Preparation and sulfate modified of *Lagenaria siceraria* (Molina) Standl polysaccharide and its immune-enhancing adjuvant activity

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ABSTRACT Herbal polysaccharides and their modifiers used as vaccine adjuvants have been widely investigated due to their safety and good immunoenhancing activity. In this study, the 50% ethanol concentration precipitated *Lagenaria siceraria*(Molina) standl polysaccharide (**LSP50**) and sulfated modified LSP50 (**sLSP50**) was prepared, and their characterization was investigated. LSP50 and sLSP50$_{-1.5}$ were used as vaccine adjuvants to immunize chickens, and the strength and type of immune responses induced by different adjuvants were detected. Our results showed that LSP50 was homogeneous polysaccharides, and the carbohydrate content was 98.6%. The sLSP50$_{-1.5}$ with the DS value of 1.5 was optimized by response surface methodology. The sLSP50$_{-1.5}$ has both characteristics of polysaccharide functional groups and sulfate functional groups. Adjuvant activity of LSP50 and sLSP50$_{-1.5}$ showed that LSP50 and sLSP50$_{-1.5}$ could induce long-lasting and high hemagglutination (HI) titers, antigen-specific lgG-NDV antibody, splenic lymphocyte proliferation, high immune organ index. Moreover, chicken immunized with sLSP50$_{-1.5}$ showed a strong mixed Th1-type (IFN-γ and TNF-α) and Th2-type (IL-4 and IL-6) cytokines expression. Thus, these findings demonstrated that sLSP50$_{-1.5}$ as a vaccine adjuvant can induce a mixed cellular and humoral immune response and can potentially serve as an effective vaccine adjuvant for NDV antigen.

Key words: Lagenaria siceraria (Molina) Standl polysaccharide, sulfated modified, response surface method, vaccine adjuvants

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INTRODUCTION

Vaccination is one of the safest and most effective tools to prevent many infectious and chronic diseases (Gu et al., 2019; Hoare et al., 2019). To enhance the immunogenicity of the vaccine and produce a long-term and effective immune protection effect for humans or animals, a vaccine is usually mixed with an adjuvant (Dai et al., 2021; Paswan et al., 2021). Adjuvants have been used to increase the magnitude of an adaptive immune response and produce the most effective immune forms for each specific pathogen (Boravleva et al., 2020). Many strategies, including immune-enhancing natural compounds, biodegradable nanomaterials, cell factors, mineral Oil, and chemicals, have been involved as adjuvants mixed with antigens to develop effective vaccine delivery systems against infectious diseases (Moyer et al., 2016; Zhao et al., 2019). Among them, immune-enhancing extracted from natural plants and their chemical modifiers have become the focus of the development of new adjuvants (Kubo and Miyauchi, 2020; He et al., 2020). *Lagenaria siceraria* (Molina) Standl. polysaccharide extracted from *Lagenaria siceraria* (Molina) Standl. has been demonstrated to possess excellent immune enhancement (Li et al., 2018). Previously our study has shown that among the different concentrations. The 50% ethanol concentration precipitated *Lagenaria siceraria* (Molina) Standl. Polysaccharide (**LSP50**) induced the most potent immune enhancement activity. LSP50 can induce splenic lymphocyte proliferation, cooperated with ConA can stimulate T lymphocyte proliferation, and cooperated with LPS can stimulate B lymphocyte proliferation (Wusiman et al., 2016). However, herbal polysaccharides also have some disadvantageous, such as brief biological half-life and weak effects in

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vivo, which limit their clinical application. To address these limitations, further research should be conducted here to improve its activity.

Sulfate modification of polysaccharides can improve the biological activity of polysaccharides (Wang et al., 2018; Ghosh et al., 2021). Sulfuric acid groups can replace some hydroxyl groups on the macromolecular chain of polysaccharides by sulfate modification methods to form artificial synthetic compounds (Li et al., 2020). A large number of studies have shown that the antiviral, antioxidant, and immune-enhancing functions of polysaccharides have been greatly enhanced after sulfated modification (Qin et al., 2019). The degree of sulfation (**DS**) value is an important index to affect the cytotoxicity and functional changes of sulfated polysaccharides (Matari et al., 2020). The DS value indicates the degree of substitution of sulfate groups in the sulfated polysaccharides (Matari et al., 2020; Zhang et al., 2020). Typically, when the DS value is larger than 2, the cytotoxicity of sulfated polysaccharides is relatively high, while when DS value is less than 1, it is unable to produce effective biological activity due to the small number of sulfate radical functional groups, so a DS value of between 1 and 2 is considered as a better modification degree (Rui et al., 2020).

In our previous research, an immunopotentiation polysaccharide LSP50 was successfully extracted. However, its adjuvant activity and whether it can induce good cellular and humoral immune responses are unknown. In this study, to facilitate the strength of immune response induced by LSP50, the LSP50 was modified with sulfidation with the chlorosulfonic acid-pyridine method. To prepare the sLSP50 with a DS value of 1.5 and systematically explain the influence of reaction temperature, reaction time, and chlorosulfonic acid-pyridine ratio on the DS value, the response surface method was used to analyze the relationship between the three factors and DS. The second-order polynomial equation between three factors and DS value was established by response surface test, and $sLSP50_{-1.5}$ with a DS value of 1.5 was prepared using the second-order polynomial equation under optimal conditions. We hypothesized that the adjuvant activity of LSP50 could be significantly improved after sulfated modification. Therefore, LSP50 and sLSP50 were used as vaccine adjuvants mixed with the H9N2 antigen. The hemagglutination inhibition (**HI**) titers, antigen-specific lgG-NDV, immune organ index, splenic lymphocyte proliferation effect, and cytokine secretion were determined to evaluate the immune strength and type of immune response.

MATERIALS AND METHODS

Materials

Lagenaria siceraria (Molina) Standl. was collected from Xinjiang Kashgar County, and the dried was pulverized over 60 mesh screening seals. aluminum (Alum) Adjuvant (Thermo scientific, Waltham), Inactivated NDV antigen (Beijing Zhongke Yueyang Technology Co. LTD, China), CCK-8 (Beijing boasen Biotechnology Co. Ltd, China), ELISA kit (Shanghai Jianglai Industrial Limited By Share Ltd, China).

Preparation of LSP50 and sLSP50

The *Lagenaria siceraria* (Molina) Standl. was collected from Kashgar County in Xinjiang, PR China. The crude 50% ethanol concentration precipitated *Lagenaria siceraria* (Molina) Standl. polysaccharide was extracted by water decoction and ethanol precipitation, and protein was removed by the Sevag' method. Then, the crude polysaccharide was further processed using a macroporous resin AB-8 column (Tianjin Damao Chemical Reagent Factory, China) for decoloration. The decolorized polysaccharides were further separated by the DEAE-52 column (Beijing Solaibao Technology Co. Ltd, China) with gradient elutions (deionized water, 0.1, 0.2, 0.5 M NaCl solutions). Water separated part was collected and purified with a Sephadex G-100 column (Beijing Solaibao Technology Co. Ltd) to obtain pure and homogeneous LSP50. The polysaccharide content of LSP50 was determined by phenol sulfuric acid method, and protein content was determined by the BCA kit (Wusiman et al., 2019).

The sLSP50 was prepared by the chlorosulfonic acid-pyridine method, and the free compounds were removed by dialysis bags (MWCO = 3500 D, Beijing Solaibao Technology Co., Ltd) according to a previous study (Wusiman et al., 2019; Malik et al., 2020).

Experimental Design of RSM

Three-level, three-variable box−behnken design was applied to optimize the conditions of the DS value for the preparation of sLSP50. These three factors and their levels were described in Table 1, and the range of levels was determined through the pre-test. Experimental data were analyzed using software Design Expert Version 8.06, and the variables were coded according to the following equation:

$$Y = A_0 + \sum_{i=1}^{3} A_i X_i + \sum_{i=1}^{3} A_{ii} X_i 2 + \sum_{i=1}^{2} \sum_{j=i+1}^{3} A_{ij} X_i X_j$$

In this equation, Y is the predicted response, A0, Ai, Aii, and Aij are represented the model constant, linear coefficient, quadratic coefficient, and interaction coefficient, the X1, X2, and X3 correspond to the independent

Table 1. Levels and code of variables of the DS used in Box −Behnken design.

			Coded levels		
Variable	Uncoded	Coded	−1	0	1
Reaction temperature (°C)	X_1	A	50	65	80
Reaction time (min)	X_2	B	60	120	180
Chlorosulfonic acid-pyridine ratio	X_3	C	1:14	1:5	1:3

Abbreviation: degree of sulfation.

variables, respectively (Gu et al., 2018; Ji et al., 2020). The sufficiency of the selected models to predict the Response function (Y) was determined by the application of analysis of variance (**ANOVA**). (Gu et al., 2018).

Identification of Sulfated Modified LSP50

A standard curve was constructed with sodium sulfate, and the degree of sulfation (DS) was calculated according to the equation. DS = (1.62 × S%)/(32-1.02 × S%), S% represents the mass fraction of sulfur element (Malik et al., 2020). FT-IR spectra of the LSP50 and sLSP50 were recorded by KBr pellets method (Liu et al., 2021).

Animal Immunization

Inactivated NDV antigen purchased from the Harbin Pharmaceutical Group Bio-vaccine Co. LTD (Harbin, China). Animal experiments were conducted in strict accordance with the guide for the care and use of laboratory animals, Xinjiang Agricultural University IACUC (Approval ID: PTA2020034). One-day-old HY-LINE Variety Brown chickens purchased from Tiankang Poultry farm (Urumqi, China) were randomly divided into 5 groups (n = 25). After centralized feeding for 1 wk average titer of maternal antibody was determined by hemagglutination inhibition (HI) assay before immunization and the value was 2.0 (Log2). The chickens were intramuscular immunized with 200 μL of the mixture of LSP50/NDV (1:1 = V_{LSP50}:V_{NDV}), sLSP50$_{-1.5}$/NDV (1:1 = $V_{sLSP50\text{-}1.5}$:V_{NDV}), Alum/NDV (Alum Adjuvant, 1:1 = V_{Alum}:V_{NDV}), free NDV, and PBS. Chickens were boosted with identical doses, and strengthen immunity after two weeks. All of the chickens were sacrificed at d 7, 14, and 21 after the second immunization.

Determination of HI Titer and Antibodies in Serum

HI titer of sera was determined by hemagglutination inhibition methods at d 7, 14, and 21 after the final vaccination according to a previous study (He et al., 2015). Antigen-specific IgG antibodies in serum were determined by ELISA on d 7, 14, and 21 after the final immunization conditions as the reference (Gu et al., 2019). The absorbance value at 450 nm (OD450) was read using an ELISA plate reader (Thermo Scientific).

Splenocyte Proliferation Assay

Splenic lymphocytes were isolated from immunized chicken on d 7, 14, and 21 after the final immunization. Splenocytes (2.5×10^5 cells/mL) were re-suspended in complete medium and re-stimulated with NDV antigen (50 μg/mL), then incubated for 48 h. Cells in the blank cells group were used as control. CCK-8 assay was used to assess cell proliferation at OD450 nm (Luo et al., 2017).

Determination of the Immune Organ Index

On d 21 after the second immunization, chicken (n = 5) immune organs spleen, thymus, and bursa of fabricius were collected, and weighed quickly. The chicken immune organ index was calculated. Immune organ index = (Immune organ weight/body weight) × 100% (Gu et al., 2019).

Determination of Cytokines in Serum

The secretion level of the Th1 cytokine (TNF-α and IFN-γ), and Th2 cytokine (IL-4 and IL-6) in serum were detected at d 7, 14, and 21 after final immunization by ELISA kits (Shanghai Jianglai Industrial Co. Ltd., China) according to the manufacturer's instructions.

Histopathological Analysis

Spleen, thymus, and bursa of fabricius were collected from the immunized chickens and fixed with 4% paraformaldehyde on d 21 after the second immunization (n = 3). Then the fixed organs were performed by hematoxylin and eosin (**HE**) staining.

Statistical Analysis

Quantitative data were expressed as means ± SEM. Statistical significance was analyzed using One-way ANOVA analysis with Turkey's test. A probability value $P < 0.05$ was considered statistically significant.

RESULTS AND DISCUSSION

Extraction, Isolation, and Purification of LSP50

The crude polysaccharides in *Lagenaria siceraria* (Molina) Standl. was isolated by hot-water extraction, ethanol precipitation, deproteinization, and decolorization. The yield of crude LSP50 was about 10.8% of the dried material. To purify and isolate crude polysaccharides, crude LSP50 was subjected to column chromatography by DEAE-52 and Sephadex G-100, respectively. The first fraction was collected by DEAE-52 column accounted for 69.4% of the total LSP50 content (Figure 1A), and the main part of the LSP50 was further purified through Sephadex G-100 column (Figure 1B). The carbohydrate content of purified LSP50 was 98.6%, and protein content was 0.8%.

Optimization of the Procedure by RSM

The design consisted of 17 experimental points, was displayed in Table 2. As shown in Table 2, the DS of sLSP50$_{-1.5}$ ranged from 0.695 to 2.677. By using multiple

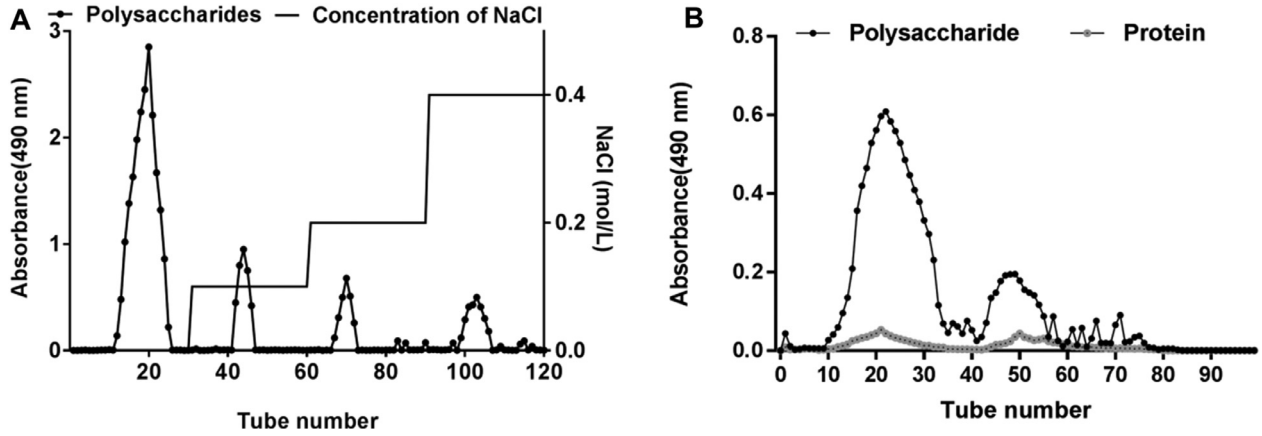

Figure 1. (A) Elution curve of LSP50 on a DEAE-52 column. (B) Elution curve of LSP50 on a Spendex-100 column.

Table 2. Box−Behnken design matrix of the three variables in coded units and response values for the DS (n = 5).

	Levels of independent factors			Response DS	
Run	A	B	C	Practical acquired DS	Predicted acquired DS
1	80	120	1:14	1.127	1.110
2	50	180	1:5	1.454	1.451
3	80	180	1:5	2.677	2.592
4	80	120	1:3	2.454	2.553
5	65	120	1:5	2.279	2.213
6	65	120	1:5	2.243	2.213
7	65	180	1:14	1.167	1.269
8	50	120	1:3	1.339	1.356
9	65	60	1:3	1.654	1.552
10	80	60	1:5	1.484	1.487
11	65	120	1:5	2.282	2.213
12	65	120	1:5	2.18	2.213
13	50	60	1:5	0.884	0.969
14	65	180	1:3	2.594	2.580
15	65	120	1:5	2.083	2.213
16	65	60	1:14	0.695	0.710
17	50	120	1:14	0.747	0.648

Abbreviation: degree of sulfation.

regression analysis on the experimental dates, a second-order polynomial equation was constructed as follows: $Y = 2.21+0.41A+0.40B+0.54C+0.16AB+0.18AC+0.12BC-0.35A^2-0.24B^2-0.45C^2$.

To test the accuracy of the regression equation, ANOVA analysis of the F-test was used to evaluate the significance of the *P*-value of each coefficient. The corresponding variables become more effective as the *P* -value becomes smaller (Gu et al., 2018; Ji et al., 2020). In Table 3, the model F-value was 79.80 ($P < 0.0001$), indicating that the model was significant. The *P*-values of coefficients A, B, C, AB, AC, BC, A2, B2, and C2 were significantly lower than 0.05, indicating the validity of each coefficient. The F-value and *P*-value of "lack of-fit" was 2.67 and 0.1831, respectively, which shows that the "lack-of-fit" was insignificant relative to the pure error (Ji

Table 3. Statistic analysis of variance for the experimental results of the BBD.

Source	Sum of squares	Df	Mean square	F value	*P*-value Prob > F
Model	7.01	9	0.78	64.81	< 0.0001 significant
A	1.38	1	1.38	114.52	< 0.0001
B	1.26	1	1.26	104.86	< 0.0001
C	2.32	1	2.32	192.79	< 0.0001
AB	0.097	1	0.097	8.08	0.025
AC	0.14	1	0.14	11.24	0.0122
BC	0.055	1	0.055	4.56	0.0402
A^2	0.51	1	0.51	42.85	0.0003
B^2	0.24	1	0.24	20.01	0.0029
C^2	0.84	1	0.84	70	< 0.0001
Residual	0.084	7	0.012		
Lack of Fit	0.056	3	0.019	2.67	0.1831 not significant
Pure error	0.028	4	7.00E-03		
Cor total	7.09	16			

$R^2 = 0.9881$; $R^2_{Adj} = 0.9729$; $R^2_{Pred} = 0.8673$.

et al., 2020). The determination coefficient (R^2) and the adjusted determination coefficient (R^2adj) were 0.9881 and 0.9729, respectively, which showed a good agreement between the experimental and predicted values of the DS with the goodness-of-fit of the regression equation.

Simulation of DS Values

The reaction conditions were calculated using the second-order polynomial equation above to prepare the sLSP50 with a DS value of 1.5. Considering the operability and convenience in the production, the optimum condition ware modified. As shown in Table 3, the DS value of sLSP50 prepared in the modified condition was 1.5 ± 0.08, and the error between the optimum condition results is small, which confirmed that the model designed in this study was adequate.

Infrared Spectra of LSP50 and sLSP50$_{-1.5}$

Infrared spectroscopy (**FT-IR**) can detect the functional groups of polysaccharides, which is of great importance in identifying the changes in modified polysaccharides (Thornes, 2015). FT-IR spectra of LSP50 and sLSP50$_{-1.5}$ have a strong absorption peak at 3,362.51 cm^{-1} and 3,382.59 cm^{-1}, a small band at 2,931.76 cm^{-1} and 2,925.99 cm^{-1}, consistent with the presence of hydroxyl groups (-OH) and C-H stretching, respectively. Absorption peaks observed at 1,417.07 cm^{-1} and 1,419.13 cm^{-1} of LSP50 and sLSP50$_{-1.5}$ were consistent with the presence of carbonyl groups. The broad band at 1,609.75 and 1,614.43 cm^{-1} were assigned to the bending vibration of the water. The spectral characteristics in the region ranging from 1,300 to 1,000 cm^{-1} are consistent with C-O and C-C stretching vibrations in polysaccharides. In addition to the similar absorption peaks of LSP50 and sLSP50$_{-1.5}$ above, sLSP50$_{-1.5}$ has an absorption peak at 1,262.03 cm^{-1}, which is the characteristic absorption peak of S = O stretching vibration (Liang et al., 2018; Wusiman et al., 2019). These results indicated that LSP50 and sLSP50$_{-1.5}$ possessed typical absorption peaks of polysaccharides, and the sulfate radical functional group was successfully modified on sLSP50$_{-1.5}$.

HI Titer and Specific Antibody Response

The antibody content of immune animals can be determined by detecting the serum hemagglutination inhibition titer, then reflecting the strength of the immune response induced by the vaccine (Liu et al., 2019; Reed et al., 2016). As shown in Figure 3A, LSP50/NDV and sLSP50$_{-1.5}$/NDV could induce long-lasting and significantly higher HI titers from d 7 to d 21 than the NDV group ($P < 0.05$). There was no significant difference between LSP50/NDV and sLSP50$_{-1.5}$/NDV and Alum/NDV on days 7 to 21 ($P > 0.05$).

The anti-NDV IgG titers were also determined to evaluate the antigen-specific antibody responses induced by LSP50 and sLSP50$_{-1.5}$ as adjuvants. As shown in Figure 3B, LSP50/NDV and sLSP50$_{-1.5}$/NDV could induce long-lasting and significantly higher antigen specific IgG-NDV titers from d 7 to d 21 than NDV group ($P < 0.05$). There was no significant difference between LSP50/NDV and sLSP50$_{-1.5}$/NDV and Alum/NDV on d 7 and d 14 ($P > 0.05$).

Spleen Lymphocyte Proliferation and Immune Organ Index

Spleen lymphocyte proliferation was measured following re-stimulation with NDV antigens on d 7, 14, and 21 after the final vaccination. As shown in Figure 4A, the group sLSP50$_{-1.5}$ induced the highest lymphocyte proliferation index. The lymphocyte proliferation index values of the LSP50 and sLSP50$_{-1.5}$ groups were significantly higher than those of the Alum/NDV, NDV, and PBS groups on d 7 and 21 ($P < 0.05$). The proliferation index of the sLSP50$_{-1.5}$ group was significantly higher than the LSP50 group on d 21 ($P < 0.05$).

The immune organ index is an important index reflecting the body's immune status. As shown in Figure 4B, the sLSP50$_{-1.5}$/NDV group exhibited high thymus, spleen, and bursa of fabricius organ index compared to PBS control groups. The spleen index of sLSP50$_{-1.5}$/NDV was significantly higher than that of the Alum and NDV groups ($P < 0.05$). The results suggested that sLSP50$_{-1.5}$ adjuvants could improve the immune function of the immunized chickens.

Cytokine Secretion

The LSP50, sLSP50$_{-1.5}$, and Alum adjuvant-based vaccine groups induced higher levels of IFN-γ and TNF-α (Th1-type cytokines) and IL-4 and IL-6 (Th2-type cytokines) than the PBS control group ($P < 0.05$). As shown in Figure 5, the sLSP50$_{-1.5}$ adjuvant-based vaccine group induced significantly higher levels of IFN-γ and TNF-α than the Alum group ($P < 0.05$). There was no significant difference between the sLSP50$_{-1.5}$ and Alum groups for IL-4 and IL-6 cytokines levels ($P > 0.05$). These results indicated that sLSP50$_{-1.5}$ as a vaccine adjuvant could induce the mixture secretion of Th1- and Th2-mediated immune responses.

Histology Analysis

The thymus, spleen, and bursa of fabricius were collected on d 21 after the second immunization. The collected organs were performed by hematoxylin and eosin (HE) staining. As shown in Figure 6, Compared with NDV and PBS groups, LSP50/NDV and sLSP50$_{-1.5}$/NDV groups had significantly thicker thymic cortex, higher cell density, larger interlobular septum, and significantly increased thymic corpuscle volume and number. The LSP50/NDV, sLSP50$_{-1.5}$/NDV, and Alum groups had substantially more splenic corpuscles in number, larger in size, and clear boundaries between red

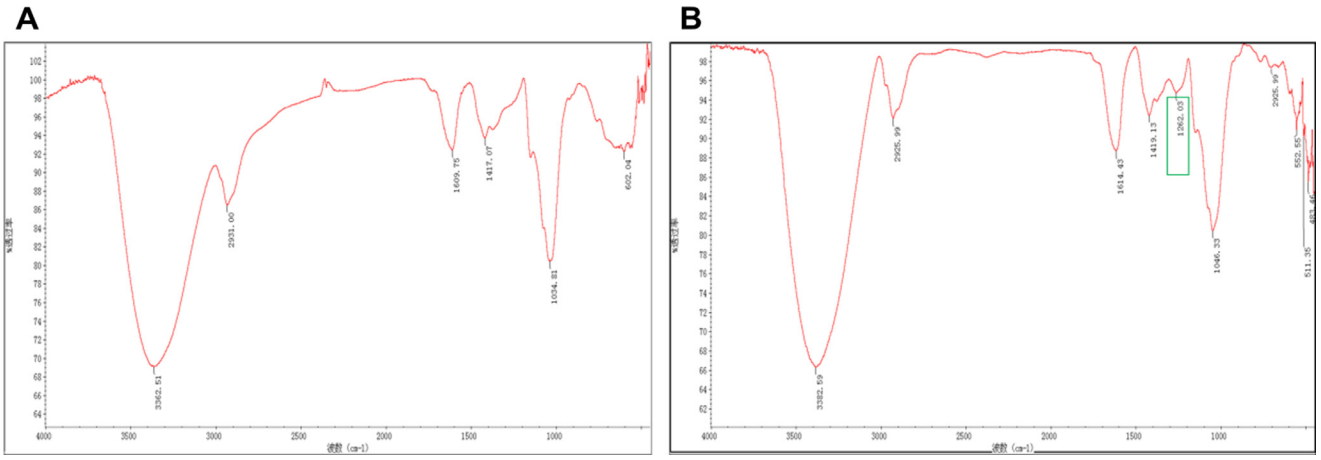

Figure 2. FT-IR analysis of (A) LSP50 and (B) sLSP50$_{-1.5}$.

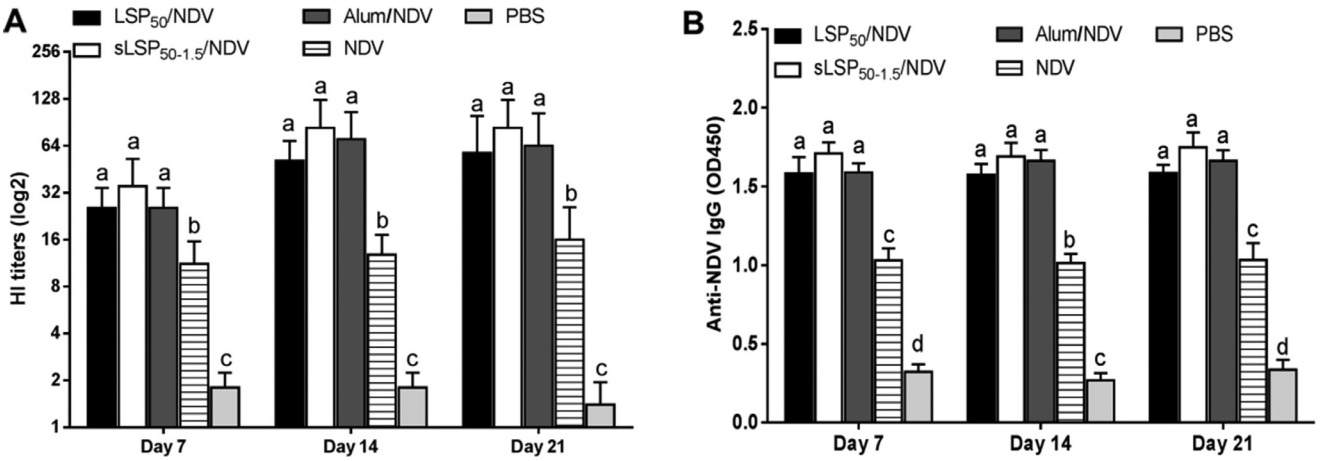

Figure 3. (A) The expression of HI titers. (B) Specific lgG-NDV antibody expression level. [a–d] Bars with different superscripts differed significantly ($P < 0.05$), n = 5.

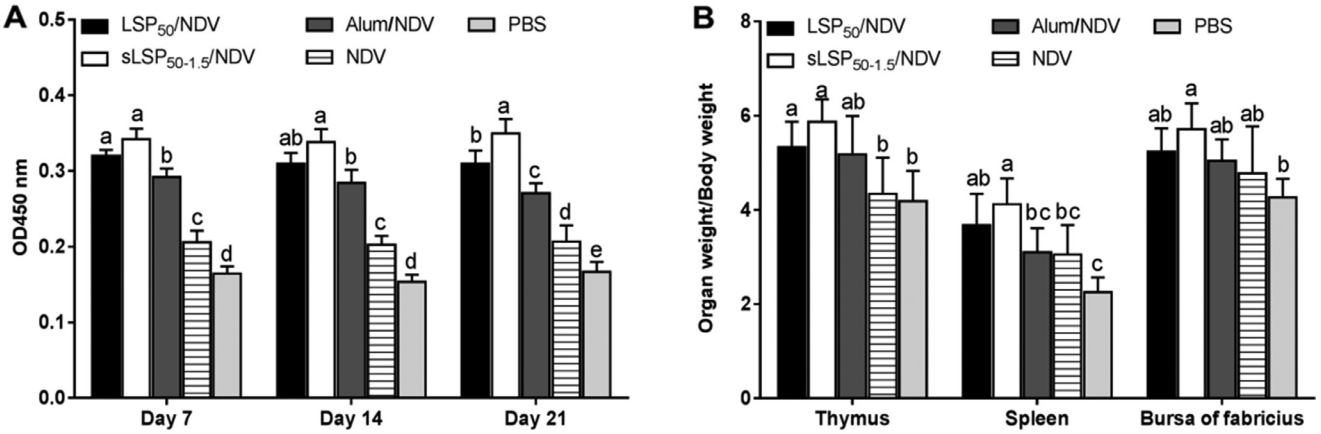

Figure 4. (A) Splenic lymphocyte proliferation. (B) The immune organs index of spleen, thymus, and bursa of fabricius of immunized chickens. [a-e] Bars with different superscripts differed significantly ($P < 0.05$), n = 5.

and white pulp. The area of a single lymphoid follicle in the bursa of Fabricius increased significantly with the more regular arrangement and filtering. The differentiation between the vesicular cortex and medulla is clearer, and the cortical ratio increases. The results showed no obvious toxicities or inflammatory infiltrates in all the experimental groups, and LSP50 and sLSP50$_{-1.5}$ had a good immune-enhancing effect.

DISCUSSION

Vaccination remains the most effective and economically prudent strategy to counter the threat posed by virus (Li et al., 2019; Boravleva et al., 2020). Current commercial adjuvants, such as Alum adjuvants or Oil emulsion adjuvants, induced strong humoral immune responses but with a poor cellular immune response or

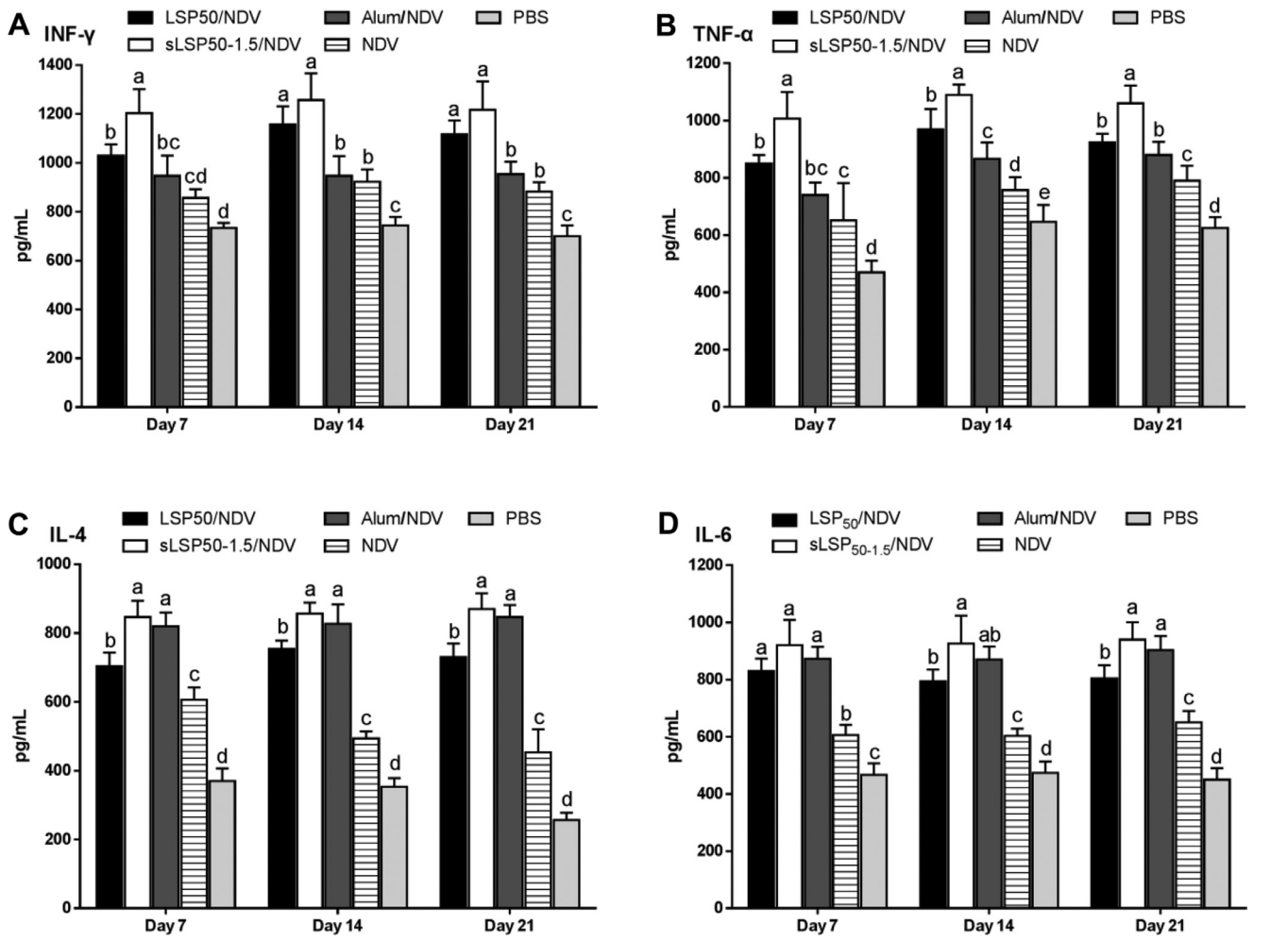

Figure 5. The levels of cytokines IFN-γ (A), TNF-α (B), IL-4 (C), and IL-6 (D) in serum from immunized chickens after the final vaccination were determined by ELISA kits. [a-d] Bars with different superscripts differed significantly ($P < 0.05$), n = 5.

significant toxic side effects (Reed et al., 2016). The cellular immune response is crucial for the host defense system against infection (Thornes, 2015). Moreover, it has been reported that in addition to humoral immunity, effective cellular responses also play a crucial role in protection against NDV, avian flu, and other virus infection (Liu et al., 2019; Pleidrup et al., 2014). Therefore, preparing a vaccine adjuvant that can induce an effective mixture of cellular and humoral immune responses is critical. Herbal polysaccharides and sulfate-modified polysaccharides have been widely investigated due to their safety, lower prices, and good adjuvants activity (Tan et al., 2019; Rui et al., 2020). LSP has been demonstrated to possess excellent immune enhancement and is widely used as an immune-stimulator (Reed et al., 2016). In our previous research, the 50% concentration precipitated polysaccharide LSP50 can induce strong immune enhancement activity in vitro (Wusiman et al., 2016). However, herbal polysaccharides also have some disadvantageous, such as brief biological half-life and weak effect in vivo. To address these limitations, chemical modification methods were applied to enhance their biological activity.

In this study, LSP50 was isolated and purified from the *Lagenaria siceraria* (Molina) Standl., and sulfated modified LSP50 was prepared with the chlorosulfonic acid-pyridine method. The crude LSP50 from dried material was about 10.8%, and the carbohydrate content of purified LSP50 was 98.6% (Figure 1). The response surface test for sLSP50 showed that the influence factors on the DS value could be expressed by the second-order polynomial equation, $Y = 2.21+0.41A+0.40B+0.54C+0.16AB+0.18AC+0.12BC-0.35A^2-0.24B^2-0.45C^2$ (Table 3). The ANOVA study for the regression equation results shows that the model F-value was 79.80 ($P < 0.0001$), coefficient (R^2), and adjusted determination coefficient (R^2adj) were 0.9881, and 0.9729. The P-values of A, B, C, AB, AC, BC, A2, B2, and C2 were lower than 0.05. The above results indicate that the effect of each factor on the DS value is significant, and the regression equation of this model fits well. The DS value and infrared spectra of sLSP50$_{-1.5}$ showed that the DS value of sLSP50$_{-1.5}$ was 1.5 ± 0.08 under modified conditions (Table 4), and the sLSP50$_{-1.5}$ has both characteristic polysaccharide functional groups and sulfate functional groups, indicating that the hydroxyl group on the sLSP50$_{-1.5}$ was successfully replaced by sulfate functional group (Figure 2).

Vaccines are the best defense against infectious diseases (Rapaka et al., 2021). The NDV vaccine usually defends

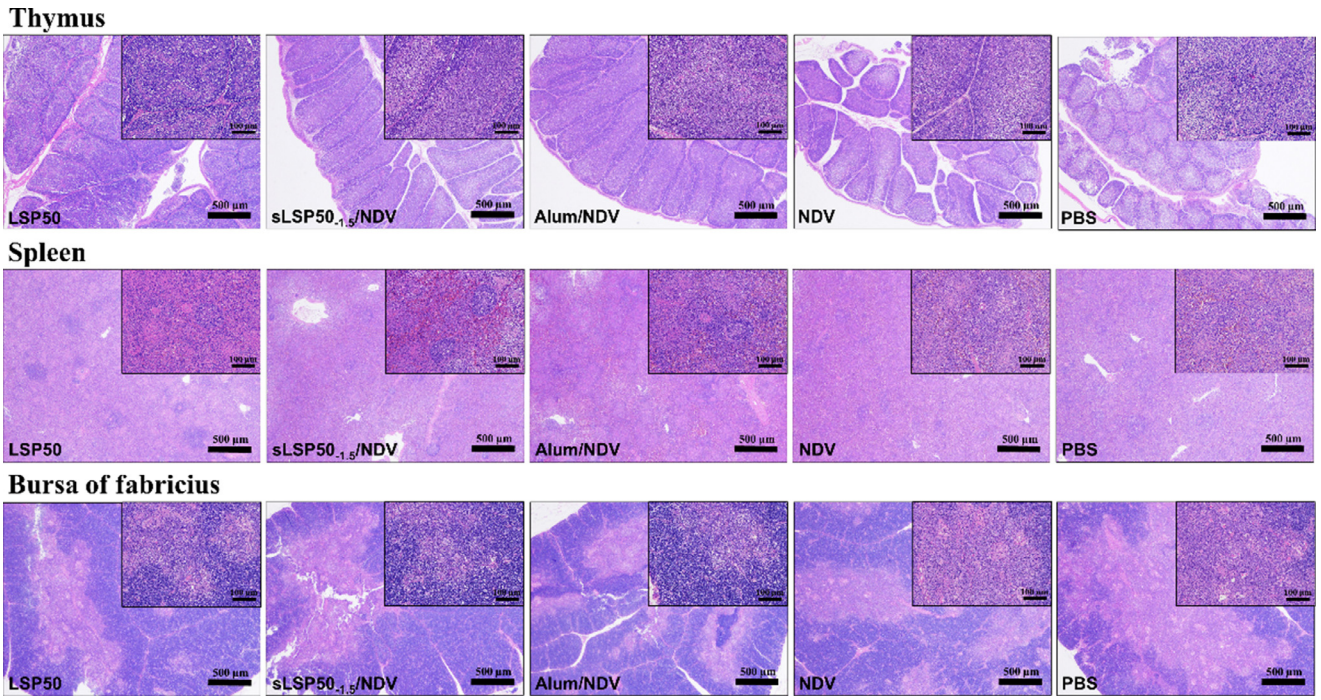

Figure 6. Results of histology analysis of bursa of fabricius (A), spleen (B), and thymus (C) at 21 days after the final immunization (200×, HE, scale bar was 100 μm).

against NDV infection, but due to poor immunogenicity of vaccine antigen, adjuvants need to be added to enhance the immune effect of NDV vaccine (Reed et al., 2016). However, commercial adjuvants such as Alum and Oil adjuvants are limited in their clinical application due to their defects, such as the inability to induce effective cellular immune responses or significant toxic and side effects (Martión et al., 2019; Deng et al., 2020).

Developing safe and effective adjuvants to induce strong humoral and cellular immune responses is necessary. In this study, the adjuvant effects of the LSP50 and sLSP50$_{-1.5}$ for NDV vaccine were evaluated. It was found that LSP50/NDV and sLSP50$_{-1.5}$/NDV induced long-lasting and high HI titer, antigen-specific lgG-NDV (Figures 3A and B), and high splenic lymphocyte proliferation effect, immune organ index, and immune-enhancing effect (Figures 4A, B, and Figure 6). Furthermore, in all treatment groups, sLSP50$_{-1.5}$/NDV increased the significantly higher secretion of Th1-type cytokines IFN-γ and TNF-α and Th2-type cytokines IL-4 and IL-6 compared to Alum/NDV group (Figure 5). Alum adjuvant induces strong Th-2 polarized humoral immune responses (Tan et al., 2019). As such, these data suggested that the sLSP50$_{-1.5}$/NDV induced a strong Th-2 type immune response and mediated an effective Th-1 type immune response. Histology analysis of immune organs from sLSP50$_{-1.5}$/NDV immunized chickens indicated the safety of the sLSP50$_{-1.5}$/NDV formulations. These results show that the adjuvant activity of LSP50 was enhanced after sulfate modification, and the sLSP50$_{-1.5}$ provides an excellent alternative for the adjuvant development of the NDV vaccine.

Table 4. Optimized values obtained by constraints applied on DS (n = 5).

Variable and response	Optimum condition	Modified condition
A (℃)	68.41	68
B (min)	131.57	132
Chlorosulfonic : Acid-pyridine	9:100	9:100
DS value	1.5	1.5 ± 0.08

Abbreviation: degree of sulfation.

CONCLUSIONS

To our knowledge, this is the first study to synthesize, characterize, and in vivo evaluation of the LSP50 and sLSP50$_{-1.5}$. LSP50 was a homogenous polysaccharide with a polysaccharide content of 98.6%. The quadratic regression equation $Y = 2.21+0.41A+0.40B+0.54C+0.16AB+0.18AC+0.12BC-0.35A^2-0.24B^2-0.45C^2$ can be used to optimize the DS value of sLSP50. Both LSP50, and sLSP50$_{-1.5}$ had polysaccharide functional groups and sLSP50$_{-1.5}$ had sulfate absorption peaks, indicating that LSP50 was successfully modified. Between the 2 polymers, sLSP50$_{-1.5}$ immunized chicken can induce high HI antibody titer, antigen-specific lgG-NDV, splenic lymphocyte proliferation effect, immune organ index, and Th1 and Th2-type cytokines secretion. The acquired data on sLSP50$_{-1.5}$ formulation potentially serves as a novel and effective vaccine adjuvant in chicken to induce specific immune responses against infections and diseases.

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DISCLOSURES

The authors declare that the research was conducted in the absence of any commercial or financial relationship that could be construed as a potential conflict of interest.

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