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RESEARCH ARTICLE

Determination of Antioxidant and Immune Responses with Bile Acids Supplementation in Geese

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Abstract

This study investigated the impact of varying levels of bile acids (BAs) on the immune and antioxidant functions in the geese. A total of 168 male Hortobágyi geese, aged 28 days, were randomly assigned to four groups: the control group received a basic diet, while the other groups received diets supplemented with 75 mg/kg, 150 mg/kg, and 300 mg/kg of BAs. The trial lasted for 35 days, after which samples were collected for the analysis of antioxidant and immune indicators. Results showed that, compared to the control group, the supplementation of BAs in the feed did not significantly affect the indices of immune organs or serum immune levels ($P>0.05$). However, a supplementation with 300 mg/kg BAs significantly increased the expression of *IL-10* mRNA in the geese's spleens. Regarding antioxidant indicators, the addition of 150 mg/kg and 300 mg/kg BAs significantly enhanced the serum levels of Superoxide Dismutase (SOD), Glutathione Peroxidase (GSH-Px), and Total Antioxidant Capacity (T-AOC) ($P<0.05$), as well as upregulated the expression of liver *Nrf2*, *GPX-1* and *SOD1* mRNA. In conclusion, within the range of BAs supplementation used in this experiment, the improvement in geese's immune status was limited. Although higher levels of BAs might influence the immune status, the supplementation significantly enhanced the antioxidant capacity in serum and liver, with an optimal addition level of 150 mg/kg.

Keywords: Antioxidant, Bile acids, Geese, Immune response

INTRODUCTION

The Hortobágyi goose, developed by the Hungarian Hortobágyi Goose Joint Stock Company, stands out as a kombine breed for meat, down, and egg production. It stands out, experiencing a growing market demand in Central Asia and Europe, with a notable surge in popularity, especially in China [1]. The Hortobágyi goose is primarily raised in Heilongjiang and Yunnan provinces in China. It typically produces 40-50 eggs annually, with each egg weighing around 170-190 g. The breed also has a strong growth rate, with adult geese reaching 6-8 kg, and males weighing up to 12 kg by the end of the breeding cycle. In recent years, goose farming systems, driven by economic benefits and environmental protection pressures, have been shifting from traditional mixed water-land farming to modern, intensive, land-based farming [2]. In modern high-density, enclosed rearing conditions, animals face increased stress, leading to reduced immune strength, weakened antioxidant capacity, impaired digestive function, decreased feed conversion rates, and higher morbidity and mortality rates [3,4]. The growth stage from gosling to market readiness is particularly crucial for geese, as their antioxidant and immune responses during this period significantly influence their overall health and development. The antioxidant and immune systems serve as crucial pillars in maintaining animal health, with the antioxidant system combating cellular damage caused by free radicals, and a robust immune system effectively warding off diseases [5,6]. Both systems are indispensable for coping with environmental stress and promoting growth. One potential strategy to reduce stress in geese is the supplementation of BAs, which have been shown to alleviate stress responses and improve overall resilience. Therefore, implementing appropriate strategies to reduce the stress response in geese has become a pressing issue to address.

Bile acids (BAs), as amphipathic steroidal compounds, feature a unique molecular structure where hydroxyl and carboxyl groups on the side chain create a hydrophilic surface, while alkyl groups and hydrocarbon nuclei form a hydrophobic surface [7]. This special molecular configuration endows BAs with both hydrophilic and lipophilic properties, enabling them to effectively

emulsify lipids, forming oil-water mixtures, and increase the contact area with lipases, thereby accelerating fat digestion. The digestion products, encapsulated in BAs micelles, are absorbed by the villi in the small intestine [8,9]. Extensive research indicates that regulating BAs plays a significant role in improving lipid metabolism [10,11]. BAs function as potent antimicrobial agents. Their binding with *farnesol X receptor (FXR)* regulates the expression of antimicrobial substances in the gut, preventing bacterial overgrowth by activating the defense system of the small intestine. BAs receptors present in intestinal endothelial cells, immune cells, and epithelial cells contribute to the immunomodulation of the gut mucosa, playing a crucial role in maintaining intestinal immunity [12,13]. Moreover, BAs can suppress the expression of nuclear factor κB (nf-κb) by upregulating the expression of *FXR*, thus reducing inflammatory responses and enhancing immunity [14]. While current applications of BAs are predominantly in aquatic animals, for instance, adding 130 mg/kg of BAs to improve serum immunity in Thinlip mullet or *Litopenaeus vannamei* [15,16]. *NF-E2 related factor 2 (Nrf2)* is a critical transcription factor that regulates the expression of various antioxidant genes. BAs indirectly enhance cellular antioxidant capacity by affecting the *Nrf2* pathway [17]. In a previous study on broilers, dietary supplementation with BAs increased antioxidant enzyme activities and reduced oxidative stress by regulating *Nrf2* expression through binding with Kelch-like ECH-associated protein 1 (keap1) [18]. Multiple studies also show that appropriate addition of BAs to the diet can enhance serum levels SOD and GSH-Px [15,19]. However, the impact of BAs on the immune and antioxidant status of geese remains unexplored. This research aims to determine the effect of dietary BAs supplementation on the antioxidant capacity and immunity of geese, thereby providing foundational data for the application of BAs in goose diets.

Material and Methods

Ethical Statement

All animal procedures were performed according to guidelines provided by the China Council on Animal Care. All animal experiments were approved by the Animal Care and Use Committee of West Anhui University (Approval no: SYDW-P20210823021).

Experimental Design and Diet

The BAs used in this experiment were purchased from Zhengzhou Shangshui Biotechnology Co., Ltd, Henan Province, China. These BAs comprised 15% cholic acid, 44% hyodeoxycholic acid, and 40.50% chenodeoxycholic acid. A total of 168 male Hortobágyi geese, aged 28 days, were purchased from the Xiangtiange Farm in Ma'anshan City, Anhui Province, China. The geese were randomly allocated to four groups, each receiving a diet with different levels of BAs (0, 75, 150, and 300 mg/kg, respectively). The addition level of BAs in each group in the experiment was determined according to previous studies and through pre-experiments [20,21]. Each group consisted of six replicates, with each replicate comprising seven geese. The trial lasted 35 days. The selected intervention period (28-63 d) spans from the end of brooding to the market weight stage, which is the critical period for growth in geese. At 28 days, geese have developed some immunity from early vaccinations and maternal antibodies. However, other potential variables, such as diet, environmental conditions, and health status, were carefully controlled to minimize any confounding effects on the experimental results. The feed formulation strictly adhered to the recommendations of NRC 1994 [22], with appropriate modifications made according to the nutritional needs of goose rearing in China *(Table 1)*. All geese in the experiment were raised in an enclosed environment with a stocking density of 0.5 m^2 per goose. The ambient temperature was maintained at around 15°C. The geese were exposed to natural daylight during the day [23]. Throughout the experiment, the geese had unrestricted access to feed and water, and their health status and vaccination records were regularly monitored. The following vaccinations were administered during the study: At 28 days of age: H5+H7 inactivated vaccine (Qingdao Yibang Biotechnology Co., Ltd, Qingdao, China). At 40 days of age: Goose peritonitis + *E. coli* inactivated vaccine (Shandong Binzhou Wohua Biotechnology Co., Ltd, Binzhou, China).

Sample Collection

At the end of the experiment (63 d), the geese from each replicate underwent an 8-h fast, and their weight was measured. One goose close to the average weight was selected from each replicate for further analysis. A 5 mL blood sample was collected from the wing vein, and serum was prepared and stored in a -20°C freezer for subsequent analysis. Subsequently, the geese were euthanized using cervical dislocation, and the thymus, bursa of Fabricius, and spleen immune organs were excised and weighed. The immune organ index was calculated (immune organ index = weight of immune organ, g/live weight of the animal, kg) [24,25].

Serum Immune and Antioxidant Indexes

The antioxidant indexes of the serum were determined following the instructions of the test kits provided by Shanghai Renjie Biotechnology Co., Ltd., Shanghai, China. The total antioxidant capacity (TAC) of the serum was measured using the Fe^{2+} reduction method. Glutathione Peroxidase (GSH-Px) activity was assessed using the enzymatic colorimetric method. Catalase (CAT) activity was determined by the ammonium molybdate

Table 1. *Composition and nutrient level of experiment diets (air-dry basis)*

Ingredients	Content (%)	Nutrient Level	Content (%)
Corn	67.92	CP	16.00
Soybean meal	24.90	ME (MJ/kg)[b]	12.40
Soybean oil	2.00	CF	2.56
Lys	0.09	Ca	0.79
Met	0.09	P	0.51
Premix[a]	5.00	Lys	0.90
Total	100.00	Met+Cys	0.66
		Thr	0.63

[a] *One kilogram of the premix contained the following: Fe: 100 mg, Cu: 8 mg, Mn: 120 mg, Zn: 100 mg, Se: 0.4 mg, Co: 1.0 mg, I: 0.4 mg, Vit. A: 8330 IU, Vit. B_1: 2.0 mg, Vit. B_2: 0.8 mg, Vit. B_6: 1.2 mg, Vit. B_{12}: 0.03 mg, Vit. D_3: 1440 IU, Vit. E: 30 IU, Biotin: 0.2 mg, Folic acid: 2.0 mg, Pantothenic acid: 20 mg, Niacin acid: 40 mg*
[b] *Nutrient levels were all calculated values*

Table 2. *Primer sequences for genes used in RT-qPCR*

Genes	Primer Sequence 5′-3′	Genbank	Amplification Length
IL-6	F:AAGCATCTGGCAACGACGATAAGG	XM_048070285.1	90
	R:TGTGAGGAGGGATTTCTGGGTAGC		
IL-10	F:TGCCAGTCGGTGTCGGAGATG	XM_048071022.1	81
	R:CTGGTGGTGCTCGCTGTTCTTG		
SOD-1	F:ATCCTGAGGGCAAGAAGCA	XM_013192917.1	188
	R:TTTACCCAGGTCATCGCTTT		
GPX-1	F:GCAAGGGGTACAAGCCCAACT	XM_013201826.1	178
	R:GATGATGTACTGCGGGTTGGTC		
CAT	F:TGTAGAGGAAGCAGGAAGGC	XM_013194546.1	98
	R:AAGACCAGGATGGGTAGTTGC		
Nrf2	F:CGCCTTGAAGCTCATCTCAC	D_49365.1	176
	R:TTCTTGCCTCTCCTGCGTAT		
β-actin	F:TCCGTGACATCAAGGAGAAG	XM_013174886.1	144
	F:TCCGTGACATCAAGGAGAAG		

method. Total Superoxide Dismutase (T-SOD) activity was evaluated using the xanthine oxidase method, and the content of Malondialdehyde (MDA) was measured by the thiobarbituric acid method.

Immune indexes were analyzed using Enzyme-Linked Immunosorbent Assay (ELISA) kits (Shanghai Renjie Biotechnology Co., Ltd., Shanghai, China). These tests measured the concentrations of Immunoglobulin A (IgA), Immunoglobulin Y (IgY), Immunoglobulin M (IgM), and inflammatory markers including Interleukin-6 (IL-6) and Interleukin-10 (IL-10), in the serum.

Gene Expression

RNA was extracted from the liver and spleen using Trizol (Invitrogen, Carlsbad, CA, USA). Subsequently, total RNA was transcribed into cDNA using a reverse transcription kit (TransGen, Beijing, China). The concentration and purity of the cDNA were measured using a Nanodrop 1000 spectrophotometer (Thermo Fisher Scientific, Waltham, MA, USA). Real-time PCR to evaluate the expression levels of target genes was performed using the TransGen TB Green kit (TransGen Bio Inc., Beijing, China) and a QuantStudio 5 fluorescence quantitation system (Thermo Fisher Scientific, MA, USA). The target genes included IL6, IL10, SOD1, GPX1, CAT, with β-actin serving as the internal reference gene. The primer sequences for these genes are listed in *Table 2*. Data analysis was performed using the relative quantification method ($2^{-\Delta\Delta CT}$).

Data Analysis

Following initial processing using Excel 2019, the experimental data were analyzed using SPSS statistical software (version 26.0, SPSS Inc., Chicago, USA) for One-Way ANOVA. Group differences were evaluated through

Tukey's multiple comparison analysis to determine the significance of differences, with statistical significance denoted by P<0.05. The results are reported as mean values ± SEM.

Results

Immune Organ Indexes

During the entire study period, no deaths occurred in any of the experimental groups. The effects of different levels of BAs on the immune organ indexes of geese are displayed in *Table 3*. It was observed that, within the specified range of BAs concentrations for this experiment, there was no significant effect on the spleen index, bursa of Fabricius index, or thymus index in geese (P>0.05).

Serum Immune Indexes

To further assess the impact of BAs on the immune status of geese, we measured the levels of immune factors in the serum *(Fig. 1)*. The analysis revealed a decreasing trend in the level of IL-6 with increasing BAs concentrations. For IL-10, the group receiving 300 mg/kg BAs showed the highest level. The 150 mg/kg BAs group had the highest levels of serum IgA and IgM, and there was a trend of increasing IgG levels with higher BAs supplementation. However, no significant differences were observed between the groups (P>0.05).

Spleen Immune Gene Expression

The effects of different levels of BAs on the expression of *IL-6* and *IL-10* mRNA in the spleen are shown in *Fig. 2*. It was observed that 300 mg/kg of BAs significantly increased the expression of *IL-10* mRNA in the liver (P<0.05). Additionally, a significant difference was noted between the 75 mg/kg and 300 mg/kg BAs groups (P<0.05).

Serum Antioxidant Indexes

As illustrated in *Fig. 3*, it is apparent that serum levels of CAT and MDA did not exhibit significant differences across all groups. The supplementation of BAs at levels ranging from 75 mg/kg to 300 mg/kg significantly increased serum levels of SOD and GSH-Px (P<0.05), with no significant differences observed among the various BAs supplementation groups. Notably, serum SOD levels continuously increased, peaking at 150 mg/kg for GSH-Px. Additionally, the supplementation with 150 mg/kg or 300 mg/kg BAs significantly enhanced serum TAC levels (P<0.05).

Table 3. *The effects of supplement BAs on the immune organ indexes of geese*

Items	Treatment				P-value
	0 mg/kg	75 mg/kg	150 mg/kg	300 mg/kg	
Spleen index, g/kg	0.73±0.08	0.64±0.07	0.70±0.07	0.67±0.09	0.858
Bursa of Fabricius index, g/kg	2.49±0.26	2.73±0.84	2.67±1.15	1.71±0.26	0.102
Thymus index, g/kg	0.56±0.03	0.64±0.09	0.51±0.05	0.58±0.04	0.425

[a,b,c] *Values within a row with different superscripts differ significantly at P<0.05*

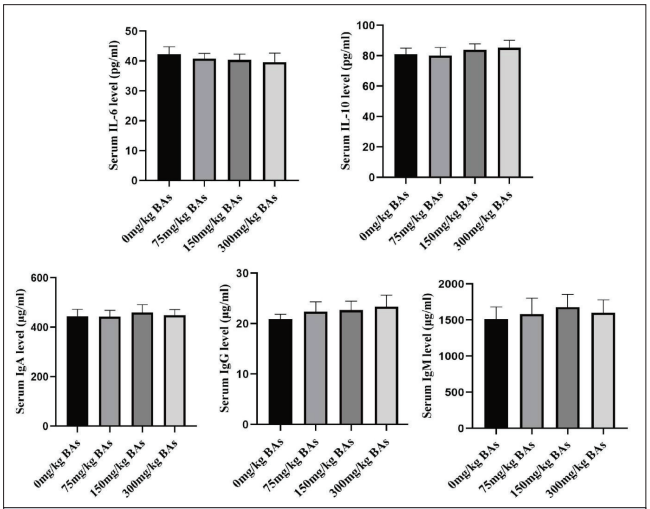

Fig 1. Effects of different BAs levels on serum immune indexes. The analysis of the impact was conducted using one-way ANOVA, with group differences deemed significant at P<0.05. Distinct means, labeled as a, b, and c, are significantly different when the P<0.05

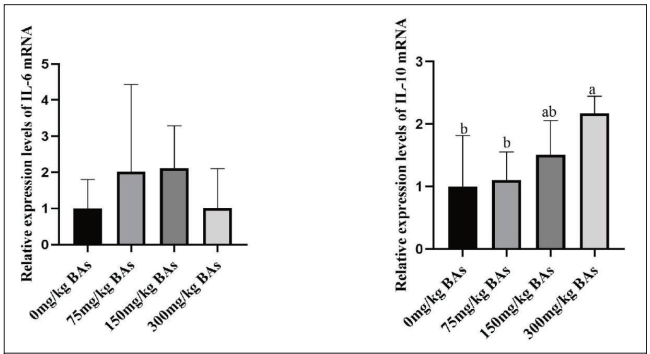

Fig 2. Effects of different BAs levels on spleen immune gene expression. The analysis of the impact was conducted using one-way ANOVA, with group differences deemed significant at P<0.05. Distinct means, labeled as a, b, and c, are significantly different when the P<0.05

Liver Antioxidant Gene Expression

The *Fig. 4* presents the expression of antioxidant genes in the liver across different groups. Similar to the serum

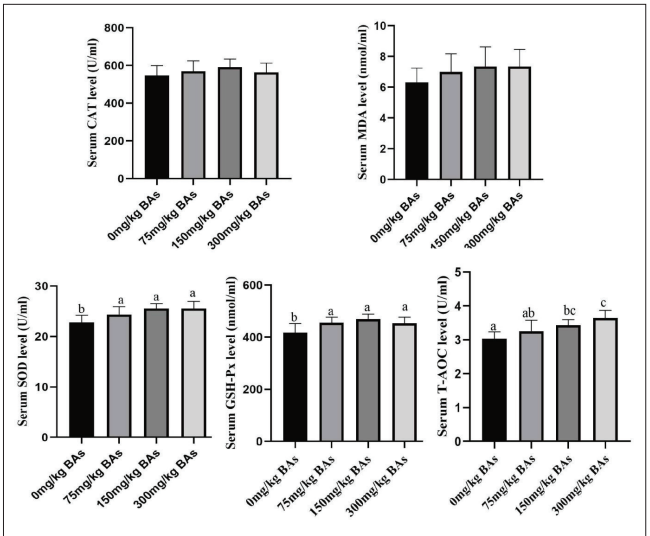

Fig 3. Effects of different BAs levels on serum antioxidant indexes. The analysis of the impact was conducted using one-way ANOVA, with group differences deemed significant at P<0.05. Distinct means, labeled as a, b, and c, are significantly different when the P<0.05

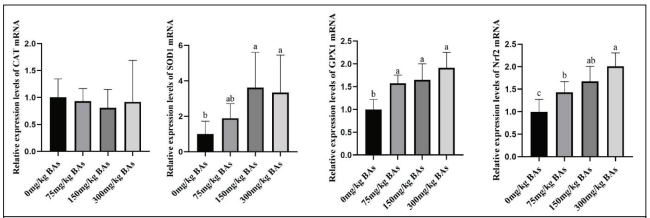

Fig 4. Effects of different BAs levels on Liver antioxidant gene expression. The analysis of the impact was conducted using one-way ANOVA, with group differences deemed significant at P<0.05. Distinct means, labeled as a, b, and c, are significantly different when the P<0.05

antioxidant levels, there were no significant differences in the expression of *CAT* mRNA in the liver among the groups (P>0.05). However, supplementation with 150 mg/kg or 300 mg/kg of BAs significantly increased the expression of *SOD1*, *GPX-1*, and *Nrf2* mRNA in the liver (P<0.05).

Discussion

Maintaining an optimal level of immune activity is crucial for the healthy growth of animals in poultry farms [26]. Immune organs, including the thymus, spleen, and bursa of Fabricius, play crucial roles in lymphocyte production, immune response [27,28]. In this study, we investigated the effects of BAs supplementation on the immune status of geese by measuring immune organ indices and serum immunoglobulins. We found no significant differences in the immune organ indices across different levels of BAs. This suggests that at the concentrations used, BAs may not have a direct effect on the development or function of these primary immune organs. These results align with some previous studies, where BAs did not significantly affect immune organ indices, which might indicate that their immune-modulating effects are not primarily exerted on immune organs themselves [29]. In contrast, studies have shown that BAs can influence immune responses, particularly within the gut, rather than the immune organs per se. For instance, BAs have been found to modulate gut-associated lymphoid tissue and influence immune cell responses in the gut. This suggests that the immune effects of BAs may be more localized, acting on mucosal immunity rather than systemic immune organs [30]. Furthermore, regarding humoral immunity, our study did not observe any significant changes in serum IgA, IgM, or IgG levels. This is consistent with other studies in which BAs did not significantly affect immunoglobulin levels in serum [31]. However, some research has shown that BAs can elevate intestinal IgA levels, particularly in animals with altered gut microbiota or those exposed to high-fat diets [28]. These studies suggest that BAs may play a more significant role in regulating gut immunity rather than circulating immunoglobulin levels. One notable finding in our study was the increased expression of IL-10 in the spleen at 300 mg/kg of BAs supplementation. This is in line with previous studies showing that BAs can reduce pro-inflammatory cytokines such as IL-6, while enhancing anti-inflammatory cytokines like IL-10 [32]. The increase in IL-10 expression in the spleen may indicate a local immune modulation effect by BAs, potentially through interactions with immune cells in the spleen. This is an area that requires further investigation, as the precise mechanisms of BAs in regulating immune responses at the molecular level are not fully understood.

Under normal circumstances, the body can neutralize surplus free radicals through its enzymatic antioxidant defense system, providing protection against oxidative damage. The assessment of oxidative damage involves measuring the activities of antioxidant enzymes and the content of MDA [33]. TAC, serving as a comprehensive indicator of the antioxidant system, reflects the cumulative effect of antioxidants in the body. Generally, higher TAC values within a certain range indicate better antioxidant capabilities of the organism [34]. One of the primary physiological functions of BAs is their antioxidant activity. Numerous studies have shown that BAs have beneficial effects on the body's redox balance. For instance, BAs can influence the PI3K/Akt signaling pathway, closely related to cellular survival, proliferation, and antioxidant defense mechanisms [35]. Serum antioxidant indicators most directly reflect the oxidative stress state of animals, and a certain amount of BAs can significantly increase serum levels of SOD and GSH-Px in pigs [36]. Further studies indicate that adding BAs to the diet of aquatic animals can effectively prevent oxidative damage and improve the health status of farmed fish [37]. This experiment demonstrates that adding BAs to the diet of geese

significantly enhances the antioxidant capacities of SOD, GSH-Px, and TAC in serum. These results underscore that BAs have the potential to improve the serum antioxidant capacity in geese.

Playing a crucial role in digestion, absorption, substance metabolism, and immune response within the animal body, the liver stands as a vital organ in livestock and poultry. Exposed to endogenous toxins delivered via the portal vein, the liver is a primary site of oxidative stress [38]. The *Nrf2* pathway plays a crucial role in combating oxidative stress and inflammation. *Nrf2*, identified over a decade ago as an essential transcription activator of antioxidative genes, exerts significant antioxidative protective effects. It is activated by many phenolic antioxidants, whose antioxidative activities are largely regulated through the *Nrf2* mechanism [39]. Upon oxidative stress stimulation, *Nrf2* binds to the antioxidant response element, regulating Phase I detoxifying enzymes and heme oxygenase-1, among other antioxidative enzymes to combat the cytotoxicity caused by oxidative stress, ultimately restoring cellular redox homeostasis [40]. In our experiment, a significant upregulation trend in liver *Nrf2* mRNA expression was observed with the addition of BAs to geese diets. Similarly, in chicken diets, the addition of BAs can regulate *Nrf2* expression by binding to *Keap1*, thereby reducing oxidative stress. It is noteworthy that some studies suggest that oxidative interference can hinder *Keap1*-mediated *Nrf2* ubiquitination but does not disrupt the *Nrf2*/*Keap1* binding [41]. Therefore, it can be inferred that under severe oxidative stress, the probability of *Nrf2* dissociating from *Keap1* and entering the nucleus to activate antioxidative genes increases. Furthermore, *Nrf2* directly controls glutamate-cysteine ligase and glutathione synthetase to regulate GSH levels. Beyond GSH synthesis, *Nrf2* also plays a role in maintaining GSH [42]. By regulating the transcription of many ROS-detoxifying enzymes, *Nrf2* reduces ROS production by improving mitochondrial function and reducing oxidative stress. Mitochondria, critical sites of ROS production within cells, can have reduced ROS production with BAs maintaining mitochondrial integrity and function [43]. This study also found that 150 mg/kg BAs or 300 mg/kg BAs increased liver *Nrf2* expression, subsequently enhancing the expression of *SOD1* and *GPX-1*. This indicates that BAs supplementation effectively improved the antioxidative state of the liver.

In summary, we conclude that the expression levels of *IL-10* in the spleen increased with the supplementation of 300mg/kg BAs in geese diets, while the expression levels of *SOD1*, *GPX-1*, and *Nrf2* genes in the liver were significantly enhanced with 150 mg/kg or 300 mg/kg BAs. Consequently, this led to an increase in the levels of serum SOD, GSH-Px, and TAC. Therefore, the antioxidative status of geese can be effectively improved with BAs. The inclusion of exogenous BAs in the diet of geese can ameliorate their stress status, with the optimal supplementation level being 150 mg/kg.

Declarations

Availability of Data and Materials: The original data of the paper are available upon request from the corresponding author (G. Xu).

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Ethical Statement: All animal procedures were performed according to guidelines provided by the China Council on Animal Care. All animal experiments were approved by the Animal Care and Use Committee of West Anhui University (Approval no: SYDW-P20210823021).

Competing Interests: The authors declared that there is no conflict of interest.

Declaration of Generative Artificial Intelligence (AI): The author declare that the article tables and figures were not written or created by AI and AI-assisted technologies.

Author Contributions: JC and LY contributed to the design of this study. JC, LY and GX participated in the sample collection, data analysis. GX provided funding and analytical tools. JC wrote the original draft. All authors contributed to data collection and discussion.

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