm. The phagocytic index was calculated as follows: phagocytic index = body weight/(liver weight + spleen weight) × K1/3. The carbon clearance (K) was calculated as follows: K = (lgOD1-OD2)/T2-T1. OD1 and OD2 were the optical density at 600 nm of the supernatant at T1 and T2, respectively.

***Table 1**. Composition of basal diet*

Composition	0-3 w	4-6 w
Corn (%)	52.71	59.01
Soybean (%)	40.00	33.80
Soybean oil (%)	3.00	3.00
Calcium hydrophosphate (%)	1.90	1.25
mountain flour (%)	1.10	1.80
Salt (%)	0.37	0.37
Trace element premix (%)[a]	0.50	0.50
Multi-vitamin (%)[b]	0.20	0.20
methionine (%)	0.19	0.07
lysine (%)	0.05	0.03
Choline chloride (CC) (50%)	0.20	0.16
Metabolic energy (MJ/Kg)[c]	12.71	12.78
Crude protein (%)[d]	21.37	18.99
Calcium (%)	1.08	1.03
Available phosphorus (%)	0.62	0.55
Lysine (%)	1.25	1.10
methionine (%)	0.54	0.39

[a] *Provided the following per kilogram of diet: Vit. A (β-carotene), 1500 IU; Vit. D, 1250 IU; Vit. E (dl-α-tocoperol), 15 IU; Vit. K, 2.2 mg; Vit. B_1, 1.5 mg; Vit. B_2, 8.0 mg; Vit. B_6, 2.5 mg; Vit. B_{12}, 0.011 mg; niacin, 44.00 mg; D-pantothenic acid, 11.00 mg; folic acid, 0.9 mg; D-biotin, 0.11 mg; choline, 550 mg*
[b] *Fe, 80.00 mg; Cu, 8.00 mg; I, 0.35 mg; Se, 0.15 mg; Zn, 80.00 mg*
[c,d] *measured value*

T Lymphocyte Transformation Rate

A sterile collection of 5 mL blood was performed from chicken hearts on days 14, 28, and 42. Heparin (1 mL) was used for anticoagulation. The blood samples were allowed to stand for 1 h and then 6 mL of lymphocyte separation solution was added (the ratio of blood and separation solution is 1:1). Next, the sample was centrifuged for 20 min at 3000 rpm to absorb the middle cloudy white blood cell layer, washed three times with Hank's solution, and centrifuged again for 10 min at 2000 rpm. The living

cells (trypan blue staining) were resuspended and the number of cells was adjusted to 3 $\times 10^6$ per mL using RPMI1640 culture medium (Gibco, Los Angeles, CA, USA). Thereafter, a certain dose of phytohemagglutinin (PHA) (sigma, St. Louis, MO, USA) was added with a final concentration of 10 μg/mL. After giving a slight shaking, the cells were cultured in a 5% CO_2 incubator at 37°C for 72 h (Thermo, Waltham, MA, USA).

Then, the cells were centrifuged at 1000 rpm for 10 min, and the supernatant was discarded. The precipitate cell (1 mL) was placed on a clean slide and fixed with methanol. After washing with PBS and drying, cells were stained with Giemsa for no less than 3 h. Finally, the number of transformed cells in 200 lymphocytes was observed and counted under the microscope.

Newcastle Disease Virus Antibody Titer

The birds were administrated the Newcastle disease virus (NDV) vaccine (La Sota) at day 1 (intranasal) and day 14 (intramuscular). And on days 14, 28, and 42, 10 broilers were randomly selected from each group. Blood was collected from these chickens and serum was separated by centrifugation. The haemagglutination inhibition (HI) assay was performed using 96-well V-shaped haemagglutination platelets. The titer of antibodies was measured by log2.

Statistical Analysis

Data were reported as means and analyzed by one-way analysis of variance (ANOVA) using SPSS 17.0. The significance of differences among different groups was evaluated by least significant difference (LSD) post-hoc multiple comparisons test.

Results

The effects of APS treatment on growth performance were evaluated through FI, BWG, and FCR and are shown in *Table 2*. No significant differences were found in the FI of broilers among different treatment groups (P>0.05). Compared to the control group, the APS caused positive effects on BWG and FCR of other groups (P<0.05). A quadratic decrease in FCR was observed in groups supplemented with APS. The highest ADG and lowest FCR values were observed in the 2.5 mg APS group. These data suggest that APS improves the broilers' growth performance.

To explore the effect of APS on phagocytic ability, India ink was injected into the peripheral vein of broilers and the carbon clearance level was determined on days 14 and days 28 *(Table 3)*. It was demonstrated that chicks administered with APS possessed a faster carbon clearance rate, which revealed a higher phagocytosis activity than that of the control group. The highest phagocytosis index (9.48) on day 14 was observed in the group administered with 2.5 mg APS (P<0.05). The same tendency was also observed in the 2.5 mg group on day 28; the highest phagocytosis index was 11.13 (P<0.05). On day 42, 2.5 mg and 5 mg APS administered groups showed an improvement in the phagocytosis index (P<0.001), and the highest index was 14.77. These data suggest that APS can improve the phagocytosis ability of peritoneal macrophages in broilers.

To further explore the effect of APS on cellular immunity, the lymphocyte transformation rate after PHA stimulation was measured. The T lymphocyte transformation rate at different ages of broilers in each group is displayed in *Table 4*. We found no significant difference in the T

***Table 2**. Effect of APS administration on growth performance of broilers*

Items	APS Dosage (mg)				P-value		
	0	2.5	5	10	ANOVA	Linear	Quadratic
FI	82.25±1.049	82.28±0.748	82.23±0.940	82.33±1.017	1.000	0.964	0.969
BWG	43.18±1.083 [a]	49.02±1.070 [b]	45.93±0.920 [a]	45.96±1.092 [a,b]	0.008	0.274	0.011
FCR	1.90±0.023 [a]	1.68±0.025 [b]	1.79±0.025 [c]	1.79±0.027 [c]	0.000	0.069	0.000

Means that do not share similar letter in row are significantly different, P≤0.05.

***Table 3.** Phagocytic index of macrophages in peripheral blood of broilers*

Items	APS Dosage (mg)				P-value		
	0	2.5	5	10	ANOVA	Linear	Quadratic
14d	6. 76±0.434 [a]	9.48±0.166 [b]	6.82±0.387 [a,c]	6.76±0.825 [a,c]	0.002	0.259	0.013
28d	8.18±0.848 [a]	11.13±0.819 [b]	9.29±0.582 [a,b]	8.45±0.442 [a,c]	0.029	0.744	0.013
42d	10.08±0.249 [a]	14.77±0.329 [b]	11.10±0.271 [c]	10.83±0.335 [a]	0.000	0.749	0.000

Means that do not share similar letter in row are significantly different, P≤0.05

Table 4. *Lymphocyte transformation rate of broilers*

Items	APS Dosage (mg)				P-value		
	0	2.5	5	10	ANOVA	Linear	Quadratic
14d	9.14±0.283 [a]	11.64±0.180 [b]	10.90±0.396 [b]	8.90±0.106 [a,c]	0.000	0.236	0.000
28d	12.92±0.595 [a]	15.91±1.301 [b]	14.1±0.415 [a,b]	13.83±0.351 [a,b]	0.075	0.791	0.045
42d	17.9±0.465 [a]	22.81±0.378 [b]	20.38±0.404 [c]	20.31±0.182 [c]	0.000	0.010	0.000

Means that do not share similar letter in row are significantly different, P≤0.05

Table 5. *Antibody titers to Newcastle disease virus vaccine (log2)*

Items	APS Dosage (mg)				P-value		
	0	2.5	5	10	ANOVA	Linear	Quadratic
14d	5.5±0.141	5.7±0.106	5.5±0.115	5.3±0.082	0.135	0.130	0.093
28d	7.5±0.124 [a]	8.0±0.124 [b]	7.7±0.118 [a,b]	7.7±0.141 [a,b]	0.078	0.604	0.063
42d	7.5±0.141 [a]	8.2±0.129 [b]	7.7±0.103 [a,c]	8.0±0.124 [b,c]	0.004	0.089	0.126

Means that do not share similar letter in row are significantly different, P≤0.05

lymphocyte transformation rate among the 5 mg and 10 mg APS administered groups on day 14 and 28 (P>0.05). However, on day 42, all of the APS administered groups (2.5 mg, 5 mg, 10 mg) showed marked improvement in T lymphocyte transformation in the peripheral blood (P<0.001) compared to the control group. Between the three APS administered groups, the 2.5 mg group showed significant improvement in the blood T lymphocyte conversion rate during the study period (P<0.05). These data suggest that APS can improve the peritoneal T lymphocyte transformation in broilers.

We measured the antibody titer to Newcastle disease virus (NDV) vaccine in the serum. As show in *Table 5*, there was no significant difference among the groups in the NDV antibody titer on day 14 (P>0.05). However, on day 28 and day 42, groups with APS dose of 5 mg and 10 mg showed increased NDV antibody titer at a certain extent (P>0.05) compared with the control group. Moreover, the 2.5 mg group showed a significant increase in NDV titer of broilers (P<0.05). These data suggest that APS can improve the humoral immunity in broilers at a certain extent.

Discussions

The biological activity of polysaccharides as an immune-enhancer is not only related to their molecular weight, solubility, viscosity, and chemical structure, but also closely related to the route of administration, dosage, animal species, and other factors [13,14]. In this research, the water-soluble APS extracted from the stems and leaves of alfalfa during pregnancy was non-starch polysaccharide, which was given to broilers through nasal drip after autoclaving. Experimental results suggest that water-soluble APS given intranasal can improve the growth performance and immune ability of broilers.

Macrophages are very important to the immune system of the body. They play the functions of monitoring, defense, antigen presentation, and regulation and elimination of apoptotic cells in the body, and are the first line of defense of the body's immune system [15,16]. The enhancement of phagocytic rate or phagocytic ability is one of the most significant characteristics of activated macrophages, which helps to improve the body's defense and anti-infection ability. In this research, our data demonstrate that APS could increase the phagocytic activity of macrophages. Our results are consistent with previous studies that suggested that bioactive polysaccharides could enhance the phagocytic activity of macrophages [17,18].

T lymphocyte proliferation is a direct indicator reflecting the characteristics of cellular immune function. Generally, lymphocytes can be divided and proliferate after being stimulated by corresponding antigen substances and produce antibodies or lymphoid factors against related antigens. Therefore, lymphocytes are the most important immune effector cells in animals [19,20]. The results of this study show that different dosages of APS could promote the proliferation of T lymphocytes in chicken peripheral blood, indicating that APS has immune potentiation. However, this effect was found to be more significant in the low dosage group (2.5 mg) than in the medium (5 mg) and high (10 mg) dosage groups, indicating that APS can promote the proliferation of peripheral blood T lymphocytes but its effect depends on the dosage and that the appropriate dose is an important factor affecting its immune enhancement effect. These data were further verified by other plant polysaccharides with suitable dosage on the proliferation activities of murine spleen lymphocytes [21].

The titer of Newcastle disease HI antibody in broiler serum was determined by erythrocyte agglutination test at different growth stages. It can be seen from the results

of the titer of the HI antibody in the serum on day 14 that the enhancement effect on the humoral immune function of broilers is not obvious. On day 28 and day 42, the 2.5 mg group showed enhanced humoral immune function. There is a certain difference between the results of NDV HI antibody across the three stages. Part of the difference may be due to the interference of the maternal antibody of NDV in the younger chicken, resulting in the insignificant increase of the HI titer of NDV by APS [22].

In the present research, chicks administered with different doses of APS demonstrated a positive effect on their growth performance, phagocytic ability, T lymphocyte proliferation, and NDV antibody titer. However, when different doses of APS were given to broilers through a nasal drip, they did not display dose dependence for the above indicators. This may be attributed to the complex structure of APS. As they are complex biological macromolecule material, polysaccharides could interact with each other to create a network structure and absorb water molecules when the concentration is increased, which could affect their biological activity [23].

In conclusion, intranasally administered APS at a dose of 2.5 mg had positive effects on broiler performance. It stimulated phytohemagglutinin-induced T-lymphocyte transformation and increased phagocytic activity without adversely affecting Newcastle disease virus antibody production. The immunomodulatory effects of APS on poultry production are likely to enhance the response the birds' diseases resistance ability.

Declarations

Availability of Data and Materials: The data and materials set during the current study are available from the corresponding author (S. Zhang) on reasonable request.

Funding Support: This work was supported by the Leading Talents of Scientific and Technological Innovation in the Central Plains (No. 224200510024), National Natural Science Foundation of China (No. 32202763), the Program for Innovative Research Team (in Science and Technology) in University of Henan Province (No. 22IRTSTHN026).

Competing of Interest: The authors declare no competing financial interest.

Ethical Statement: Experimentation with animals was approved by the Experimental Animal Management Methods of Xinxiang Medical University (Approval number: 201206078) and followed Henan Authority's Experimental Animal Regulations.

Author's Contribution: Y. Dong and S. Zhang designed this study. Y. Dong and L. Wang executed the experiment and analyzed the data. All authors interpreted the data, critically revised the manuscript for important intellectual contents and approved the final version.

Reference

1. Yu Y, Shen M, Song Q, Xie J: Biological activities and pharmaceutical applications of polysaccharide from natural resources: A review. *Carbohydr Polym,* 183, 91-101, 2018. DOI: 10.1016/j.carbpol.2017.12.009

2. Mukherjee S, Jana S, Khawas S, Kicuntod J, Marschall M, Ray B, Ray S: Synthesis, molecular features and biological activities of modified plant polysaccharides. *Carbohydr Polym,* 289:119299, 2022. DOI: 10.1016/j.carbpol.2022.119299

3. Chen F, Huang G: Preparation and immunological activity of polysaccharides and their derivatives. *Int J Biol Macromol,* 112, 211-216, 2018. DOI: 10.1016/j.ijbiomac.2018.01.169

4. Swierczewska M, Han HS, Kim K, Park JH, Lee S: Polysaccharide-based nanoparticles for theranostic nanomedicine. *Adv Drug Deliv Rev,* 99 (Pt A): 70-84, 2016. DOI: 10.1016/j.addr.2015.11.015

5. Yin M, Zhang Y, Li H: Advances in research on immunoregulation of macrophages by plant polysaccharides. *Front Immunol,* 10:145, 2019. DOI: 10.3389/fimmu.2019.00145

6. Li CX, Liu Y, Zhang YZ, Li JC, Lai J: Astragalus polysaccharide: A review of its immunomodulatory effect. *Arch Pharm Res,* 45 (6): 367-389, 2022. DOI: 10.1007/s12272-022-01393-3

7. Li W, Hu X, Wang S, Jiao Z, Sun T, Liu T, Song K: Characterization and anti-tumor bioactivity of astragalus polysaccharides by immunomodulation. *Int J Biol Macromol,* 145, 985-997, 2020. DOI: 10.1016/j.ijbiomac.2019.09.189

8. Li M, Du X, Yuan Z, Cheng M, Dong P, Bai Y: Lentinan triggers oxidative stress-mediated anti-inflammatory responses in lung cancer cells. *Mol Cell Biochem,* 477 (2): 469-477, 2022. DOI: 10.1007/s11010-021-04293-0

9. Chen S, Wan C, Ma Y, Zhang K, Wang F, Shen S: Study on the quality of mixed silage of rapeseed with alfalfa or myriophyllum. *Int J Environ Res Public Health,* 20 (5): 3884, 2023. DOI: 10.3390/ijerph20053884

10. Jonker A, Yu P: the role of proanthocyanidins complex in structure and nutrition interaction in alfalfa forage. *Int J Mol Sci,* 17 (5):793, 2016. DOI: 10.3390/ijms17050793

11. Zhang C, Li Z, Zhang CY, Li M, Lee Y, Zhang GG: Extract methods, molecular characteristics, and bioactivities of polysaccharide from alfalfa *(Medicago sativa* L.). *Nutrients,* 11 (5):1181, 2019. DOI: 10.3390/nu11051181

12. Zhang S, Zhu C, Xie H, Wang L, Hu J: Effect of gan cao *(Glycyrrhiza uralensis* Fisch) polysaccharide on growth performance, immune function, and gut microflora of broiler chickens. *Poult Sci,* 101 (10):102068, 2022. DOI: 10.1016/j.psj.2022.102068

13. Wusiman A, Rexiati S, Aziz M, Cheng X, Mai Z, Abulaiti A, Wutikuer A, Rozi P, Abuduwaili A, Abula S: Preparation and sulfate modified of *Lagenaria siceraria* (Molina) Standl polysaccharide and its immune-enhancing adjuvant activity. *Poult Sci,* 101 (11):102112, 2022. DOI: 10.1016/j.psj.2022.102112

14. Yang X, Wei S, Lu X, Qiao X, Simal-Gandara J, Capanoglu E, Wozniak L, Zou L, Cao H, Xiao J, Tang X, Li N: A neutral polysaccharide with a triple helix structure from ginger: Characterization and immuno-modulatory activity. *Food Chem,* 350:129261, 2021. DOI: 10.1016/j.foodchem.2021.129261

15. Wynn TA, Chawla A, Pollard JW: Macrophage biology in development, homeostasis and disease. *Nature,* 496 (7446): 445-455, 2013. DOI: 10.1038/nature12034

16. Yan ZQ, Hansson GK: Innate immunity, macrophage activation, and atherosclerosis. *Immunol Rev,* 219, 187-203, 2007. DOI: 10.1111/j.1600-065X.2007.00554.x

17. Ghosh S, Khatua S, Dasgupta A, Acharya K: Crude polysaccharide from the milky mushroom, *Calocybe indica,* modulates innate immunity of macrophage cells by triggering MyD88-dependent TLR4/NF-kappaB pathway. *J Pharm Pharmacol,* 73 (1): 70-81, 2021. DOI: 10.1093/jpp/rgaa020

18. Xie Y, Wang L, Sun H, Wang Y, Yang Z, Zhang G, Jiang S, Yang W: Polysaccharide from alfalfa activates RAW 264.7 macrophages through MAPK and NF-kappaB signaling pathways. *Int J Biol Macromol,* 126, 960-968, 2019. DOI: 10.1016/j.ijbiomac.2018.12.227

19. Datta S, Sarvetnick N: Lymphocyte proliferation in immune-mediated diseases. *Trends Immunol,* 30 (9): 430-438, 2009. DOI: 10.1016/j.it.2009.06.002

20. Heinzel S, Marchingo JM, Horton MB, Hodgkin PD: The regulation of lymphocyte activation and proliferation. *Curr Opin Immunol,* 51, 32-38, 2018. DOI: 10.1016/j.coi.2018.01.002

21. Cao LZ, Lin ZB: Comparison of the effects of polysaccharides from wood-cultured and bag-cultured *Ganoderma lucidum* on murine spleen lymphocyte proliferation *in vitro*. *Yao Xue Xue Bao,* 38 (2): 92-97, 2003.

22. Bertran K, Lee DH, Criado MF, Balzli CL, Killmaster LF, Kapczynski DR, Swayne DE: Maternal antibody inhibition of recombinant Newcastle disease virus vectored vaccine in a primary or booster avian influenza vaccination program of broiler chickens. *Vaccine,* 36 (43): 6361-6372, 2018. DOI: 10.1016/j.vaccine.2018.09.015

23. Hosseini SM, Emam-Djomeh Z, Negahdarifar M, Sepeidnameh M, Razavi SH, Van der Meeren P: Polysaccharide type and concentration affect nanocomplex formation in associative mixture with beta-lactoglobulin. *Int J Biol Macromol,* 93 (Pt A): 724-730, 2016. DOI: 10.1016/j.ijbiomac.2016.09.037