The Journal of Veterinary Medical Science

FULL PAPER

Physiology

Effect of fisetin and probiotic supplementation on erythrocyte osmotic fragility, malondialdehyde concentration and superoxide dismutase activity in broiler chickens exposed to heat stress

Ngozi Ejum OGBUAGU[1)]*, Tagang ALUWONG[1)], Joseph Olusegun AYO[1)] and Victory Osirimade SUMANU[1)]

[1)]Department of Veterinary Physiology, Faculty of Veterinary Medicine, Ahmadu Bello University, Zaria, P.O. Box 2555, Nigeria

ABSTRACT. The aim of the study was to evaluate effects of fisetin and probiotic on erythrocyte osmotic fragility (EOF), malondialdehyde (MDA) and superoxide dismutase (SOD) in broiler chickens exposed to heat stress. Sixty day-old broilers were divided into: Group I (control) given distilled water; Group II, fisetin (5 mg/kg); Group III, probiotic *Saccharomyces cerevisiae* (4.125×10^6 cfu/100 m*l*); and Group IV, fisetin (5 mg/kg) + probiotic (4.125×10^6 cfu/100 m*l*) orally for 7 days. Blood samples collected from 42-day-old birds were evaluated for EOF, serum MDA concentration and SOD activity. Percentage EOF at 0.5% NaCl was lower ($P<0.05$) in fisetin, probiotic and fisetin + probiotic groups (34.26 ± 0.98%, 35.65 ± 0.81% and 34.25 ± 1.98%, respectively) than in controls (48.42 ± 0.40%). The MDA concentrations in broiler chickens administered with fisetin (14.37 ± 1.15 nmol/*l*), probiotic (5.66 ± 1.06 nmol/*l*) and fisetin + probiotic (4.136 ± 0.58 nmol/*l*) were lower ($P<0.05$) than in controls (22.64 ± 2.95 nmol/*l*). Activities of SOD were higher ($P<0.05$) in fisetin, probiotic and fisetin + probiotic broiler chickens (6.34 ± 0.24 IU/*l*, 5.67 ± 0.09 IU/*l* and 5.93 ± 0.13 IU/*l*, respectively) than in controls (5.37 ± 0.09 IU/*l*). Fisetin + probiotic ameliorated oxidative stress changes in broiler chickens better than fisetin or probiotic alone. In conclusion, administration of fisetin or probiotic and, especially their combination, decreased EOF, lipoperoxidation and increased superoxide dismutase activity in broiler chickens exposed to heat stress.

KEY WORDS: erythrocyte osmotic fragility, fisetin, lipoperoxidation, probiotic, superoxide dismutase

J. Vet. Med. Sci.
80(12): 1895–1900, 2018
doi: 10.1292/jvms.18-0477

Received: 15 August 2018
Accepted: 17 October 2018
Published online in J-STAGE: 8 November 2018

Broilers chickens are prone to oxidative stress in adverse thermal environmental conditions such as high ambient temperature (AT) and high relative humidity (RH) [47]. High AT and high RH induce heat stress in broiler chickens, causing oxidative stress. Oxidative stress occurs as a result of increased reactive oxygen species (ROS) production, which may overwhelm or deplete the natural antioxidant defence systems of the body, resulting in cell damage and destruction [16, 17].

Stress induces increased ROS generation which leads to lipid peroxidation of cytomembranes [36]. Lipid peroxidation is the oxidative degradation of polyunsaturated fatty acids, occurring in biological membranes. It inactivates several membrane bound enzymes and impairs membrane fluidity and integrity [7]. Erythrocyte osmotic fragility (EOF) is a biomarker of oxidative membrane damage to erythrocytes [39]. Malondialdehyde (MDA) is a product of peroxidized polyunsaturated fatty acids; increased MDA content is an important indicator of lipid peroxidation [14, 29]. The activities of antioxidant enzymes such as superoxide dismutase (SOD) are used to assess tolerance of broiler chickens to stress [42]. A decrease in activities of antioxidant enzymes is an indicator of oxidative stress in broiler chickens [2, 41]. It is beneficial in determining whether oxidative stress reactions occur in cells and identification of the damaged cell [30].

Fisetin (3,3′,4′,7-tetrahydroxyflavone) is a naturally occurring flavonoid, which is found in fruits and vegetables such as strawberries, apples, persimmons, grapes and onions [23]. It has high antioxidant activity and efficiently interacts with common target sites of lipid peroxidation in cell membranes, enhancing their integrity against hypotonic lysis [34, 35]. Fisetin also has the ability to protect the erythrocytes from extracellular oxidative agents by donating electron to plasma membrane oxidoreductase; thus, maintaining the redox state of the plasma [44]. Fisetin exerts potential effects on the levels of antioxidants, reversing the

*Correspondence to: Ogbuagu, N. E.: ogbuagungozi1989@gmail.com

depleted levels of different enzymatic (SOD, catalase, glutathione peroxidase) and non-enzymatic (vitamins C and E) antioxidants to near normal levels [28]. Probiotic supplementation has been shown to modulate the dynamics of oxidants and antioxidants in the body, inhibiting lipid peroxidation [1]. It enhances serum antioxidant enzyme activities in broiler chickens [5, 45].

Probiotic improves performance, intestinal morphology and structure, immune system and, thus, performance and well-being of heat-stressed poultry [4, 15]. Although studies have been conducted on single effects of probiotic in broiler production, there is paucity of information on effects of fisetin and its combination with probiotic on heat stressed broiler chickens. We hypothesized that supplementation of fisetin and probiotic to broiler chickens exposed to heat stress would be associated with decreased erythrocyte osmotic fragility, malondialdehyde concentration and increased superoxide dismutase activity.

MATERIALS AND METHODS

Experimental site and meteorological conditions

The experiment was conducted at the Department of Physiology, Faculty of Veterinary Medicine, Ahmadu Bello University, Zaria (11°10′N, 07° 38′E), located in the Northern Guinea Savannah zone of Nigeria. It was carried out from June to July, during the early rainy season [13]. The dry-bulb and wet-bulb temperatures were recorded using the wet- and dry-bulb thermometer (Brannan®, Cumbria, U.K.) thrice per day at 07:00 hr, 13:00 hr and 18:00 hr on June 20-July 31, 2017. The RH was obtained using Osmon's hygrometric table (Narinda Scientific Industries®, Haryana, India). The temperature-humidity index (THI) was determined as an index of heat load using the following formula [43];

$THI_{broiler}=0.85\ T_{db} + 0.15T_{Wb,}$

Where $THI_{broiler}$=temperature-humidity index for broilers, T_{db}=dry-bulb temperature and T_{Wb}=wet-bulb temperature.

The dry-bulb temperature (DBT), RH and THI ranges during the study period were 25.00–36.00°C, 47.00–100.00% and 25.00–35.55, respectively; and the corresponding mean values obtained were: 31.11 ± 0.28, 79.32 ± 0.97, 30.65 ± 0.26, respectively.

Ethical clearance

Ethical approval for this work was sought and obtained from the Ahmadu Bello University Committee on Animal Use and Care (ABUCAUC), with Reference Number: ABUCAUC/2018/020.

Experimental animals, grouping and management

Sixty day-old broiler chickens, comprising both sexes and purchased from a commercial farm served as subjects. They were fed with broiler starter (day 0–28) and broiler finisher (day 29–42). Proximate analysis of the broiler diet is shown in Table 1. The birds were kept under an intensive management system. They were housed in the same pen, littered with wood shavings on the floor and having a zinc roof with cardboard ceiling. The dimension of the pen was 8.4 × 5.6 × 1.91 m, and the broiler chickens were stocked at 15 birds/m^2 [27]. The length of day light prevailing during the study period was 12 hr.

The birds were randomly divided into four groups (Group I–IV) of fifteen each. Group I, which served as the control group, was given only distilled water. Group II was administered with fisetin only at 5 mg/kg, Group III was given probiotic (*Saccharomyces cerevisiae)* only at 4.125 × 10^6 cfu/100 m*l*, while group IV (fisetin + probiotic) was co-administered with fisetin and probiotic at 5 mg/kg and 4.125 × 10^6 cfu/100 m*l*, respectively. Probiotic and fisetin were administered for first 7 days of life consecutively using 1 m*l*-tuberculin syringe. Each bird was tagged using a masking tape on the leg for identification and proper recordings. Biosecurity measures were ensured by providing footbath, foot wears and clothing for persons assisting in carrying out the experiment. The pen was not accessible to any non-essential persons, animals, other birds or rodents.

Experimental measurements

Experimental drugs and their preparation: Fisetin (Sigma-Aldrich, St. Louis, MO, U.S.A.) 100 mg was dissolved in 105 m*l* of carboxylmethylcellulose and administered at the dose of 5 mg/kg, while 1.5 m*l* from the stock of probiotic (Montajat Pharmaceuticals, Biosciences Division, Dammam, Saudi Arabia) was dissolved in 1 *l* of water and administered at a dose of 4.125 × 10^6 cfu/100 m*l* based on the method of competitive exclusion [6]. Fisetin and probiotic were administered during the morning period at 8.00 hr (GMT + 1) to avoid excessive stress on the birds.

Collection of blood and serum samples: Blood sample (3 m*l*) was collected once, on day 42, from the wing vein of each broiler chicken. Half of the quantity was placed into a test tube containing an anticoagulant, sodium ethylenediaminetetraacetate, and the other was poured into another test tube without an anticoagulant [24]. For determining EOF, blood samples collected into sample bottles containing anticoagulant ethylenediaminetetraacetate at concentration of 1.5 mg/m*l* [38] were used, while 1.5 m*l* of blood samples in sample bottles without anticoagulant were centrifuged at 3,000 g for 10 min; Serum was harvested and stored at 4°C until assayed for evaluation of serum concentration of MDA [37] and SOD activity [25].

Table 1. Proximate analysis of broiler diet during the study period

Parameters	Starter	Finisher
Crude protein	22	21
Fat (%)	7.9	6.8
Crude fibre (%)	4.3	3.0
Calcium (%)	2.0	2.0
Available phosphorus (%)	0.8	0.7
Methionine (%)	0.56	0.5
Lysine (%)	1.2	1.2
Metabolisable Energy (kcal/kg)	2,900	2,980

Source: Nutrition Laboratory, Marks Farm, Osara, Kogi State.

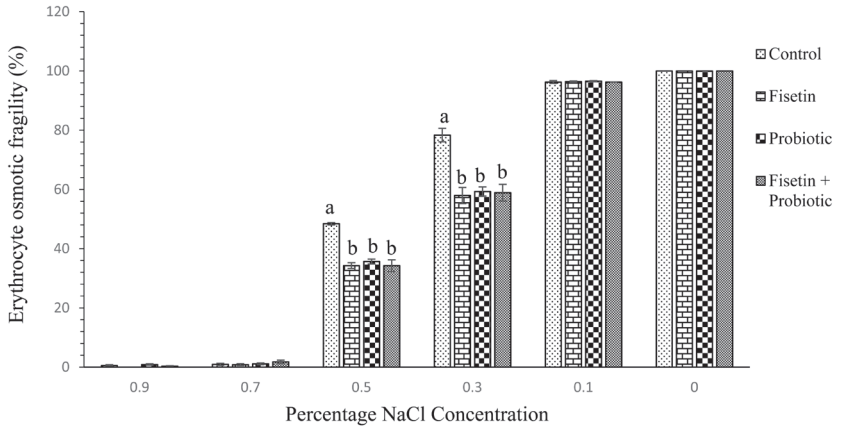

Fig. 1. Effect of fisetin and probiotic supplementation on erythrocyte osmotic fragility in broiler chickens.
[a, b]Values with different superscript letters are significantly (P<0.05) different, mean ± SEM; n=7.

Determination of EOF

The EOF test was performed as described by Oyewale [32]. Briefly, 0.02 m*l* of blood was added to six tubes, containing increasing concentrations (0, 0.1, 0.3, 0.5, 0.7 and 0.9%) of phosphate-buffered sodium chloride (NaCl) solution at pH 7.4. The tubes were gently mixed and incubated at room temperature (26–28°C) for 30 min. The content of each tube was centrifuged at 150 × g for 10 min and the supernatant decanted. Optical density (OD) of the supernatant was determined using spectrophotometer (Spectronic-20, Philip Harris Limited, Shenstone, England) at wavelength of 540 nm. Hemolysis in each tube was expressed as a percentage, taking hemolysis in distilled water (0% NaCl) as 100%:

$$\text{Percentage (\%)} = \frac{\text{Optical density of test}}{\text{Optical density of standard}} \times 100$$

Where OD=optical density.

Evaluation of malondialdehyde concentration

Serum lipid peroxidation was evaluated, based on MDA concentration as described by Janero [19]. Briefly, serum sample (100 μl) was mixed with sodium dodecyl sulphate-acetate buffer (pH 3.5) and aqueous solution of thiobarbituric acid. It was heated at 95°C for 60 min, the red pigment produced was extracted with n-butanol-pyridine mixture and estimated by measuring the absorbance at 532 nm. Tetramethoxy-propane was used as an external standard, and MDA concentration was expressed in nmol/*l*.

Evaluation of superoxide dismutase activity

The activity of superoxide dismutase (SOD) was measured using the Northwest Life Science Specialties SOD kit (NWLSS™ NWK-SOD02) based on the method of monitoring the auto-oxidation rate of hematoxylin originally described by Martin *et al*. [25], and modified to enhance reliability. The presence of SOD enzyme at specific assay pH, the rate of auto-oxidation was inhibited and the percentage of inhibition was linearly proportional to the amount of SOD present within a specific range. Sample SOD activity was determined by measuring ratios of auto-oxidation rates in the presence and absence of the sample and expressed as McCord-Fridovich "cytochrome c" units.

Statistical analysis

The data obtained were expressed as mean ± standard error of the mean, using Graph Pad Prism 4.0 for Windows (San Diego, CA, U.S.A.). The values obtained were analyzed using one-way analysis of variance (ANOVA), followed by Tukey's *post-hoc* test. Values of P<0.05 were considered significant [41].

RESULTS

Erythrocyte osmotic fragility

The EOF decreased significantly (P<0.05) at 0.5% NaCl, in the fisetin, probiotic and fisetin + probiotic groups (34.26 ± 0.98%, 35.65 ± 0.81% and 34.25 ± 1.98%, respectively), when compared with that of the control group (48.42 ± 0.40%). At 0.3% NaCl, the EOF in the groups treated with fisetin (57.94 ± 2.72%), probiotic (59.29 ± 1.55%) and fisetin + probiotic (58.91 ± 2.82%) also decreased significantly (P<0.001), when compared with that of the control group (78.33 ± 2.29%) (Fig. 1).

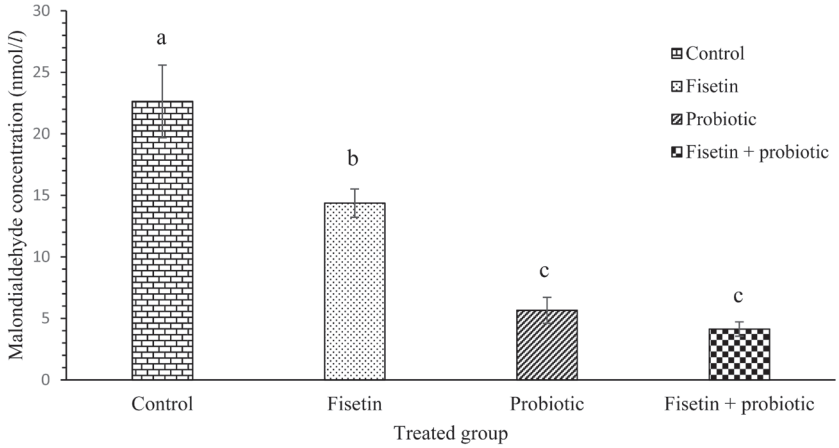

Fig. 2. Effect of fisetin and probiotic supplementation on malondialdehyde concentration in broiler chickens.
[a–c]Values with different superscript letters are significantly (P<0.05) different, mean ± SEM; n=7.

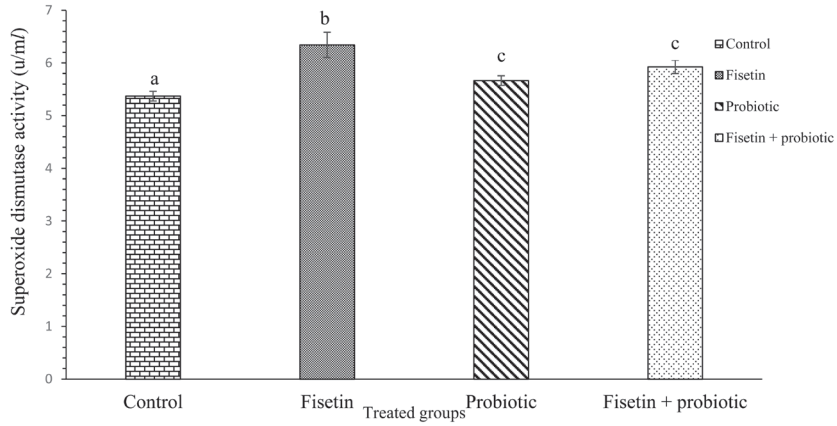

Fig. 3. Effect of fisetin and probiotic supplementation on superoxide dismutase activity in broiler chickens.
[a–c]Values with different superscript letters are significantly (P<0.05) different, mean ± SEM; n=7.

Malondialdehyde concentration

The MDA concentration in broiler chickens administered with fisetin, probiotic and fisetin + probiotic (14.37 ± 1.15 nmol/*l*, 5.66 ± 1.06 nmol/*l*, 4.136 ± 0.58 nmol/*l*, respectively) were significantly (P<0.05) lower than the concentration obtained in the control group (22.64 ± 2.95 nmol/*l*). The MDA concentration in the group administered with fisetin + probiotic was significantly (P<0.05) lower than that of the control and fisetin-treated group. The concentration of MDA in probiotic and fisetin + probiotic-treated groups did not differ significantly (P>0.05; 5.66 ± 1.06 nmol/*l* and 4.136 ± 0.58 nmol/*l*, respectively) (Fig. 2).

Superoxide dismutase activity

The activities of SOD in the fisetin, probiotic and fisetin + probiotic groups (6.34 ± 0.24 IU/*l*, 5.67 ± 0.09 IU/*l* and 5.93 ± 0.13 IU/*l*, respectively) were significantly (P<0.05) higher, when compared with that of the control group (5.37 ± 0.09 IU/*l*).The activity of SOD in fisetin treated group (6.34 ± 0.24 IU/*l*) increased significantly (P<0.05), when compared to that of controls (5.37 ± 0.09 IU/*l*). Activities of SOD in probiotic and fisetin + probiotic-treated groups did not differ significantly (5.67 ± 0.09 IU/*l* and 5.93 ± 0.13 IU/*l*, respectively) (Fig. 3).

DISCUSSION

The values of DBT of 18–24°C [31], RH (65–70%) [3] and THI (20.8) [42] are conducive for raising broiler chickens. The values obtained in the present study were outside the thermoneutral zone, indicating that the broiler chickens were exposed to heat stress. In broiler chickens, such high values may result in poor performance, hyperthermia, immune suppression and high mortality [21, 40]. The administration of fisetin to broiler chickens decreased EOF and, thus, maintained the integrity of the erythrocyte membrane. The finding shows that fisetin exerted antioxidant effect on the cell membrane [10], thus protecting the cells from the

deleterious effects of heat stress-induced lipid peroxidation. The result agrees with that of Belkhiri *et al.* [9], who demonstrated that the binding of flavonoids to RBC membranes significantly inhibits lipid peroxidation, and consolidates their integrity against hypotonic lysis. The result also supports the finding that probiotic exerts antioxidant effects by protecting the cell integrity and inhibiting lipid peroxidation, thus preventing cells against adverse effects of heat stress [5]. The study showed that administration of both fisetin and probiotic has no additive or synergistic effect on the maintenance of the integrity of the cell membrane. However, Egbuniwe *et al*. [14] demonstrated that combined administration of antioxidants, betaine and ascorbic acid, reduced EOF in broiler chickens under stressful hot-dry conditions. The inconsistency may be due to fact that different antioxidants were used and the research was carried out at different periods of the year.

Oxidative stress increases the production of ROS which cause damage to the RBC membrane via lipid peroxidation [12], decreases erythrocyte diapole potential (a biophysical determinant of membrane function) and results in the formation of hemoglobin-spectrin complexes, which stiffen the membrane [20, 46], and impairs erythrocyte functions. Heat stress produces imbalance of pH in the erythrocyte environment due to loss of some ions, which adversely affect EOF [11, 33]. The result of the present study shows that impairments induced by oxidative stress were ameliorated by the administration of antioxidants, fisetin and/or probiotic, to broiler chickens exposed to heat stress. Thus, the antioxidants by consolidating the membrane integrity, protected the erythrocytes from the adverse effects of heat stress.

The findings of the study that fisetin decreased serum MDA concentration and significantly increased SOD agree with that of Iskender *et al*. [18], who reported a decrease in MDA concentration and increase in SOD activity in laying hens, administered with a flavonoid, quercetin. Thus, fisetin, a flavonoid, conferred some protection on the broiler chickens against the negative effects of oxidative stress, resulting from heat stress. Similarly, findings from the present study revealed that probiotic decreased serum MDA concentration and improved the activity of SOD, which agree with that of Bai *et al*. [8] who reported that probiotic, as an antioxidant, restores the activity of SOD and decreases serum MDA concentration in broiler chickens reared under heat stress. Probiotic has been shown to improve the negative influence of oxidative stress [22], and promotes the activities of antioxidant enzymes; thus, it scavenges excess ROS that may cause cell damage, and, consequently improves the health status of the host [26]. The results of the present study show that the administration of antioxidants, fisetin and/or probiotic, to broiler chickens exposed to heat stress protected against the adverse effects of excess ROS generation.

In conclusion, the administration of fisetin or probiotic and especially their combination decreased EOF and MDA concentration, and increased superoxide dismutase activity in broiler chickens exposed to heat stress.

COFLICT OF INTERESTS. The authors declare that there are no competing interests.

ACKNOWLEDGMENTS. The authors thank staff of the Research Laboratory of the Department of Physiology, Faculty of Veterinary Medicine, Ahmadu Bello University, Zaria, Nigeria.

REFERENCES

1. Abudabos, A. M., Alyemni, A. H. and Zakaria, H. A. H. 2016. Effect of two strains of probiotics on the antioxidant capacity, oxidative stress, and immune responses of salmonella challenged broilers. *Braz. J. Poult. Sci.* **18**: 175–180. [CrossRef]
2. Akbarian, A., Michiels, J., Degroote, J., Majdeddin, M., Golian, A. and De Smet, S. 2016. Association between heat stress and oxidative stress in poultry; mitochondrial dysfunction and dietary interventions with phytochemicals. *J. Anim. Sci. Biotechnol.* **7**: 37. [Medline] [CrossRef]
3. Akyuz, A. and Boyaci, S. 2010. Determination of heat and moisture balance for broiler house. *J. Anim. Vet. Adv.* **9**: 1899–1901. [CrossRef]
4. Al-Fataftah, A. R. and Abdelqader, A. 2014. Effects of dietary *Bacillus subtilis* on heat stressed broilers performance, intestinal morphology and microflora composition. *Anim. Feed Sci. Technol.* **198**: 279–285. [CrossRef]
5. Aluwong, T., Kawu, M., Raji, M., Dzenda, T., Govwang, F., Sinkalu, V. and Ayo, J. 2013. Effect of yeast probiotic on growth, antioxidant enzyme activities and malondialdehyde concentration of broiler chickens. *Antioxidants* **2**: 326–339. [Medline] [CrossRef]
6. Aluwong, T., Sumanu, V. O., Ayo, J. O., Ocheja, B. O., Zakari, F. O. and Minka, N. S. 2017. Daily rhythms of cloacal temperature in broiler chickens of different age groups administered with zinc gluconate and probiotic during the hot-dry season. *Physiol. Rep.* **5**: e13314. [Medline] [CrossRef]
7. Azeez, O. A., Oyagbemi, A. A. and Iji, O. T. 2013. Haematology and erythrocyte osmotic fragility indices in domestic chicken following exposure to a polyvalent iodophorous disinfectant. *Jordan J. Biol. Sci.* **5**: 99–103.
8. Bai, K., Huang, Q., Zhang, J., He, J., Zhang, L. and Wang, T. 2017. Supplemental effects of probiotic Bacillus subtilis fmbJ on growth performance, antioxidant capacity, and meat quality of broiler chickens. *Poult. Sci.* **96**: 74–82. [Medline] [CrossRef]
9. Belkhiri, F., Baghiani, A., Zerroug, M. M. and Arrar, L. 2017. Investigation of antihemolytic, xanthine oxidase inhibition, antioxidant and antimicrobial properties of Salvia verbenaca L. aerial part extracts. *Afr. J. Tradit. Complement. Altern. Med.* **14**: 273–281. [Medline] [CrossRef]
10. Belscak-Cvitanovic, A., Durgo, K., Huđek, A., Bacun-Druzina, V. and Komes, D. 2018. Overview of polyphenols and their properties. pp. 3–44. *In*: Polyphenols: Properties, Recovery, and Applications, Woodhead Publishing, Cambridge.
11. Chauhan, S. S., Celi, P., Leury, B. J. and Dunshea, F. R. 2015. High dietary selenium and vitamin E supplementation ameliorates the impacts of heat load on oxidative status and acid-base balance in sheep. *J. Anim. Sci.* **93**: 3342–3354. [Medline] [CrossRef]
12. Duranti, G., Ceci, R., Patrizio, F., Sgrò, P., Di Luigi, L., Sabatini, S., Felici, F. and Bazzucchi, I. 2018. Chronic consumption of quercetin reduces erythrocytes oxidative damage: Evaluation at resting and after eccentric exercise in humans. *Nutr. Res.* **50**: 73–81. [Medline] [CrossRef]
13. Dzenda, T., Ayo, J. O., Lakpini, C. A. M. and Adelaiye, A. B. 2013. Seasonal, sex and live weight variations in feed and water consumptions of adult captive African Giant rats (Cricetomys gambianus, Waterhouse-1840) kept individually in cages. *J. Anim. Physiol. Anim. Nutr. (Berl.)* **97**: 465–474. [Medline] [CrossRef]
14. Egbuniwe, I. C., Ayo, J. O., Kawu, M. U. and Mohammed, A. 2018. Ameliorative effects of betaine and ascorbic acid on erythrocyte osmotic

fragility and malondialdehyde concentrations in broiler chickens during the hot-dry season. *J. Appl. Anim. Res.* **46**: 380–385. [CrossRef]
15. Faseleh Jahromi, M., Wesam Altaher, Y., Shokryazdan, P., Ebrahimi, R., Ebrahimi, M., Idrus, Z., Tufarelli, V. and Liang, J. B. 2016. Dietary supplementation of a mixture of Lactobacillus strains enhances performance of broiler chickens raised under heat stress conditions. *Int. J. Biometeorol.* **60**: 1099–1110. [Medline] [CrossRef]
16. Gupta, A., Chauhan, N. R., Chowdhury, D., Singh, A., Meena, R. C., Chakrabarti, A. and Singh, S. B. 2017. Heat stress modulated gastrointestinal barrier dysfunction: role of tight junctions and heat shock proteins. *Scand. J. Gastroenterol.* **52**: 1315–1319. [Medline] [CrossRef]
17. Habibian, M., Ghazi, S., Moeini, M. M. and Abdolmohammadi, A. 2014. Effects of dietary selenium and vitamin E on immune response and biological blood parameters of broilers reared under thermoneutral or heat stress conditions. *Int. J. Biometeorol.* **58**: 741–752. [Medline] [CrossRef]
18. Iskender, H., Yenice, G., Dokumacioglu, E. I., Kaynar, O., Hayirli, A. and Kaya, A. 2016. The effects of dietary flavonoid supplementation on the antioxidant status of laying hens. *Braz. J. Poult. Sci.* **18**: 663–668. [CrossRef]
19. Janero, D. R. 1990. Malondialdehyde and thiobarbituric acid-reactivity as diagnostic indices of lipid peroxidation and peroxidative tissue injury. *Free Radic. Biol. Med.* **9**: 515–540. [Medline] [CrossRef]
20. Jewell, S. A., Petrov, P. G. and Winlove, C. P. 2013. The effect of oxidative stress on the membrane dipole potential of human red blood cells. *Biochim. Biophys. Acta* **1828**: 1250–1258. [Medline] [CrossRef]
21. Kamel, N. N., Ahmed, A. M. H., Mehaisen, G. M. K., Mashaly, M. M. and Abass, A. O. 2017. Depression of leukocyte protein synthesis, immune function and growth performance induced by high environmental temperature in broiler chickens. *Int. J. Biometeorol.* **61**: 1637–1645. [Medline] [CrossRef]
22. Kim, H. W., Kim, J. H., Yan, F., Cheng, H. W. and Brad Kim, Y. H. 2017. Effects of heat stress and probiotic supplementation on protein functionality and oxidative stability of ground chicken leg meat during display storage. *J. Sci. Food Agric.* **97**: 5343–5351. [Medline] [CrossRef]
23. Lall, R. K., Adhami, V. M. and Mukhtar, H. 2016. Dietary flavonoid fisetin for cancer prevention and treatment. *Mol. Nutr. Food Res.* **60**: 1396–1405. [Medline] [CrossRef]
24. Maini, S., Rastogi, S. K., Korde, J. P., Madan, A. K. and Shukla, S. K. 2007. Evaluation of oxidative stress and its amelioration through certain antioxidants in broilers during summer. *J. Poult. Sci.* **44**: 339–347. [CrossRef]
25. Martin, J. P. Jr., Dailey, M. and Sugarman, E. 1987. Negative and positive assays of superoxide dismutase based on hematoxylin autoxidation. *Arch. Biochem. Biophys.* **255**: 329–336. [Medline] [CrossRef]
26. Mishra, V., Shah, C., Mokashe, N., Chavan, R., Yadav, H. and Prajapati, J. 2015. Probiotics as potential antioxidants: a systematic review. *J. Agric. Food Chem.* **63**: 3615–3626. [Medline] [CrossRef]
27. Muniz, E. C., Fascina, V. B., Pires, P. P., Carrijo, A. S. and Guimarães, E. B. 2006. Histomorphology of bursa of fabricius: Effects of stock densities on commercial broilers. *Rev. Bras. Cienc. Avic.* **8**: 217–220. [CrossRef]
28. Naeimia, A. F. and Alizadeh, M. 2017. Antioxidant properties of the flavonoid fisetin: an updated review of *in vivo* and *in vitro* studies. *Trends Food Sci. Technol.* **70**: 34–44. [CrossRef]
29. Nasiroleslami, M., Torki, M., Saki, A. A. and Abdolmohammadi, A. R. 2018. Effects of dietary guanidinoacetic acid and betaine supplementation on performance, blood biochemical parameters and antioxidant status of broilers subjected to cold stress. *J. Appl. Anim. Res.* **46**: 1016–1022. [CrossRef]
30. Ognik, K. and Krauze, M. 2016. The potential for using enzymatic assays to assess the health of turkeys. *Worlds Poult. Sci. J.* **72**: 535–550. [CrossRef]
31. Olanrewaju, H. A., Purswell, J. L., Collier, S. D. and Branton, S. L. 2016. Interactive effects of ambient temperature and light sources at high relative humidity on growth performance and blood physiological variables in broilers grown to 42 day of age. *Int. J. Poult. Sci.* **15**: 394–400. [CrossRef]
32. Oyewale, J. O. 1992. Effects of temperature and pH on osmotic fragility of erythrocytes of the domestic fowl (Gallus domesticus) and guinea-fowl (Numida meleagris). *Res. Vet. Sci.* **52**: 1–4. [Medline] [CrossRef]
33. Oyewale, J., Dzenda, T. L., Akanbi, D., Ayo, J., Owoyele, O., Minka, N. and Dare, T. 2011. Alterations in the osmotic fragility of camel and donkey erythrocytes caused by temperature, pH and blood storage. *Vet. Arch.* **81**: 459–470.
34. Panche, A. N., Diwan, A. D. and Chandra, S. R. 2016. Flavonoids: an overview. *J. Nutr. Sci.* **5**: e47 [CrossRef]. [Medline]
35. Prasath, G. S., Sundaram, C. S. and Subramanian, S. P. 2013. Fisetin averts oxidative stress in pancreatic tissues of streptozotocin-induced diabetic rats. *Endocrine* **44**: 359–368. [Medline] [CrossRef]
36. Puttachary, S., Sharma, S. and Thippeswamy, T. 2015. Seizure-induced oxidative stress in temporal lobe epilepsy: Review article. *BioMed Res. Int.* **13**: 334–339.
37. Ramnath, V., Rekha, P. S. and Sujatha, K. S. 2008. Amelioration of heat stress induced disturbances of antioxidant defense system in chicken by brahma rasayana. *Evid. Based Complement. Alternat. Med.* **5**: 77–84. [Medline] [CrossRef]
38. Robertson, G. W. and Maxwell, M. H. 1993. Importance of optimal mixtures of EDTA anticoagulant:blood for the preparation of well-stained avian blood smears. *Br. Poult. Sci.* **34**: 615–617. [Medline] [CrossRef]
39. Sharma, B., Rai, D. K., Rai, P. K., Rizvi, S. I. and Watal, G. 2010. Determination of erythrocyte fragility as a marker of pesticide-induced membrane oxidative damage. *Methods Mol. Biol.* **594**: 123–128. [Medline] [CrossRef]
40. Sinkalu, V. O., Ayo, J. O. and Abimbola, A. A. 2014. Effects of melatonin on cloacal temperature and erythrocyte osmotic fragility in layer hens during the hot-dry season. *J. Appl. Anim. Res.* **43**: 52–60. [CrossRef]
41. Snedecor, G. W. and Cochran, W. G. 1994. Statistical Methods, Oxford and IBH Publishing Corp., Calcutta.
42. Surai, F. A. 2015. Antioxidant systems in poultry biology: Superoxide dismutase. *J. Anim. Res. Nutr.* **1**: 1–8.
43. Tao, X. and Xin, H. 2003. Acute synergistic effect of air temperature, humidity and velocity on homeostasis of market-size broilers. *Trans. ASAE* **46**: 491–497.
44. Tedesco, I., Carbone, V., Spagnuolo, C., Minasi, P. and Russo, G. L. 2015. Identification and quantification of flavonoids from two southern Italian cultivars of *Allium cepa* L., Tropea (Red Onion) and Montoro (Copper Onion), and their capacity to protect human erythrocytes from oxidative stress. *J. Agric. Food Chem.* **63**: 5229–5238. [Medline] [CrossRef]
45. Wang, W. C., Yan, F. F., Hu, J. Y., Amen, O. A. and Cheng, H. W. 2018. Supplementation of Bacillus subtilis based probiotic reduces heat stress-related behaviors and inflammatory response in broiler chickens. *J. Anim. Sci.* **96**: 1654–1666. [Medline]
46. Welbourn, E. M., Wilson, M. T., Yusof, A., Metodiev, M. V. and Cooper, C. E. 2017. The mechanism of formation, structure and physiological relevance of covalent hemoglobin attachment to the erythrocyte membrane. *Free Radic. Biol. Med.* **103**: 95–106. [Medline] [CrossRef]
47. Zheng, X. C., Wu, Q. J., Song, Z. H., Zhang, H., Zhang, J. F., Zhang, L. L., Zhang, T. Y., Wang, C. and Wang, T. 2016. Effects of Oridonin on growth performance and oxidative stress in broilers challenged with lipopolysaccharide. *Poult. Sci.* **95**: 2281–2289. [Medline] [CrossRef]