Rubber (*Hevea brasiliensis*) seed oil supplementation attenuates immunological stress and inflammatory response in lipopolysaccharide-challenged laying hens

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ABSTRACT This study was conducted to investigate the effect of PUFA-enriched rubber (*Hevea brasiliensis*) seed oil (**RSO**) supplementation in diets on the productive performance, plasma biochemical parameters, immune response, and inflammation in lipopolysaccharide (**LPS**)-challenged laying hens. Two hundred and forty 25-wk-old Lohmann Brown laying hens were randomly divided into 5 treatments, each including 4 replicates with 12 birds per replicate. The control group and LPS-challenged group were fed a corn-soybean-basal diet; 3 RSO-supplemented groups were fed experimental diets containing 1, 2, and 4% RSO for a feeding period of 4 wk. On the 15, 18, 21, 24, and 27 d of the RSO supplementation period of 4 wk, hens were injected intraperitoneally with LPS at 1 mg/kg body weight (challenge group and RSO-supplemented groups) or with the same amount of saline (control group). The results showed that the addition of RSO promoted laying performance by increasing egg production, total egg weight, daily egg mass, and feed intake in comparison to the LPS-challenged laying hens ($P < 0.05$). In addition, compared with laying hens stimulated with LPS, the analysis of blood cell and plasma parameters revealed that hens in RSO-supplemented groups had significantly lower levels ($P < 0.05$) of white blood cells (**WBC**), lymphocytes (**LYM**), aspartate aminotransferase (**AST**) activity, immunoglobulin A (**IgA**), triiodothyronine (**T3**), interleukin-2 (**IL-2**), and tumor necrosis factor-α (**TNF-α**). Further, RSO supplementation significantly reduced the mRNA expression of toll-like receptor 4 (**TLR4**), nuclear factor kappa-light-chain-enhancer of activated B cells (**NF-κB**), interleukin-6 (**IL-6**), and interleukin-1β (**IL-1β**) of the ileum, spleen, and liver in LPS-challenged laying hens ($P < 0.05$), suggesting that the anti-inflammatory mechanism of RSO is related to the TLR4/NF-κB signaling pathway. In conclusion, RSO supplementation in diets could improve laying performance, attenuate immunological stress, and inhibit the inflammatory response in LPS-challenged laying hens, especially at the dietary inclusion of 4% RSO. This study will provide an insight into the application of RSO to positively contribute to overall health and welfare in laying hens.

Key words: anti-inflammatory, immune activity, laying performance, polyunsaturated fatty acids, rubber seed oil

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INTRODUCTION

In the production environment, poultry is easily affected by a series of unfavorable factors, including various pathogens (Dahshan et al., 2016; Delpont et al., 2021) and non-pathogens (Zeng et al., 2014; Su et al., 2018), resulting in immunological stress and inflammatory response (Hoerr, 2010). Excessive response of poultry will further reduce feed intake, decline productive performance, damage immune function, cause intestinal inflammation, and hinder growth and development, which seriously endangers the health of poultry and brings huge economic losses to the poultry industry (Wein et al., 2017; Abbas et al., 2020). In poultry production, dietary intervention plays important role in the improvement of immunity and productive performance to prevent stress response and following economic losses. Among the ingredients of the diets, lipids can not only provide energy but also provide essential n-6 and n-3 polyunsaturated fatty acids (**PUFA**) which are involved in most biological processes, including the function of the immune system and the inflammatory response (Konieczka et al., 2017; Thanabalan and Kiarie, 2021).

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Appropriate PUFA source supplementation has been reported to be a feasible option for stabilizing productive performance, regulating immunity, and alleviating inflammation of poultry (Sijben et al., 2001; Lee et al., 2019). Al-khalaifah et al. (2020) found that the diets supplemented with four different PUFA sources, including linseed oil, echium oil, fish oil, and algal biomass, showed different effects on fatty acid composition and immune response in broiler chickens. They demonstrated that chickens fed flaxseed oil (18 g/kg) and algal product (15 g/kg) had better immune states (Al-Khalaifah et al., 2020). In addition, it has been reported that a moderate level of dietary PUFA enrichment (n-3 PUFA level of 11.5% and n-6/n-3 PUFA ratio of 2.8) helps to put together the efficiency of performance and relative immune response enhancement in infectious bursal disease-challenged broilers (Maroufyan et al., 2012a). More interestingly, Konieczka et al. (2017) revealed that compared with diets containing corn oil with a relatively high n-6/n-3 PUFA ratio of 51.10, the diets containing a mixture of linseed and fish oils with a relatively low n-6/n-3 PUFA ratio of 0.52 enhanced immunity and decreased inflammatory response in phytohemagglutinin (**PHA**)-challenged broiler chickens. Thus, it can be seen that dietary PUFA supplementation benefits the immune function and inflammatory response of poultry, but its effect was related to the type, dose, composition, and proportion of PUFA sources. Therefore, it is interesting and promising to study PUFA resources with immune and anti-inflammatory benefits for poultry.

Rubber seed oil (**RSO**) is a kind of plant oil extracted from the seeds of rubber (*Hevea brasiliensis*) trees (Azócar et al., 2010). The continuous demand for latex raw materials leads to the extensive planting of rubber trees, which makes the production of RSO large and sustainable (Zhu et al., 2014; Onoji et al., 2016). RSO is rich in PUFA, mainly linoleic acids (**LA**, C18:2n-6) and α-linolenic acids (**ALA**, C18:3n-3), accounting for 39.6−52.84% and 2.38−26% of its total fatty acids (Onoji et al., 2016). However, as typical PUFA-enriched plant oil, RSO has not been widely used in the feed industry. It is worth mentioning that compared with soybean oil commonly used in animal feed, RSO has about 3 times the ALA content of soybean oil (Wen et al., 2019; Messina et al., 2021). The report showed that ALA is an important precursor of eicosapentaenoic (**EPA**, C20:5) and docosahexaenoic (**DHA**, C22:6) that are commonly reported to have anti-inflammatory benefits (Shahidi and Ambigaipalan, 2018). What's more, the n-6/n-3 PUFA ratio of RSO was calculated to be 1.92 (Wen et al., 2019), lower than that of soybean oil 7.5 and corn oil 49.9 (Messina et al., 2021), indicating that RSO has a relatively balanced n-6/n-3 PUFA ratio (2:1 to 5:1, recommended by FAO). Accumulating studies reported that a balanced dietary n-6/n-3 PUFA ratio is contributed to modulating productive performance (Carrillo-Domínguez et al., 2005), fatty acid profile (Li et al., 2017), immune response (Maroufyan et al., 2012b), inflammation (Konieczka et al., 2017), even reproductive performance (Safari Asl et al., 2018) in poultry. Previous studies showed that RSO had no toxicity to the rats (Gandhi et al., 1990; Salimon et al., 2012). In lipopolysaccharide (**LPS**)-stimulated macrophages, RSO displayed antioxidant, immunomodulation, and anti-inflammatory properties (Liu et al., 2022). Pi et al. (2019) reported that RSO addition increased nutrient digestibility, altered serum fatty acid profile, and enhanced immune function of dairy cows. The above findings suggest that RSO has great potential to serve as a functional lipid resource, which not only provides PUFA, especially n-3 PUFA, but also could play roles in regulating productive performance, immunity, and inflammatory response.

Our previous studies showed that RSO supplementation (1, 2, 4, and 6%) to the diet of laying hens increased the contents of n-3 PUFA (ALA, DHA, and EPA) in yolks and improved the productive performance (Wen et al., 2019). However, to the best of our knowledge, the effects of supplementing RSO as a dietary intervention on the immune function and inflammatory response of laying hens and the underlying mechanisms have not been reported. Thus, in this study, laying hens were injected with LPS to establish the model of inflammation and the effect of RSO supplementation (1, 2, and 4%) on productive performance, immunity, and inflammatory response of laying hens was evaluated.

MATERIALS AND METHODS

All experimental procedures were approved by the Animal Ethics Committee of the Feed Research Institute of the Chinese Academy of Agricultural Sciences (ACE-CAAS-20190916) and performed following the guidelines.

Birds, Diets, and Managements

Two hundred and forty 25-wk-old Lohmann commercial laying hens with similar laying rates and body shapes were randomly divided into 5 treatments. Birds within each treatment were designated to 4 replicates with 12 birds per replicate. Laying hens in the control group and the LPS group were fed a corn-soybean-basal diet. The remaining three RSO treatment groups were given the experimental diet containing RSO (1, 2, and 4%). To induce the inflammatory response, hens in the LPS group and 3 RSO supplementation groups were injected intraperitoneally with LPS (1 mg/kg body weight, dissolved in saline) at nine in the morning on the 15, 18, 21, 24, and 27 d of the test, and the control group was injected with the same amount of saline at the same time.

The experimental diet adopted a corn-soybean-basal diet according to the Chinese *Feeding standard of chicken* (NY/T 33-2004) and the National Research Council (1994). For the experimental diet, RSO was added to corn-soybean meal diets at the expense of corn and soybean to produce experimental diets containing 0 (Control), 1, 2, and 4% RSO, respectively. The experimental diets had similar nutrient levels, and the composition and nutritional levels of the experimental diet are shown in Table 1. The fatty acid composition of the experimental diets is shown in Table 2. From Table 2, with the increase of the level of RSO, the level of ALA in

Table 1. Ingredients and nutrient levels of the experimental diets (air-dry basis).

Item	Experimental diets			
	Control	1% RSO[1]	2% RSO[1]	4% RSO[1]
Ingredients (%)				
Corn	66.00	63.00	59.80	51.40
Soybean meal	19.00	19.60	20.20	19.20
Dicalcium phosphate	1.10	1.10	1.10	1.10
Limestone	8.50	8.50	8.50	8.50
Salt	0.3	0.3	0.3	0.3
Fish meal (CP 64.5%)	4.00	4.00	4.00	4.00
Wheat bran	-	-	-	7.38
Rice hull powder	-	1.4	3.0	3.0
RSO[1]	0	1.00	2.00	4.00
DL-methionine	0.10	0.10	0.10	0.12
Premix[2]	1.00	1.00	1.00	1.00
Total	100.00	100.00	100.00	100.00
Nutrient levels				
ME (MJ/kg)[3]	11.31	11.33	11.32	11.33
Crude protein (%)[4]	16.55	16.56	16.55	16.56
Calcium (%)[4]	3.53	3.53	3.53	3.53
Total phosphorus (%)[4]	0.61	0.60	0.60	0.64
Nonphytate phosphorus (%)[4]	0.42	0.42	0.42	0.43
Methionine (%)[4]	0.41	0.40	0.40	0.41
Lysine (%)[4]	0.87	0.88	0.89	0.88
Methionine + Cysteine (%)[4]	0.69	0.69	0.68	0.69
Threonine (%)[4]	0.68	0.68	0.68	0.67
Tryptophane (%)[4]	0.20	0.20	0.20	0.20

[1]Abbreviation: RSO, rubber seed oil.
[2]The premix supplied the following per kilogram of diet: vitamin A, 10,000 IU; vitamin B_1, 4 mg; vitamin B_2, 5.0 mg; vitamin B_5, 40 mg; vitamin B_6, 4 mg; vitamin B_{12}, 2 mg; vitamin D_3 4,000 IU; vitamin E, 200 IU; vitamin K_3, 2.5 mg; biotin, 0.5 mg; folic acid, 3.0 mg; D-pantothenic acid, 20 mg; nicotinic acid, 20 mg; Cu, 10 mg; Fe, 100 mg; Mn, 100 mg; Zn, 100 mg; I, 1.00 mg; Se, 0.40 mg; Choline chloride, 500 mg.
[3]The value of metabolizable energy (ME) was calculated according to MEn of feedstuffs for poultry provided by NRC (1994).
[4]The numbers were analyzed values.

Table 2. The fatty acid composition of the experimental diets (%).

Fatty acids	Experimental diets (%)			
	Control	1% RSO	2% RSO	4% RSO
C14:0	1.26	1.33	0.89	1.19
C16:0	16.06	18.07	16.97	16.27
C16:1 n-7	1.47	1.74	1.01	1.52
C18:0	5.95	5.77	9.15	6.99
C18:1 n-9	24.09	26.89	29.05	26.87
C18:2 n-6 (LA)	35.51	29.70	25.42	26.03
C18:3 n-3 (ALA)	1.97	3.94	6.49	9.06
C20:0	1.01	0.43	0.85	1.05
C20:1	0.86	0.63	0.74	0.51
C20:2	1.24	1.09	1.46	1.68
C20:4 n-6 (AA)	0.44	0.42	0.28	0.29
C20:5 n-3 (EPA)	2.00	2.38	1.40	1.63
C22:5 (DPA)	0.89	0.91	0.60	0.75
C22:6 n-3 (DHA)	2.51	2.87	2.12	2.85
n-3 PUFA [1]	6.48	9.18	10.02	13.54
n-6 PUFA [2]	35.95	30.11	25.71	26.32
n-6/n-3	5.55	3.28	2.57	1.94

Abbreviations: ALA, α-linolenic acid; AA, arachidonic acid; DHA, docosahexaenoic acid; DPA, docosapentaenoic acid; EPA, eicosapentaenoic acid; LA, linoleic acid; PUFA, polyunsaturated fatty acids; RSO, rubber seed oil.
[1]n-3 PUFA = C18:3n-3 + C20:5n-3 + C22:6n-3.
[2]n-6 PUFA = C18:2n-6 + C20:4n-6.

the diet increased and the level of n-3 PUFA also increased while the n-6/n-3 PUFA ratio decreased.

Laying hens were housed in the 3-tier battery cage with 6 birds per cage (80 cm × 80 cm × 50 cm) in the enclosed chicken house. Two sequential cages with one diet trough were arranged as a replicate, and all replicates were equally distributed in different spatial directions to eliminate the influence of environment and location on the measurement indicators. The chicken house was managed with natural light and artificial lighting to provide 16 h of continuous light daily with intensity from 10 to 20 lx. The chicken house adopts positive pressure lateral ventilation. The temperature ranged from 25 ± 2°C and relative humidity was controlled at 60 to 65% throughout the experiment. The chicken house was equipped with trough feeders and nipple drinkers, and hens were allowed ad libitum access to diets (in mash form) and water during the experiment period. Eggs were picked up once a day. The chicken house was cleaned and disinfected once every 5 d, and epidemic prevention was carried out following conventional procedures.

Productive Performance

During the experiment period, total egg weight, egg number, shell-less eggs, and cracked eggs were recorded daily on a replicate cage basis. Feed intake was also measured and recorded based on a replicate weekly. After the 4-wk experiment, the egg production, total egg weight, daily egg mass, average egg weight, daily feed intake, and feed conversion ratio (**FCR**) were calculated throughout the experimental period.

Blood Sample and Biochemical Parameter

At the end of the experiment, 2 hens close to the average weight were selected from each replicate, and whole blood collected from the wing vein of hens was placed into the 5 mL ethylenediaminetetraacetic acid (**EDTA**) anticoagulation tube. The following parameters of whole blood, including white blood cells (**WBC**), red blood cells (**RBC**), hemoglobin (**HGB**), monocytes (**MON**), lymphocytes (**LYM**), neutrophils (**MEU**), and basophils (**BAS**) were analyzed using an ABX Pentra 120 hematology analyzer (Montpellier, France). At the same time, approximately 10 mL of blood samples collected from hens (2 birds/replicate) were placed in the 10 mL vacuum blood collection tube (containing heparin sodium). After 4 h at room temperature, blood samples were centrifuged at 3,500 rpm for 10 min at 4°C to obtain blood plasma. The obtained plasma samples were divided into the 1.5 mL EP tubes and stored at −20°C for testing. The plasma biochemical indices were determined with the Hitachi 7080 automatic biochemical analyzer (Tokyo, Japan) for aspartate aminotransferase (**AST**) (IFCC method), alanine transaminase (**ALT**) (IFCC method), total protein (**TP**) (BCA method), albumin (**ALB**) (BCG method), globulin (**GLOB**) (ELISA method), immunoglobulin M (**IgM**) (Immunoturbidimetry method), immunoglobulin G (**IgG**) (Immunoturbidimetry method), and immunoglobulin A (**IgA**) (Immunoturbidimetry method). The above

indicators were determined according to the manufacturer's instructions with commercial kits from the Nanjing Jiancheng Bioengineering Institute (Nanjing, China). Other biochemical parameters, including interleukin-2 (**IL-2**), tumor necrosis factor-α (**TNF-α**), triiodothyronine (**T3**), thyroxine (**T4**), insulin (**INS**), and glucagon (**GLN**) were all performed with the GC-911 gamma radioimmuno counter from Anhui USTC Zonkia Scientific Instruments Co., Ltd (Hefei, China).

Analysis of Inflammatory-Related Genes Expression

After blood collection, part of liver, spleen, and ileum tissues of hens was collected into the 1.5 mL cryotubes, plunged into liquid nitrogen immediately, and then transferred to −80°C for the next analysis. The relative expression of inflammatory-related genes, including toll-like receptor 4 (***TLR4***), nuclear factor kappa-light-chain-enhancer of activated B cells (***NF-κB***), interleukin-6 (***IL-6***), and interleukin-1β (***IL-1β***), were determined using the quantitative real-time PCR method. The total RNA of liver, spleen, and ileum samples was extracted using the Trizol reagent (Invitrogen, Carlsbad, CA, USA), and the concentration and purity of the total RNA were detected by a NanoDrop ND-2000 (Thermo Scientific, Madison, USA). The cDNA was obtained using the PrimeScript 1st Strand cDNA Synthesis Kit (Takara Bio, Shiga, Japan), and stored at −80°C for further experiments. The quantitative RT-PCR reactions were conducted with the SYBR Premix Ex Taq II (TaKaRa, Kyoto, Japan) according to the manufacturer's instruction by the ABI 7500 detection system (Applied Biosystems, Foster City, CA, USA). After amplification, the specificity of PCR products was verified by melting curve analysis and agarose gel electrophoresis. Primers of target genes (*IL-6, IL-1β, TLR4*, and *NF-κB*) and the reference gene (***GAPDH***) were listed in Table 3. The relative expression of target genes was analyzed using the $2^{-\Delta\Delta Ct}$ method.

Statistical Analysis

Data were analyzed as a completely randomized design by one way-ANOVA using the GLM procedure of SAS (SAS Institute, 2003). Replicate was used as the experimental unit for analysis of laying performance, and individual bird served as the experimental unit for blood parameters measurements. When dietary treatment was significant ($P < 0.05$), means were compared using Duncan's multiple comparison procedure. The results are expressed as mean ± SEM.

Table 3. Primers of genes analyzed by real-time PCR.

Genes	Primers (5' to 3')	Accession number
TLR4	F: AGTCTGAAATTGCTGAGCTCAAAT R: GCGACGTTAAGCCATGGAAG	NM_001030693
NF-κB	F: GTGTGAAGAAACGGGAACTG R: GGCACGGTTGTCATAGATGG	NM_205129.1
IL-1β	F: ACTGGGCATCAAGGGCTA R: GGTAGAAGATGAAGCGGGTC	NM_015297469.1
IL-6	F: TTTATGGAGAAGACCGTGAGG R: TGTGGCAGATTGGTAACAGAG	NM_204628
GAPDH	F: CCTAGGATACACAGAGGACCAGGTT R: GGTGGAGGAATGGCTGTCA	NM_204305.1

RESULTS

Effect of RSO on Productive Performance of LPS-Challenged Laying Hens

In this experiment, the egg production, the total egg weight, the daily egg mass, the average egg weight, the feed intake, and the feed conversion ratio (FCR) were determined to assess the effect of RSO on the productive performance of LPS-challenged laying hens, and results were showed in Table 4. The results showed that, compared with the control group, the LPS stimulation significantly decreased ($P < 0.05$) the egg production, total egg weight, daily egg mass, and feed intake ($P < 0.05$). Compared with the LPS-challenged group, RSO supplementation significantly increased ($P < 0.05$) the egg production, the total egg weight, the daily egg mass, and the feed intake in a dose-dependently manner. What was more noteworthy was that the egg production of the high-dose (4%) RSO supplementation group is better than that of the control group. RSO supplementation and LPS stimulation did not significantly alter average egg weight and feed conversion ratio, which were all similar to that of the control group. In conclusion, dietary RSO supplementation had a beneficial effect on the productive performance of laying hens.

Effect of RSO on Blood Cell Parameters of LPS-Challenged Laying Hens

As shown in Table 5, we found that the LPS stimulation significantly increased ($P < 0.05$) white blood cells (WBC) and lymphocytes (LYM) concentrations in comparison to the control group. However, RSO supplementation decreased WBC and LYM concentrations to a similar level as the control group. No statistical differences among groups were found in red blood cells (RBC), hemoglobin (HGB), monocytes (MON), neutrophils (MEU), and basophils (BAS) levels ($P > 0.05$).

Effect of RSO on Plasma Biochemical Parameters of LPS-Challenged Laying Hens

Figure 1 presented the blood biochemical data, including the activities of aspartate aminotransferase (AST), alanine transaminase (ALT), and the contents of total protein (TP), albumin (ALB), globulin (GLOB), immunoglobulin M (IgM), immunoglobulin G (IgG), and immunoglobulin A (IgA). It can be seen that the LPS stimulation and RSO supplementation did not affect the activity of ALT, and the content of TP, ALB, GLOB, IgM, and IgG. However, it was apparent from Figure 1 that the LPS stimulation significantly increased ($P < 0.05$) AST activity and IgA content, while RSO supplementation decreased these 2 indices to a relatively normal level.

Table 4. Effect of RSO supplementation on productive performance of LPS-challenged laying hens[1].

Item	Dietary treatments					SEM	*P*-value
	Control	LPS	1% RSO	2% RSO	4% RSO		
Egg production (%)	88.82 ± 1.52[b]	84.77 ± 1.32[c]	87.83 ± 1.75[b]	89.23 ± 1.26[ab]	92.23 ± 0.96[a]	0.63	0.0001
Total egg weight (kg)	26.26 ± 0.76[ab]	24.53 ± 0.67[c]	25.80 ± 1.19[b]	26.46 ± 0.57[ab]	27.26 ± 0.58[a]	0.26	0.0031
Daily egg mass (g/Bird)	51.09 ± 0.88[b]	48.91 ± 0.77[c]	50.64 ± 0.45[b]	51.19 ± 0.57[b]	52.44 ± 0.80[a]	0.30	0.0001
Average egg weight (g)	57.18 ± 1.19	57.85 ± 0.52	57.43 ± 1.52	57.58 ± 0.51	57.26 ± 0.41	0.19	0.8559
Daily feed intake (g/Bird)	101.25 ± 2.25[b]	95.42 ± 1.69[c]	99.76 ± 1.55[b]	102.11 ± 1.28[b]	106.05 ± 2.34[a]	0.87	<.0001
FCR	2.15 ± 0.09	2.17 ± 0.05	2.16 ± 0.09	2.15 ± 0.03	2.17 ± 0.05	0.01	0.9949

Abbreviations: FCR, feed conversion ratio; LPS, lipopolysaccharides; RSO, rubber seed oil.
[a,b,c]Different superscripts in a row indicate a significant difference ($P < 0.05$).
[1]Values are mean ± standard error (SEM) of 4 replicates per treatment for the 4-wk experimental period.

Table 5. Effect of RSO supplementation on blood cell parameters of LPS-challenged laying hens[1].

Item	Dietary treatments					SEM	*P*-value
	Control	LPS	1% RSO	2% RSO	4% RSO		
WBC (10^9/L)	43.03 ± 4.75[b]	55.37 ± 5.06[a]	46.89 ± 3.94[b]	47.42 ± 1.72[b]	47.31 ± 1.42[b]	0.89	0.0011
RBC (10^9/L)	3.09 ± 0.40	3.12 ± 0.32	3.17 ± 0.15	3.19 ± 0.31	3.12 ± 0.10	0.05	0.9685
HGB (g/L)	74.17 ± 4.75	71.67 ± 4.27	72.33 ± 2.66	74.83 ± 3.37	74.33 ± 3.72	0.69	0.5471
MON (10^9/L)	7.75 ± 0.76	7.52 ± 0.28	7.70 ± 0.34	7.62 ± 0.16	7.68 ± 0.41	0.08	0.9104
LYM (10^9/L)	13.91 ± 2.79[b]	18.80 ± 3.33[a]	14.71 ± 2.40[b]	14.71 ± 2.70[b]	13.14 ± 1.38[b]	0.57	0.0092
MEU (10^9/L)	14.52 ± 1.98	17.13 ± 3.04	15.55 ± 1.31	15.13 ± 0.37	14.94 ± 0.40	0.34	0.1229
BAS (10^9/L)	11.59 ± 3.61	12.23 ± 1.17	10.87 ± 1.88	11.27 ± 0.91	11.95 ± 0.58	0.34	0.7671

Abbreviations: BAS, basophils; HGB, hemoglobin; LYM, lymphocytes; MEU, neutrophils; MON, monocytes; RBC, red blood cells; WBC, white blood cells.
[a,b,c]Different superscripts in a row indicate a significant difference ($P < 0.05$).
[1]Values are mean ± standard error (SEM) of 4 replicates per treatment for the 4-wk experimental period.

Effect of RSO on Hormones and Inflammatory Markers of LPS-Challenged Laying Hens

The levels of insulin (INS), glucagon (GLN), triiodothyronine (T3), thyroxine (T4), interleukin-2 (IL-2), and tumor necrosis factor-α (TNF-α) in plasma of laying hens were detected at the end of the experiment. From the results (Figures 2C, 2E, and 2F), it was clear that the LPS stimulation significantly increased ($P < 0.05$) the concentration of T3, IL-2, and TNF-α. Compared with the LPS-challenged group, 3 RSO supplementation groups statistically decreased ($P < 0.05$) the content of T3, IL-2, and

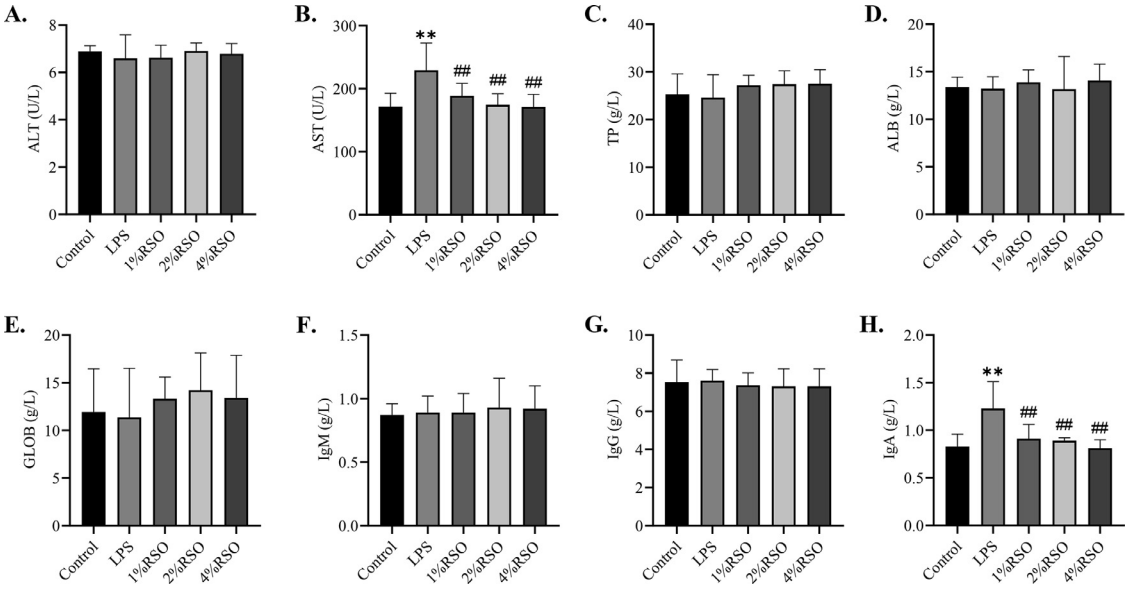

Figure 1. Effect of RSO supplementation on plasma biochemical parameters of LPS-challenged laying hens. (A) The activities of aspartate aminotransferase (AST). (B) The activities of alanine transaminase (ALT). (C) The contents of total protein (TP). (D) The contents of albumin (ALB). (E) The contents of globulin (GLOB). (F) The contents of immunoglobulin M (IgM). (G) The contents of immunoglobulin G (IgG). (H) The contents of immunoglobulin A (IgA). Values are mean ± standard error (SEM) of 4 replicates per treatment for the 4-wk experimental period. Asterisk ($^*P < 0.05$, $^{**}P < 0.01$) indicates significant differences from the control group, and pound sign ($^{\#}P < 0.05$, $^{\#\#}P < 0.01$) indicates significant differences from the LPS-challenged group. Abbreviations: LPS, lipopolysaccharide; RSO, rubber seed oil.

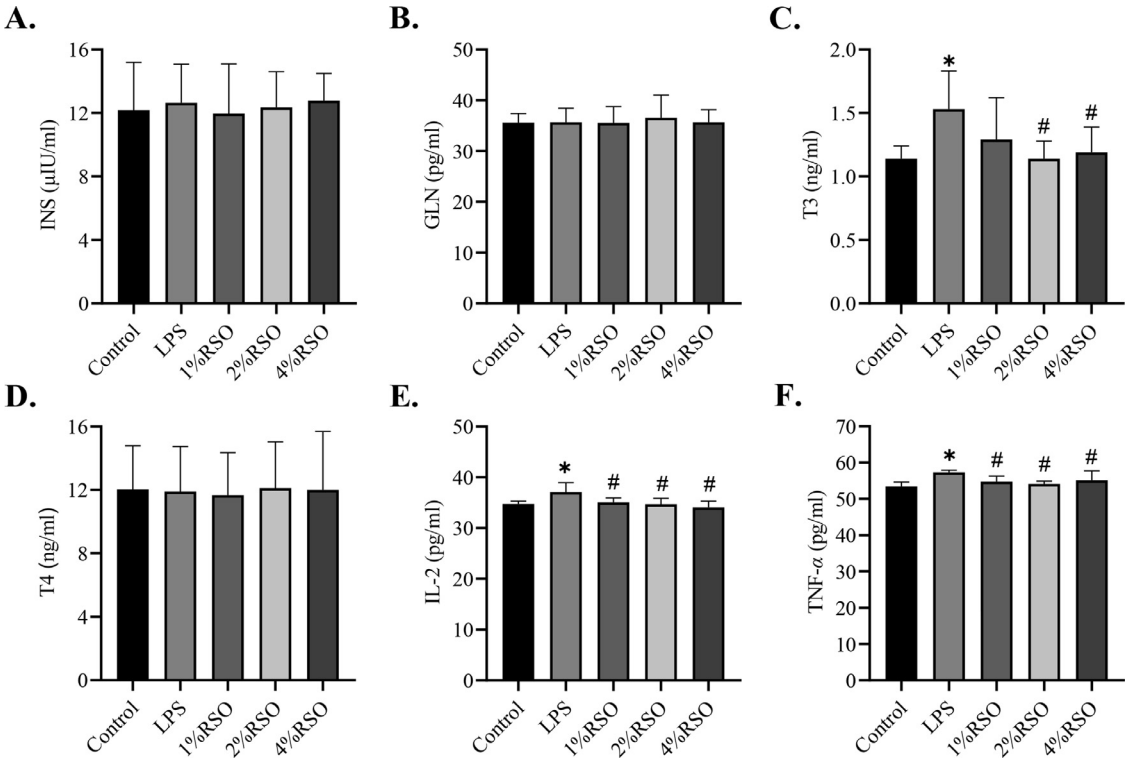

Figure 2. Effect of RSO supplementation on hormones and inflammatory markers plasma immunological parameters of LPS-challenged laying hens. (A) The levels of insulin (INS). (B) The levels of glucagon (GLN). (C) The levels of triiodothyronine (T3). (D) The levels of thyroxine (T4). (E) The levels of interleukin-2 (IL-2). (F) The levels of tumor necrosis factor-α (TNF-α). Values are mean ± standard error (SEM) of 4 replicates per treatment for the 4-wk experimental period. Asterisk ($^{*}P < 0.05$, $^{**}P < 0.01$) indicates significant differences from the control group, and pound sign ($^{\#}P < 0.05$, $^{\#\#}P < 0.01$) indicates significant differences from the LPS-challenged group. Abbreviations: LPS, lipopolysaccharide; RSO, rubber seed oil.

TNF-α to levels close to those of the control group in LPS-challenged laying hens.

Effect of RSO on Inflammatory-Related Genes Expression of LPS-Challenged Laying Hens

The inflammatory-related genes, including *TLR4, NF-κB, IL-1β*, and *IL-6*, were determined, and the results were shown in Figure 3. Compared with the control group, LPS stimulation increased the mRNA expression of *TLR4, NF-κB, IL-1β*, and *IL-6* genes in the ileum and liver of laying hens ($P < 0.05$, Figures 3A−3H), while RSO supplementation decreased the expression of these inflammatory-related genes in LPS-challenged laying hens ($P < 0.05$). Similar effects were found in the spleen of laying hens that RSO significantly reduced the increase of *TLR4, NF-κB*, and *IL-1β* gene expression stimulated by LPS ($P < 0.05$, Figures 3I−3L). These results indicated that RSO supplementation reduced inflammatory-related gene expression of TLR4/NF-κB signaling pathway in LPS-challenged laying hens.

DISCUSSION

The productive performance effects of PUFA-enriched plant oil on laying hens had been a topic of debate. Cabrera et al. (2006) found that PUFA sources supplemented in the diet, such as 3% sunflower oil and 3% rice oil, could increase egg weight and yolk weight. Kostogrys et al. (2017) revealed that adding 1% pomegranate seed oil to the diet significantly increased feed intake, feed conversion, laying rate, and mean egg mass in laying hens. However, some studies have found that the addition of PUFA-enriched vegetable oil, such as olive oil (Zhang and Kim, 2014) and hazelnut oil (Cetıngul and Inal, 2009), to the laying hen diets, has no significant effect on the laying performance of laying hens, and even some research results showed that the addition of PUFA in the diet had a negative effect on laying hens (Liu et al., 2020a; Wang et al., 2016). The reasons for these differences may be related to differences in composition and dose of dietary PUFA, lipid sources, laying hen breeds, and nutrient levels. In previous work, adding 4% RSO to the basal diet of laying hens increased the egg production rate, daily egg production, and feed intake of laying hens by 6.52, 5.12, and 9.03%, respectively (Wen et al., 2019). In this present study, in LPS-challenged laying hens, RSO supplementation (1, 2, and 4%) to the diets also increased the egg production, total egg weight, daily egg mass, and feed intake, which is consistent with the results of Wen et al. (2019). From the results of productive performance of laying hens (Table 4) and the fatty acid composition of the experimental diets (Table 2), we could see that the improvement of productive performance was positively correlated with the addition of RSO. For the experimental diet, as the level of RSO increased, the level of PUFA in the diet gradually increased and the level of n-3 PUFA also

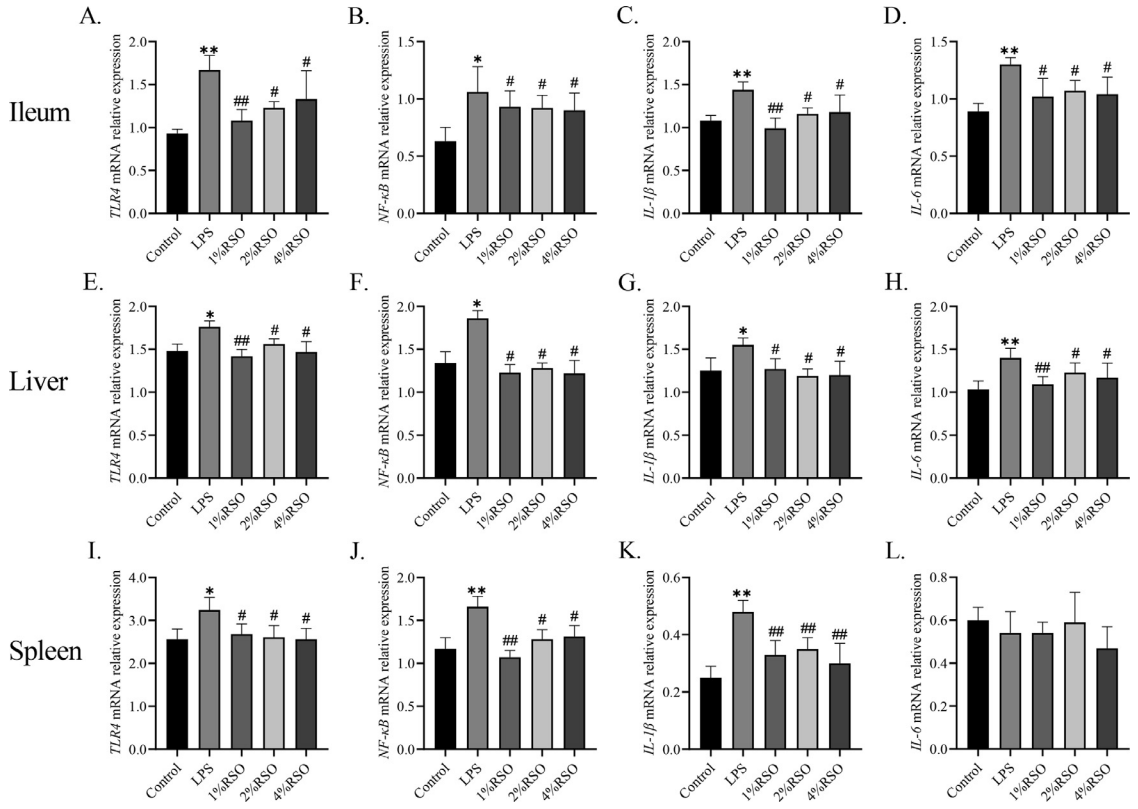

Figure 3. Effect of RSO supplementation on inflammatory-related genes expression of LPS-challenged laying hens. (A–D) The levels of mRNA expression of *TLR4*, *NF-κB*, *IL-1β*, and *IL-6* in the ileum. (E–H) The levels of mRNA expression of *TLR4*, *NF-κB*, *IL-1β*, and *IL-6* in the liver. (I–L) The levels of mRNA expression of *TLR4*, *NF-κB*, *IL-1β*, and *IL-6* in the spleen. Values are mean ± standard error (SEM) of 4 replicates per treatment for the 4-wk experimental period. Asterisk ($^{*}P < 0.05$, $^{**}P < 0.01$) indicates significant differences from the control group, and pound sign ($^{\#}P < 0.05$, $^{\#\#}P < 0.01$) indicates significant differences from the LPS-challenged group. Abbreviations: LPS, lipopolysaccharide; RSO, rubber seed oil.

gradually increased. Our previous study also found that RSO is rich in phenols and flavonoids (authors' unpublished data). Accumulating researchers have reported that phenols and flavonoids showed positive effects on the productive performance, immunity, and inflammatory response of laying hens (Yuan et al., 2016; Scicutella et al., 2021). Thus, we speculated that the addition of RSO to the diet could not only supplement PUFA but also supplement other bioactive components, such as phenols and flavonoids, which could all help alleviate the immunological stress and inflammatory response of LPS-induced laying hens, thereby helping to improve laying performance.

Lipopolysaccharide (LPS), a major outer surface membrane component present in almost all Gram-negative bacteria, is recognized as a microbe-associated molecular pattern (**MAMP**) molecule and initiates transduction of ligand-specific perception, subsequent signaling cascades, and the activation of an immune response in animal organisms (Nürnberger et al., 2004; Mgcina et al., 2015). Excessive LPS stimulation can cause physiological stress, which has been widely applied to establish experimental models of immunological stress and systemic inflammatory response in laying hens (Nie et al., 2018; Gu et al., 2022). Regarding immunological stress and inflammation, the parameters of immune cells, plasma immunoglobulins, levels of hormone, and inflammatory factors are important indicators reflecting the physiological state of animal organisms (Kany et al., 2019; Yuan et al., 2020). In the process of the immune response, the white blood cells (WBC) are one of the most important immune cells and a critical indicator to reflect the level of the immune response (Eldridge, 2021). In addition, plasma immunoglobulins protect the body against invading bacteria and viruses and play a key role in the immune system of animals (Mathew et al., 2021). Besides, in recent years, numerous studies have demonstrated that hormones, such as triiodothyronine (T3) and thyroxine (T4) play particularly important roles in various physiopathological processes of animal organisms, which include oxidative stress, inflammatory response, immunological stress, etc. (Mancini et al., 2016; Gan et al., 2020). Also, plasma aminotransferases, including aspartate aminotransferase (AST) and alanine aminotransferase (ALT), have been regarded as a reliable and sensitive marker of liver damage and inflammation (Kim et al., 2008; Xiong et al., 2017). Moreover, inflammatory factors are critical and commonly used in the assessment of inflammatory status or immune response, including TNF-α (Brenner et al., 2014). TNF-α has been identified as a major regulator of inflammatory responses and is known to be involved in the pathogenesis of some inflammatory and immune diseases (Bradley, 2008). During gram-negative bacteria infection, monocytes are triggered to produce large quantities of TNF-α in response to LPS (Van Der Bruggen et al., 1999). Therefore, in this experiment, the

LPS challenge was used to stimulate laying hens to establish experimental models of immunological stress and inflammatory response in laying hens, and effects of dietary RSO supplementation were evaluated by detecting parameters of immune cells, plasma immunoglobulins, hormone levels, and inflammatory factors.

In this study, LPS stimulation elicited significant fluctuations in levels of immune cells, immunoglobulins, hormone levels, and inflammatory factors in laying hens, suggesting LPS injection efficiently stimulated immunological stress and inflammatory response in laying hens. As expected, dietary RSO supplementation significantly alleviated the immunological stress and inhibited the systemic inflammatory response in LPS-challenged laying hens, as evidenced by reduced immune cells (white blood cells and lymphocytes), triiodothyronine (T3) levels, IgA contents, AST activities, and TNF-α levels to near control levels. Consistent with our results, Gandhi et al. (1990) observed that the WBC content of the RSO supplementation group was lower in rats compared with the peanut oil supplementation group. Similarly, Liu et al. (2015) showed that dietary supplementation of PUFA-enriched fish oil dramatically reduced TNF-α levels and AST activities compared with supplementation of corn oil in LPS-challenged mice. A similar finding was also reported by Nayak et al. (2018) that dietary supplementation with PUFA-enriched algae downregulated the expression of TNF-α mRNA, modulated immune function, and improved resistance to streptococcal infection in zebrafish. Pi et al. (2019) found that dietary RSO addition to the basal diet had no significant effect on plasma IgA content but significantly reduced the concentration of proinflammatory cytokines (TNF-α and INF-γ) in serum in dairy cows, thereby helping to alleviate inflammation of dairy cows. The possible reason was that there are differences in PUFA requirement and physiological metabolism between poultry and ruminants. In summary, this study suggested that RSO, as PUFA-enriched plant oil, could be used in the diet to alleviate immunological stress and relieve the inflammation of laying hens.

The beneficial effects of dietary RSO supplementation on the immunity and inflammatory response of LPS-challenged laying hens were closely related to the improvement of the production performance of hens. In the present study, compared with the control group, LPS stimulation significantly reduced the egg production, total egg weight, daily egg mass, and feed intake of laying hens ($P < 0.05$), whereas RSO supplementation increased these productive indexes in LPS-challenged laying hens. These results suggested that RSO supplementation can alleviate the reduction in productive performance induced by LPS stimulation. The possible reason was that RSO supplementation could inhibit the mRNA expression of inflammatory genes in immune organs such as the liver and spleen of laying hens, thereby reducing the inflammatory response of laying hens and reducing the secretion of plasma immune factors, to maintain a normal physiological state and productive performance of laying hens. In line with our study, many studies have reported practical application of n-3 and n-6 PUFA in poultry diets and their beneficial effects on productive performance, inflammatory response, and immunity (Maroufyan et al., 2012a; Alagawany et al., 2019).

For the natural immune system, TLR4, as one of the most important receptors to recognize pathogenic microorganisms, is mainly localized on the surface of many immune cells, such as monocytes, macrophages, dendritic cells, and neutrophils (Oliveira and Reygaert, 2022). TLR4 could specifically recognize LPS and trigger the innate immune response of immune cells (Alexander and Rietschel, 2001). TLR4 then recruits adaptor proteins and activates two distinct signaling pathways (the MyD88-dependent pathway and the TRIF-dependent pathway), which are all mediate the NF-κB activation (Kawai and Akira, 2010). Activation of NF-κB subsequently induces the expression of related inflammatory genes, thereby promoting the synthesis and release of cytokines and inflammatory mediators, such as *IL-1β* and *IL-6*, leading to inflammatory responses (Kordjazy et al., 2018). According to reports, dietary intake of PUFA and its deposition and metabolism in the body play an important role in the inflammatory response of animals (Calder, 2013; Zaloga, 2021). Our previous studies have found that RSO supplementation increased DHA and EPA contents of yolk of laying hens (Wen et al., 2019). Our results were consistent with Pi et al. (2019), who demonstrated that RSO supplementation elevated the proportion of conjugated linoleic acid (**CLA**) and α-linolenic acid (ALA) in the rumen of dairy cows. The accumulation and metabolism of PUFA in animals contribute to the anti-inflammatory of organisms. In detail, for example, DHA and EPA could replace arachidonic acid (**AA**, n-6) in the cell membrane, and competitively combine cyclooxygenase (**COX**) and lipoxygenase (**LOX**) to reduce the production of inflammatory mediators derived from AA, and therefore reduce inflammation response (Maroon and Bost, 2006). It has been reported that PUFA could mediate the TLR4/NF-κB signaling pathway to regulate the inflammatory response in animals, and reduce the production of inflammatory cytokines such as TNF-α, IL-1β, and IL-6 (Fan et al., 2020; Liu et al., 2020b). In the current study, we found that LPS stimulation significantly ($P < 0.05$) increased the mRNA expression of *TLR4, NF-κB p65, IL-1β*, and *IL-6* genes in the liver, ileum, and spleen of laying hens (Figure 3). However, dietary RSO supplementation significantly ($P < 0.05$) reduced the LPS-stimulated increase in the mRNA expression of *IL-6, NF-κB*, and *TLR4* genes in laying hens. Thus, it was concluded that RSO supplementation could inhibit the expression of inflammation-related genes through the TLR4/NF-κB signaling pathway, thereby alleviating inflammation in LPS-challenged laying hens.

In conclusion, this study demonstrated that adding RSO to the diet could dose-dependently improve the laying performance in LPS-challenged laying hens. When the RSO level was 4%, the laying performance of the laying hens was pretty good. In addition, dietary RSO

supplementation could regulate the biochemical profile, attenuate immunological stress, and inhibit the systemic inflammatory response in LPS-challenged laying hens. This study will provide an insight into the positive contribution of RSO as feed oil to the overall health and welfare in laying hens.

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DISCLOSURES

The authors declare that there is no conflict of interest.

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