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Effect of Dietary Salicin Standardized Extract from *Salix alba* Bark on Oxidative Stress Biomarkers and Intestinal Microflora of Broiler Chickens Exposed to Heat Stress

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Abstract: The implication of heat stress (HS) in the oxidative stress phenomenon and its related diseases in chickens has been widely reported. Salicin is a precursor for the synthesis of salicylic acid and aspirin obtained from the bark of *Salix alba*, with an undeniable anti-inflammatory effect. However, little attention has been paid to salicin's antioxidant/oxidative stress-reducing effect compared to its well-known anti-inflammatory effect. The purpose of the study was to investigate the effect of dietary salicin standardized extract from *Salix alba* bark (SAB) on oxidative stress biomarkers and intestinal microflora of broiler chickens exposed to heat stress. In our study, chickens (14 days) were randomly allocated to three treatment groups (SAB0; SAB25; SAB50), each of which included five replicates with eight birds per replicate. Broilers were exposed to heat stress (32 ± 2 °C) between 14 and 42 days. The liver tissues were collected to analyze oxidative stress biomarkers (total antioxidant capacity, glutathione, catalase, superoxide dismutase). The intestinal content was collected and measurements of the intestinal microbial population were performed (*E. coli*, staphylococci, lactobacilli). Results indicated that liver malondialdehyde and protein carbonyls activity decreased ($p < 0.05$) in SAB50 treatment concomitantly with linearly increased total antioxidant capacity, and glutathione concentration. Dietary supplementation with SAB reduced ($p < 0.05$) the abundance of staphylococci and increased the number of lactobacilli. Taken together, SAB possesses an advantageous effect on liver oxidative status and the balance of intestinal microflora in broilers exposed to heat stress. These findings provide new insight into the potential use of salicin standardized extract from *Salix alba* bark for liver damage prevention and dysbiosis related to heat stress.

Keywords: broiler chicken; salicin; *Salix alba* bark; heat stress; oxidative stress; microbiota

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1. Introduction

Heat stress (HS) is an environmental factor that impacts broiler chickens' health and production. As global warming intensifies and breeds become more sensitive (due to genetic improvement to respond to the demands for meat quantity), economic damage related to heat stress in poultry increase repeatedly [1,2]. When broilers are exposed to heat stress, the production of reactive oxygen species (ROS) increases, while the activities of antioxidant enzymes and the capacity to scavenge the free radicals decrease, a phenomenon called oxidative stress. Oxidative stress is associated with damage to demanding lipids, proteins, and DNA, and disrupts redox homeostasis, leading to decreased meat quality and increased tissue damage [3,4]. Heat stress has been observed to decrease endogenous

enzymatic (superoxide dismutase, SOD; catalase, CAT) and non-enzymatic antioxidant (glutathione, GSH) levels and increase lipid and protein oxidation markers in liver tissue and meat [5]. As metabolically active organs, liver and intestinal tissues are very sensitive to HS, such disruption is directly conducive to decreased performance and augments the proneness to disease [5,6]. In the intestine of broiler chickens, an imbalance between harmful and commensal bacteria and a disturbance of the intestinal barrier (a critical element of the gut-liver axis) were observed during heat stress [6]. The link between liver and gut bacteria is acknowledged by several studies [7,8] and continues to evolve. It has been reported that alteration of the gut microbiota leads to increased gut permeability, which causes the activation of various inflammatory pathways in the liver [7]. Due to the negative effects of HS, exploring nutritional solutions to reduce heat stress has become an important research goal.

Endogenous antioxidants (e.g., superoxide dismutase, SOD, catalase, CAT, glutathione peroxidase, GPx) represent the beginning point of antioxidant protection, but during HS their levels are insufficient; therefore, supplementation with exogenous antioxidants is required. Supplementation of chicken feed with exogenous antioxidants, such as vitamins [9], minerals [10], herbal extracts [11,12], and other bioactive compounds [13], is an encouraging solution to mitigate the detrimental effects of HS. Natural antioxidants obtained from leaves, shrubs, and barks could be a safe and easy substitute for chemical additives. Salicin is among the natural compounds extracted from plants. Salicin is a precursor for the synthesis of salicylic acid and aspirin with undeniable anti-inflammatory effects. Recently, however, other biological properties of salicin have been investigated [14]. However, compared to its known anti-inflammatory activity, little attention has been paid to the antioxidant/oxidative stress-reducing effects of salicin. Salicin is the active constituent obtained from the bark of *Salix* species (about 400 species) that can be used in medicine. The addition of *Salix* sp. bark to broiler diets has been shown to support the balance of gut microflora [15], improve performance, and decrease the panting rate [16]. However, to the best of our knowledge, no study has investigated the effect of salicin from the bark of *Salix alba* on the oxidative status of broiler chickens under heat stress. The purpose of the study was to investigate the effect of dietary salicin standardized extract from *Salix alba* bark on liver oxidative stress biomarkers and intestinal microflora of broiler chickens subjected to heat stress.

2. Materials and Methods

2.1. Birds, Diets, and Experimental Design

The procedures concerning animal care, handling, and sampling were conducted under the approval (No. 52/30.07.2014) of the Ethics Committee of the National Research and Development Institute of Animal Biology and Nutrition, Romania. A total of one hundred and twenty 1-day-old Cobb broilers were housed in digestibility cages. Broilers were fed a commercial diet based on corn and soybean meal until 14 days of age. After 2 weeks of acclimation, chicks were randomly assigned into 3 treatment groups (5 replicates/each group with 8 birds per replicate). Three treatment groups were designated as follows: the control group (SAB0), in which birds were fed a basal diet; the experimental groups, in which birds were fed a basal diet supplemented with 25 g *Salix alba* bark extract (SAB)/100 kg diet containing 0.006% salicin (SAB25); and a basal diet supplemented with 50 g *Salix alba* bark extract/100 kg diet (SAB50) containing 0.012% salicin (Table 1). *Salix alba* bark extract was purchased as a powder from a company in China (Changsha Vigorous-Tech Co., Ltd., Changsha, China) containing salicin as an active principle (25% salicin). During the entire experimental period (d 14–d 42), broilers were exposed to heat stress (32 $\pm$ 2 °C). The environmental control systems automatically controlled the temperature and relative humidity (Viper Touch computer). A lighting schedule of 23 h light/day was followed. Water and feed were offered ad libitum.

Table 1. Diet structure of experimental diets (%).

Ingredients (%)	Grower Phase (14–35 Days)			Finisher Phase (36–42 Days)		
	SAB0	**SAB25**	**SAB50**	**SAB0**	**SAB25**	**SAB50**
Corn	62.00	61.97	61.95	60.50	60.47	60.45
Soybean meal	26.58	26.58	26.58	25.46	25.46	25.46
Gluten	4.00	4.00	4.00	6.00	6.00	6.00
Oil	2.50	2.50	2.50	3.75	3.75	3.75
Salix alba bark extract (25% salicin)	-	0.025	0.050	-	0.025	0.050
Calcium carbonate	1.40	1.40	1.40	1.33	1.33	1.33
Monocalcium phosphate	1.36	1.36	1.36	1.13	1.13	1.13
Salt	0.37	0.37	0.37	0.33	0.33	0.33
Methionine	0.26	0.26	0.26	0.25	0.25	0.25
Lysine	0.48	0.48	0.48	0.20	0.20	0.20
Choline	0.05	0.05	0.05	0.05	0.05	0.05
Premix	1.00	1.00	1.00	1.00	1.00	1.00
Total ingredients	100	100	100	100	100	100
Chemical analysis						
Total polyphenols, mg/g GAE	1.70	1.92	2.27	1.69	1.81	2.24

Diet and premix structure published by [17].

2.2. Sample Collection

To evaluate the antioxidant effect, samples of *Salix alba* bark were used to test its effect on in vitro-induced lipid peroxidation (LPO) compared with a synthetic antioxidant, vitamin E. At 42 days, broilers were sacrificed by dislocating of the cervical spine. After exsanguination and evisceration, the whole gastrointestinal tract was rapidly removed. Samples of the intestinal content were collected and kept at −20 °C for microflora measurement. Liver tissues were sampled and stored frozen at −80 °C until used for the analysis of biomarkers of oxidative stress (total antioxidant capacity, TAC; glutathione, GSH; catalase, CAT; superoxide dismutase, SOD; thiobarbituric acid reactive substances, TBARS; and protein carbonyls, PCOs).

2.3. Chemical Analysis

Iron-induced lipid oxidation—Iron-induced lipid oxidation was performed according to the method described previously by [18,19]. Meat samples collected from broilers fed a conventional diet were used to obtain a homogenate as described previously [18]. In three 10 mL plastic tubes, 2 mL of homogenate was transferred. Then, 1000 mg L^{-1} methanolic extract of SAB (tube 2) and 500 µM vitamin E (tube 3) were added to two of them in a total volume of 4 mL. After this, 0.4 mL of peroxidation mix was added to each of the three tubes. The peroxidation solutions consisted of 0.2 mL FeCl2 (100 µM) and 0.2 mL ascorbic acid (500 µM). The mixture was incubated at 37 °C for 60 min and used to quantify thiobarbituric acid reactive substances (TBARS) expressed as mg/kg malondialdehyde (MDA). The inhibition of in vitro-induced lipid peroxidation (LPO) was calculated using the following formula:

$$\% \textbf{ Inhibition LPO} = \left(conc\ MDA_{\ peroxidized\ meat} - conc\ MDA_{peroxidized\ meat+SAB/Vitamin\ E}\right) * 100) / \text{ conc MDA}_{peroxidized\ meat}$$

2.4. Oxidative Stress Biomarkers in Liver

Preparation of liver homogenate—To obtain the liver homogenate, 1 g of liver tissue was weighted and homogenized with 10 mL of potassium phosphate buffer (66 mM, pH 7.2) including 1 mM EDTA. After centrifugation (10,000× *g* for 15 min at 4 °C), the supernatant was collected and used for the analysis of total antioxidant capacity (TAC), glutathione (GSH), catalase (CAT), superoxide dismutase (SOD), thiobarbituric acid reactive substance (TBARS), and protein carbonyls (PCOs).

Total antioxidant activity (TAC) analysis—The assay of TAC was performed based on the capacity of buffered aqueous extracts of the liver in scavenging 2,2- diphenyl-1-picrylhydrazyl free radical (DPPH) [20]. Specifically, 20 µL of the liver extract was homogenized with 480 µL of potassium phosphate buffer (22 mM, pH 7.4). To the resulting mixture was added 500 µL of DPPH solution (0.1 mM). The mixture was stored in a dark room for 30 min and centrifuged (3 min, 10,000× *g*). The absorbance of the mixture was recorded at 520 nm and was converted to mmol/L 6-hydroxy-2,5,7,8-tetramethylchroman-2-carboxylic acid (Trolox) from a Trolox standard curve.

Glutathione (GSH) assay—GSH was determined using a spectrophotometric method according to [21]. An aliquot volume of 20 µL of the liver extract was removed to a plastic tube and mixed well with 5% trichloroacetic acid (TCA). Then, 650 µL potassium phosphate buffer (pH 8.0) and 330 µL of 5,5 dithiobis-2-nitrobenzoate (DTNB) were added. The samples were stored for 45 min in a dark room. The absorbance of solutions was recorded at 412 nm and the GSH concentration was obtained using a calibration curve. The results were expressed as µmol/g tissue.

Catalase activity (CAT) assay—CAT activity was performed as according to [22]. A volume of 10 µL diluted liver extract was mixed with 2980 µL of potassium phosphate buffer (pH 7.4). The solutions were stored for 10 min at 37 °C and then 10 µL of hydrogen peroxide was added. The absorbance was recorded at 240 nm for 3 min. Catalase activity was calculated using the molar extinction coefficient of H_2O_2 and was expressed as U/mg tissue.

Superoxide dismutase activity (SOD)—Superoxide dismutase (SOD) was assayed using a commercial enzymatic kit (FlukaTM, Charlotte, CA, USA) following the manufacturer's protocol. SOD activity was expressed as U/g tissue.

Thiobarbituric acid reactive substance assay (TBARS)—Thiobarbituric acid reactive substances (TBARS) were evaluated by a spectrophotometric method using malondialdehyde (MDA) as standard [23]. The absorbance of samples was read using a spectrophotometer (Jasco V-530, Japan Servo Co. Ltd., Tokyo, Japan) at 532 nm and the results were expressed as mg MDA/kg tissue (liver).

Protein carbonyls (PCOs)—PCOs were assayed by the DNPH-based method [24]. A volume of 50 µL liver extract was mixed with 50 µL TCA (20%) and kept in the water-ice bath for 15 min. The mixture was centrifuged at 15,000× *g* for 10 min at 4 °C. The supernatant obtained was mixed with 500 µL of 2,4-dinitrophenylhydrazine (DNPH) dissolved in 2.5 N HCl and maintained at room temperature for 1 h. After incubation, the mixture was homogenized with a vortex and centrifuged (15,000× *g* for 5 min, 4 °C). The supernatant was mixed with 1 mL TCA, then vortexed and centrifuged. The supernatant was transferred and mixed with 1 mL of ethanol-ethyl acetate (1:1, *v*/*v*); the solution was vortexed, and centrifuged. This washing step was repeated twice. The resulting supernatant was homogenized with 1 mL of 5 M urea (pH 2.3) and maintained at 37 °C for 15 min. Consequently, the solutions were centrifuged and the absorbance was read at 375 nm. The calculation of PCOs concentration was based on the molar extinction coefficient. The results were expressed as nmol DNPH/mg protein. The protein concentration was calculated by reading the absorption at 280 mm and using the bovine serum albumin (BSA) as standard.

2.5. Intestinal Microbial Population Measurements

The intestinal content collected was used to determine the intestinal microbial population. The populations of *Escherichia coli*, staphylococci, and lactobacilli were determined as described previously [12,25]. The colonies were counted with a Scan 300 colony counter (Interscience, Paris, France). The results were indicated as log base 10 colony-forming units (CFU)/gram of intestinal contents.

2.6. Statistical Analysis

The analyses were performed using Addinsoft statistical software [26] (version 2022.3.1). The effect of dietary treatments on tested parameters was determined using one-way analysis of variance (ANOVA) followed by Tukey's multiple-range test. Graphs were drawn

using Prism-GraphPad software v. 9.03 (San Diego, CA, USA). Statistical significance was considered as $p < 0.05$. Correlations between antioxidant capacity, lipid and protein oxidation, and intestinal microflora were performed using Pearson's correlation coefficient analysis. The significant correlations are indicated as follows: * $p < 0.05$; ** $p < 0.01$; *** $p < 0.001$; **** $p < 0.0001$.

3. Results

3.1. Effect of Salicin Standardized Extract from SAB on In Vitro Induced Lipid Peroxidation

Table 2 shows the effect of *Salix alba* bark on in vitro-induced lipid peroxidation (LPO) of meat compared to vitamin E. Vitamin E is a well-known antioxidant, being used as a standard for several analytical methods to highlight the antioxidant capacity. As expected, peroxidized meat treated with synthetic vitamin E had the highest percentage of LPO inhibition, vitamin E being a more effective antioxidant than *Salix alba* bark in retarding lipid peroxidation.

Table 2. Effect of dietary salicin standardized extract from SAB on in vitro-induced lipid peroxidation of meat compared to vitamin E.

Item	TBARS (mg/kg)	% Inhibition of LPO
Meat with induced LPO	1.58 ^{a}	-
Meat with induced LPO and SAB (1000 mg/kg)	1.39 ^{b}	11.60 ^{b}
Meat with induced LPO and vitamin E (500 µM)	1.19 ^{c}	24.21 ^{a}
SEM	0.547	0.813
p-value	<0.0001	<0.0001

$^{a–c}$ Means within a column with no common superscript differ ($p < 0.05$). LPO—lipid peroxidation.

3.2. Oxidative Stress Biomarkers

To evaluate the effect of dietary salicin standardized extract from SAB on the oxidative status of the liver in heat-stressed broilers, we determined the total antioxidant capacity (TAC), the activities of CAT, SOD, and GSH levels (Table 3).

Table 3. Effect of dietary salicin standardized extract from SAB on liver oxidative stress biomarkers of broiler chickens.

Item	Groups			SEM	*p*-Value
	SAB0	SAB25	SAB50		
TAC (mmol Trolox/L)	0.86 ^{a}	0.98 ^{a}	1.12 ^{b}	0.027	0.001
CAT (U/mL)	1848.60	1788.38	1596.81	0.438	0.4346
GSH (µmol/g tissue)	3.138 ^{a}	3.66 ^{ab}	4.05 ^{b}	0.179	0.012
SOD (U/g tissue)	2085.8	2187.3	2208.7	0.137	0.8161

^{a,b} Means within a column with no common superscript differ ($p < 0.05$). Abbreviations: TAC—total antioxidant capacity; CAT—catalase; GSH—glutathione; SOD—superoxide dismutase.

Compared with the SAB0 group, TAC activity and GSH concentration were increased in the liver of heat-stressed broilers fed SAB50 (Table 3). Liver CAT and SOD activity did not differ significantly among the groups.

Figure 1 shows the effects of SAB on liver concentrations of TBARS and PCOs. Liver malondialdehyde was decreased ($p < 0.05$) both in SAB25 and SAB50 treatment compared to SAB0. Protein carbonyls (PCOs) decreased only in the SAB50 group. Notably, the higher level of SAB50 significantly decreased the PCOs activity compared to the lower one.

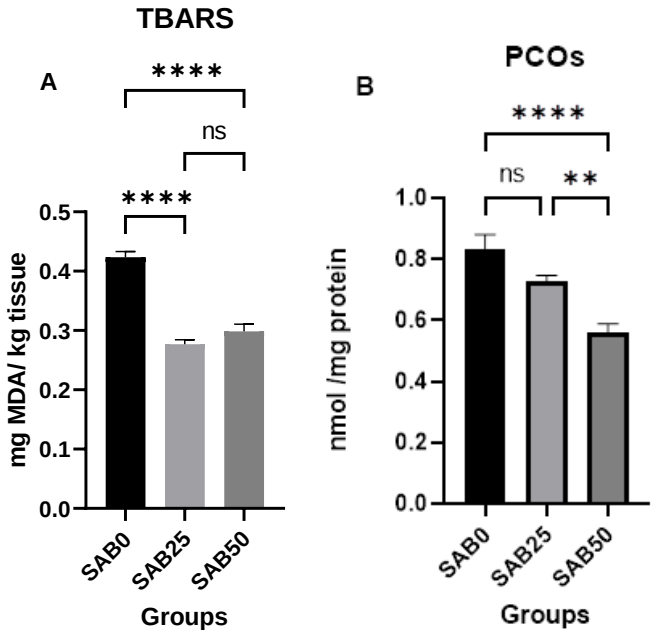

Figure 1. Effects of dietary salicin standardized extract from SAB on liver concentrations of (**A**) thiobarbituric acid reactive substances (TBARS), and (**B**) protein carbonyls (PCOs) in broiler chickens. Bars represent means ± SEM. Abbreviations: SAB0—basal diet, without SAB; SAB25—basal diet + 25 g SAB/100 g diet; SAB50—basal diet + 50 g SAB/100 g diet; ** $p < 0.01$; **** $p < 0.0001$.

3.3. Intestinal Microbial Population Measurements

Figure 2 shows the effects of dietary salicin standardized extract from SAB on intestinal microflora in broiler chickens. *E. coli* did not show any difference between treatments. The number of staphylococci was significantly lower in SAB25 and SAB50 compared to SAB0. Lactobacilli were significantly higher in groups fed supplements with SAB compared to those fed a non-supplemented diet (SAB0). *E. coli*:lactobacilli was significantly lower in SAB 25 and SAB50 compared to SAB0.

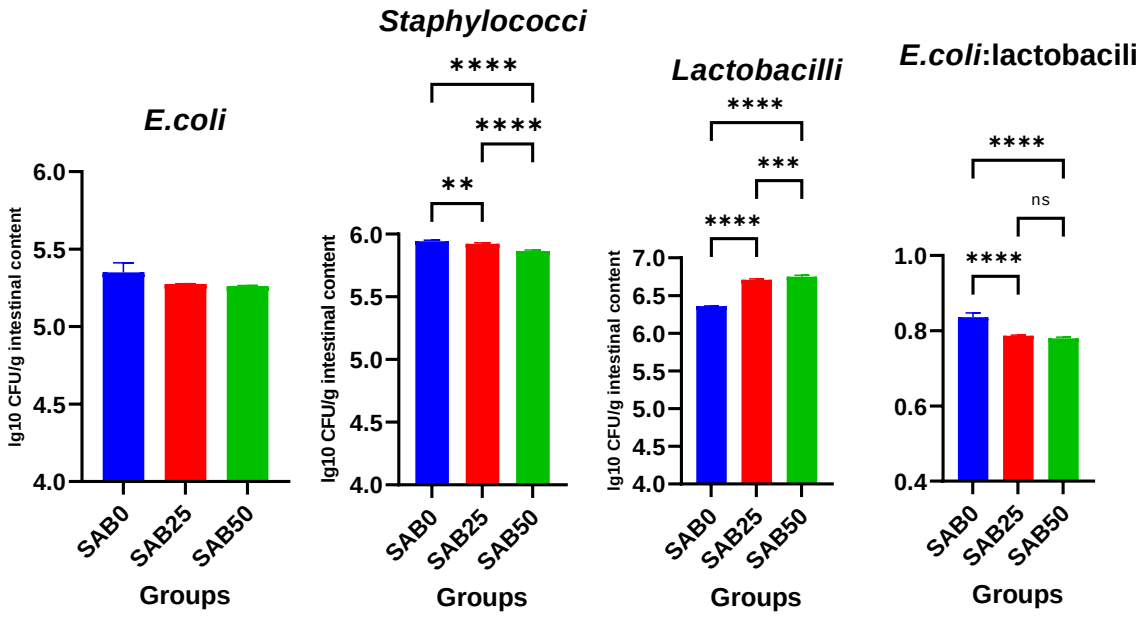

Figure 2. Effects of dietary salicin standardized extract from SAB on intestinal microflora in broiler chickens. Bars represent means ± SEM. Abbreviations: SAB0—basal diet, without SAB; SAB25—basal diet + 25 g SAB/100 g diet; SAB50—basal diet + 50 g SAB/100 g diet; ** $p < 0.01$; *** $p < 0.001$; **** $p < 0.0001$.

3.4. Correlation between Antioxidant Capacity, Lipid and Protein Oxidation Biomarkers, and Intestinal Microflora

TAC showed positive correlation with lactobacilli and negative correlation with TBARS, PCOs, and staphylococci. According to Figure 3, TBARS was negatively correlated with TAC and lactobacilli and positively correlated with PCOs and staphylococci. Pearson's correlation showed a positive correlation of PCOs with TBARS and staphylococci and a negative correlation with TAC and lactobacilli. *E. coli* was found to be negatively correlated with lactobacilli. Regarding the staphylococci content, a positive correlation was found with TBARS and PCOs and a negative correlation with TAC and lactobacilli. In addition, lactobacilli were negatively correlated with TBARS, PCOSs, *E. coli*, and staphylococci.

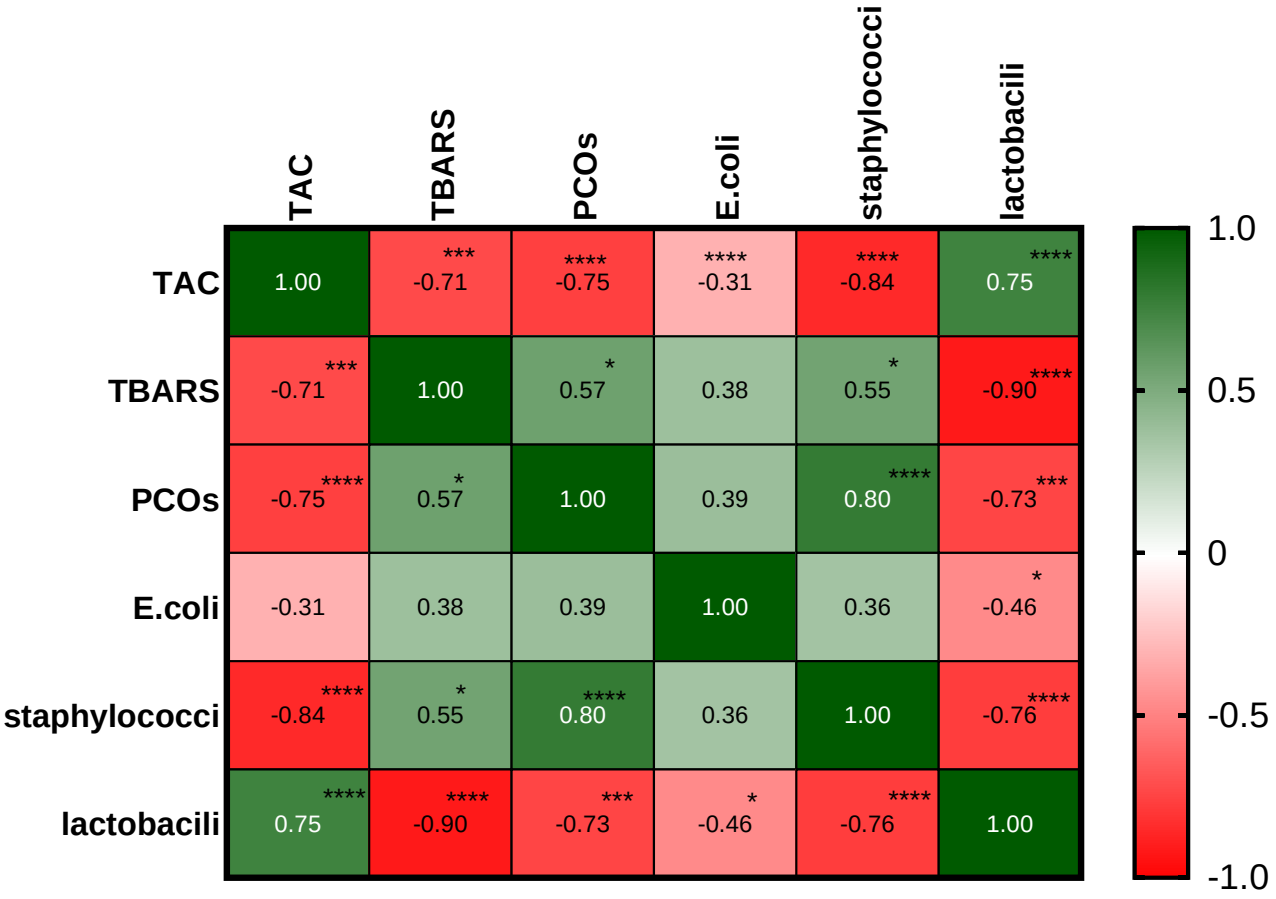

Figure 3. Pearson's correlations between antioxidant capacity, lipid and protein oxidation biomarkers in liver and intestinal microflora. Each cell contains the correlation coefficient (r^2) and statistical level of significance (* $p < 0.05$; *** $p < 0.001$; **** $p < 0.0001$). The negative correlations are highlighted with green and the positive correlations are highlighted with red. The darker the color is, the higher the correlation between variables. Abbreviations: TAC—total antioxidant capacity; TBARS—thiobarbituric reactive species; PCOs—protein carbonyls.

4. Discussion

4.1. Effect of Salicin Standardized Extract from Salix Alba Bark on In Vitro-Induced Lipid Peroxidation

The bark of *Salix alba* is known for its content of salicin, a β-glucoside compound with anti-inflammatory properties that have been demonstrated in in vitro and in vivo studies. However, in the present study, we have shown that the bark of *Salix alba* possesses antioxidant activity in vitro, exhibiting an LPO inhibitory effect of 11.60%, which is almost half that of vitamin E. However, considering that it is an antioxidant standard and SAB is a natural product, this percentage proves that SAB has a significant antioxidant capacity.

These findings are in agreement with several studies [27–29]. Moreover, recent evidence has demonstrated that the biological effects of willow bark extract are not exclusively due to salicylates, but also to the synergism between different bioactive compounds such as chlorogenic acid and salicin derivatives [29].

4.2. Oxidative Stress Biomarkers

The endogenous antioxidant system is the first line of defense against oxidation in the body. In general, under HS conditions, an excess of RS is formed, which decreases the activity of antioxidants (CAT, GSH, SOD) and, accordingly, leads to a disturbance of the equilibrium between antioxidant and oxidative systems in broiler chickens. In this study, feeding with SAB50 increased the concentration of GSH, a tripeptide produced by the body that participates in the body's defenses to combat oxidative stress. GSH may exhibit antioxidant properties in several ways: it detoxifies H_2O_2 and lipid peroxides with the help of glutathione peroxidase (GSH-Px); provides an electron to H_2O_2 for conversion to H_2O and O_2; and protects lipid membranes from oxidative stress by transferring protons [30,31]. In this study, the increase in GSH concentration means that the antioxidant capacity of the body has increased, which was statistically demonstrated by the increase in TAC. These results support the beneficial effects of SAB on the liver of heat-stressed broiler chickens. Overall, this evidence showed for the first time that dietary supplementation with SAB50 can enhance the antioxidant status of broiler chickens by increasing GSH and TAC levels in the liver and protecting heat-stressed chickens from oxidative stress. These results are consistent with those of other studies [17] when broilers raised under thermoneutral conditions were supplemented with 0.05% *S. alba* bark. However, the specific antioxidant mechanism of SAB needs further research. Thus, supplementation of broiler diets with salicin from *Salix alba* bark could explain the increase in GSH levels in the liver with increasing SAB dose. According to some authors [32], an extract of *Salix alba* bark containing 2% salicin increased antioxidant status and inhibited lipid peroxidation in rats. In another study on rats with rheumatoid arthritis, salicin exposure (240 mg) could improve the level of GSH, SOD, and CAT in joint tissues [33]. Although *Salix alba* extracts are commonly standardized to salicin, the same authors showed that those effects are due to both salicin and other compounds in the extracts such as salicylates, polyphenols, and flavonoids, and their interaction may also contribute to the overall antioxidant effect. Polyphenols such as flavonoids improved the expression of γ-glutamylcysteine synthetase and increased the intracellular GSH concentration in muscle [34]. However, the increase only of GSH activity in the liver must be further studied. A possible explanation for the fact that the activity of the other enzymes did not increase may be that the body did not have to call on all the resources to fight the stress because there were enough endogenous and exogenous antioxidants.

As a result of an overwhelmed antioxidant defense system, rises in the levels of MDA and PCOs were recorded in the liver of broiler chickens reared under heat-stress [35,36]. The explanation is that the presence of ROS leads to both protein and lipid oxidation. In heat stress, the formation of reactive oxygen species (ROS) and reactive nitrogen species (RNS) causes protein side chain oxidation that gives rise to reactive aldehydes and ketones known as protein carbonyls (PCOs) [37]. Protein carbonyls are the most commonly used biomarker for protein oxidative damage in tissues since they highlight cellular damage caused by different forms of ROS [38]. As mentioned above, the presence of RS also leads to lipid oxidation. Malondialdehyde (MDA) is the end-product derived from the cleavage of polyunsaturated fatty acids by ROS processes in cells [39]. Interestingly, in our study, SAB administration reduced TBARS and PCOs levels in the liver of broiler chickens reared under heat-stress. The observation made was probably due to the capacity of metabolites of SAB as agonists to induce the synthesis of antioxidants such as GSH (electron donor in peroxides reduction) in the present study, improving oxidative status, and, correspondingly, retarding the process of lipid and protein oxidation in the liver tissue. On the mechanism, SAB has been shown to activate the Nrf2 pathways, which induces the transcription of antioxidant

genes [40–42]. Through those mechanisms, *Salix alba* bark can manage oxidative stress, which altogether may decrease the generation of ROS, plausibly explaining the lower levels of TBARS and PCOs. Studies have shown that the treatment with 50 and 100 mM salicin downregulated oxidative stress induced by advanced glycation end-products (AGEs) in SW1353 human chondrocytes, significantly reducing the ROS level in a dose-dependent manner [43]. Others [33] showed that salicin (240 mg) stabilizes the oxidative stress in rats with rheumatoid arthritis by increasing nuclear factor (erythroid-derived 2)-like 2 (Nrf2) translocation and increasing the expression of heme oxygenase-1 (HO-1). Moreover, the activation of NRf2 plays an important antioxidant role in reducing oxidative stress and inflammation in organs such as the liver, kidneys, and intestine [44,45]. To the best of our knowledge, no studies have investigated the effect of a dietary salicin standardized extract from *Salix alba* bark on the oxidative status of broilers under heat stress. Thus, the present findings show for the first time that, in addition to its beneficial anti-inflammatory effect, salicin from a standardized *Salix alba* bark extract may improve oxidative status in heat-stressed broilers.

4.3. Intestinal Microbial Population Measurements

Oxidative stress related to HS affects gut microbiota composition and causes barrier disruption and dysbiosis in broilers [46,47]. In fact, the gut barrier is strengthened by the commensal bacteria existing in the gut (e.g., *Lactobacillus, Bifidobacterium*) which prevents the colonization of opportunistic pathogens (e.g., *E. coli*, staphylococci, coliforms, and *Clostridium*). These actions are efficient to protect against pathogens [48]. On the other hand, *E. coli* and staphylococci are continuously reported as the main pathogens involved in many intestinal and extra-intestinal disease conditions in poultry. It is a Gram-negative Bacillus which triggers serious health problems including yolk sac infection, respiratory tract infection, etc., and is responsible for considerable economic losses in the poultry industry [49]. *Staphylococcus* genus included bacteria that colonize the surface of the skin and mucous membranes of poultry leading to different diseases, which usually occurs when the natural immunity is destabilized (e.g., stress factors) [50]. Such diseases lead to economic losses resulting from decreased weight gain, mortality, and condemnation at slaughter.

In the present study, dietary administration of SAB positively changed the gut microbiota of heat-stressed broiler chickens, reducing the number of colony units of staphylococci and increasing the abundance of commensal bacteria such as lactobacilli. The natural supplement, SAB, can be metabolized by gut microbiota in new and active small molecules, which possess active pharmacological effects. For instance, the pharmacokinetics of SAB implies a hydrolyzation of salicin to salicyl alcohol, which is further oxidized to salicylic acid (a major metabolite of salicin). The latter, salicylic acid, is converted to salicylic acid and gentisic acid, which are excreted as glucuronides [51]. Some authors [52] identified mono- and oligosaccharides (sucrose, raffinose, and stachyose) and aromatic phytochemicals (triandrin, catechin, salicin, and picein) as antibacterial metabolites of SAB, which showed a high antibacterial effect of *Salix alba* bark water extract on the growth of *S. aureus*. Other authors [53] showed that possibly salicin and salicylic acid have a main contribution to the antimicrobial effect against *Staphylococcus aureus* of the extracts. Studies showed that oral administration of salicin from *Salix alba* bark (100 and 200 mg per body weight, administered daily through oral gavage, 7 days) had an anti-inflammatory effect in DSS-treated mice, improving the recovery in *Lactobacillus* and *Bifidobacterium* populations during the short period of treatment [54]. The mechanism by which the metabolites of SAB act positively on the gut microflora can be by favoring the growth of beneficial bacteria and inhibiting pathogenic bacteria, thereby playing a dual regulatory role in gut microbiota composition. Many herbal medicines were studied to increase beneficial bacteria and reduce harmful bacteria levels, thereby playing a dual regulatory role [55].

4.4. Correlation between Liver Oxidative Biomarkers and Intestinal Microflora

Increasing evidence indicates a crucial role for the gut microbiota in maintaining liver function through modulation of the gut–liver axis [56]. The gut–liver axis defines the mutual interaction between the gut and its microbiota on one hand and the liver on the other. Indeed, bile acids formed in the liver control the microbiota, and intestinal products manage bile acid synthesis, and glucose and lipid metabolism in the liver. A variety of liver diseases are characterized by alterations in the intestinal flora, and some of the altered species have been considered predictive of liver disease outcomes. In addition, it is increasingly evident that oxidative stress leads to disruption of the gut–liver axis (dysbiosis leads to the activation of various inflammatory pathways in the liver) and further to chronic liver disease [7,57]. Interestingly, the present study demonstrated the close link between gut microbiota and liver oxidative status. Present findings showed a strong positive correlation between lactobacilli and TAC and showed that the increase in lactobacilli in the intestine improved the antioxidant status of the liver. Improvement of the intestinal microbiota (decreasing staphylococci and increasing lactobacilli abundance) was attributed to the increase in TAC and the reduction of MDA and PCOs levels in the liver. Little evidence was found regarding the correlation between the gut microbiota and the oxidative state of the liver, if we consider its importance, especially in the context of exposure to a stressor, which in our case would be heat stress. Several studies [58] showed that the redox mechanisms of *Lactobacillus* spp. may take part to the downregulation of these ROS-forming enzymes. Moreover, *Lactobacillus* spp. has been studied to modulate oxidative stress via Nrf-2 and nuclear factor kappa B (NF-kB). In this study, we showed that improving TAC suppresses lipid and protein oxidation of liver tissue, from which we can infer that high hepatic levels of TAC delay oxidative stress in heat-stressed broilers and restores the RS/antioxidant equilibrium. In the case of the TAC–liver oxidative protection relationship, considerable evidence was found [59,60].

5. Conclusions

HS is a serious stressor inflicting oxidative stress and affecting poultry production. In this study, it was confirmed that doses of 25 and 50 mg/100 kg SAB containing 0.006% and 0.012% salicin exerts a positive effect on counteract oxidative stress damage of the liver in broilers exposed to heat stress due to stimulation of GSH synthesis and increased antioxidant capacity. Dietary supplementation with SAB increases beneficial bacteria (lactobacilli) levels and reduces harmful bacteria (staphylococci) abundance in the intestine of heat-stressed broiler chickens, thereby possibly playing a dual regulatory role. The increase in lactobacilli and the decrease in staphylococci due to the supplementation with SAB led to the improvement of the oxidative status of the liver of chickens raised under heat stress. The novel results presented in this study provide new insight into the potential use of salicin standardized extract from *Salix alba* bark for liver damage prevention and dysbiosis related to heat stress.

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