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Evaluation of changes in tonic immobility, vigilance, malondialdehyde, and superoxide dismutase in broiler chickens administered fisetin and probiotic (*Saccharomyces cerevisiae*) and exposed to heat stress

Victory Osirimade Sumanu*, Tagang Aluwong, Joseph Olusegun Ayo, Ngozi Ejum Ogbuagu

Department of Veterinary Physiology, Ahmadu Bello University, Zaria, Nigeria

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ABSTRACT

The study evaluated changes in tonic immobility (TI), vigilance behavior, malondialdehyde (MDA), and superoxide dismutase (SOD) in broiler chickens administered fisetin and probiotic. Sixty Arbor Acre breed of broiler chickens at day old, allotted into 4 groups of 15 birds each, were used. Group I (control) was given only sterile water; group II, fisetin (5 mg/kg); group III, probiotic (4.125×10^6 cfu/100 mL); and group IV, fisetin + probiotic. At weeks 3, 4, and 5, thermal environment parameters, TI and vigilance behavior, were recorded each day at 07:00 h, 13:00 h, and 18:00 h. The vigilance at self-righting during each TI test was observed in broiler chickens and ranked as (1) fearlessness, (2) slightly fearful, and (3) fearfulness. MDA concentration and SOD activity in breast muscle were determined using a spectrophotometer. Temperature-humidity index (25.55-35.30) was outside the thermoneutral zone (20.8) for broiler chickens above 3 weeks of age. Week 3 had the longest ($P < 0.05$) duration of TI in the controls, but the shortest ($P < 0.05$) duration was recorded in the fisetin + probiotic group. At week 4, the shortest ($P < 0.05$) TI duration was recorded in the probiotic-supplemented group, while the longest ($P < 0.05$) TI was obtained in the control group. The shortest ($P < 0.05$) TI duration was recorded at week 5 in the probiotic and fisetin + probiotic groups, compared with that of the controls. The highest ($P < 0.05$) TI duration was recorded at 13:00 h in the controls, but the shortest ($P < 0.05$) was obtained at 18:00 h in the probiotic and fisetin + probiotic groups. At week 3, the vigilance behavior ranking recorded in the probiotic, fisetin + probiotic, and fisetin broiler chickens were lower ($P < 0.05$) than that obtained in the control group, which was the highest. At week 4, the highest ($P < 0.05$) vigilance behavior ranking was obtained in the control group. At week 5, the lowest ($P < 0.05$) vigilance behavior ranking was recorded in the probiotic-supplemented group, but the highest ($P < 0.05$) value was obtained in the control group. At 7:00, 13:00, and 18:00 h, there was no significant difference ($P > 0.05$) in the vigilance behavior ranking in the control, fisetin, probiotic, and fisetin + probiotic groups of broiler chickens. The MDA concentration in broiler chickens administered probiotic, either alone or in combination with fisetin, was lower ($P < 0.05$) compared with that of the controls. The activity of SOD was higher ($P < 0.05$) in fisetin + probiotic broiler chickens than in the control group. Probiotic and/or fisetin ameliorated the behavioral stress response in the broiler chickens via oxidative stress mechanism, as evidenced by increase in SOD activity and decrease in MDA concentration in the breast muscle of the chickens. In conclusion, administration of probiotic, either alone or in combination with fisetin, decreased duration of TI and vigilance behavior ranking, increased SOD activity, and lowered MDA concentration in broiler chickens exposed to heat stress.

Introduction

Tonic immobility (TI) and vigilance behavior in birds are used to evaluate fear or stress level (Suzuki et al., 2013; Campo et al., 2014). The negative effects of fearfulness on performance may be linked to the

* Address for reprint requests and correspondence: Victory Osirimade Sumanu, DVM, MSc, Department of Veterinary Physiology, Ahmadu Bello University, Zaria, Nigeria. Tel: +2348172611742.
E-mail address: devicpet@gmail.com (V.O. Sumanu).

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hypothalamus-pituitary-adrenal axis response. Fear and stress are similar because fear elicitation, just like stress, involves adrenergic, dopaminergic, and cholinergic systems, involved in the stress response by poultry, especially in predator or prey-like interactions (Wilson et al., 2015). They are used to determine the adaptability of birds to thermal environmental stress factors (Wang et al., 2014; Egbuniwe et al., 2016, 2018). Fear reactions are produced in stress, especially during predator defense (Sinkalu et al., 2016). Increase in concentration of malondialdehyde (MDA), which is an index of lipoperoxidative damages caused by reactive oxygen species (ROS) to cell membranes, occurs in responses to stressful situations in broiler chickens, including heat stress (Peng et al., 2012; Chen et al., 2013; Egbuniwe et al., 2017). Antioxidant enzymes, such as superoxide dismutase (SOD), scavenge ROS in cells via a chain-reaction mechanism (Bai et al., 2016; Utama et al., 2016; Saleh et al., 2018), and protect the cell membranes from lipid peroxidation (Egbuniwe et al., 2016, 2017). Thus, the activity of SOD alters in stress situations (Bai et al., 2016). During heat stress, the body generates excessive ROS, resulting in oxidative stress (Lin et al., 2006; Egbuniwe et al., 2016). Broiler chickens may cope with certain levels of oxidative stress due to heat stress, but functions of body cells may be impaired by ROS-induced lipid peroxidation (Puttachary et al., 2015; Rajkumar et al., 2018). Antioxidants are molecules that slow or prevent the oxidation of other molecules (Tian et al., 2018). Antioxidants, including probiotic and fisetin (Aluwong et al., 2017; Youns et al., 2017), are widely used as ingredients in dietary supplements, and their supplementation provides beneficial effects against stress-induced tissue damage (Delles et al., 2014; Sun et al., 2015).

In general, probiotics improve intestinal microbial balance, provide protection against gut pathogens, and modulate immune system (Song et al., 2013) and may serve as promising alternative to antibiotics in broiler chicken production, especially in stressful situations (Jahromi et al., 2016; Sugiharto et al., 2017; Gatrell et al., 2018). The meat of broiler chickens raised using probiotics is wholesome and free of antibiotic residues that pose danger to human health (Sahil et al., 2017; Johnson et al., 2018). The emergence of new probiotic products has spurred the increasing number of research groups, exploring new probiotic strains and potential novel health functions of probiotics (Aluwong et al., 2013; Sahil et al., 2017). Probiotics are increasingly added as supplements to animal feeds such as those for ducks and broiler chickens (Sugiharto et al., 2017). Fisetin (3,3′,4′,7-tetrahydroxyflavone) is a flavonoid present in fruits and vegetables, including cucumbers, onions, strawberries, and grapes (Chen et al., 2015; Youns et al., 2017; Sandeep et al., 2018). It is a plant secondary metabolite that exhibits antioxidant, anti-inflammatory, antibacterial, antiviral, immunostimulating, antiaging, antiproliferative, anticarcinogenic, and neuroprotective activities (Khan et al., 2013; Prakash et al., 2013; Yong et al., 2016). It exerts its antioxidant effects by scavenging ROS and prevents or retards the oxidation of substrates by ROS (Sandeep et al., 2018). It enhances behavioral responses, attenuates inflammatory, oxidative, and toxic responses in cells (Chuang et al., 2014; Yuan et al., 2015). It is a chemopreventive or chemotherapeutic agent in cancer. Fisetin is a promising and potent antioxidant (Youns et al., 2017; Sundarraj et al., 2018), which may be beneficial against heat stress.

The aim of the study was to evaluate changes in TI, vigilance behavior, and concentration of MDA and activity of SOD in the breast muscle of broiler chickens administered fisetin and probiotic and exposed to heat stress.

Materials and methods

Experimental site, location, and period

The experiment was conducted on broiler chickens at the Department of Veterinary Physiology, Ahmadu Bello University, Zaria (11° 10′N, 07° 38′E), located in the Northern Guinea Savannah zone of Nigeria. The broiler chickens were reared under natural, thermal microenvironment conditions, without artificial control. They were, thus, exposed to natural conditions of fluctuating ambient temperature (AT) and relative humidity (RH). The study was carried out during the early rainy season from June to July, 2017, characterized by high AT and high RH (Ayo et al., 2011; Dzenda et al., 2013).

Flock management

Apparently, healthy day-old broiler chicks (Arbor Acres, n = 60), composed of both sexes and purchased from a reputable hatchery, Zartech Farms, Ibadan (07° 22′N, 03° 58′E), Nigeria, served as subjects. They were kept in a poultry pen under an intensive management system, and the floor was provided with wood shavings. The poultry pen comprised concrete floor, cement block, and aluminum roofing with cardboard ceiling. The pen dimension was 8.4 m × 5.6 m × 1.91 m and the stocking density of the broiler chicks was 15 birds/m^2 (Aluwong et al., 2017). The broiler chicks were given access to broiler starter (day 0-28) and broiler finisher (day 29-42), produced by Hybrid Feeds, Kaduna, Nigeria, and water *ad libitum*. The feed composition and proximate analysis of the feeds (Nutrition Laboratory, Mark Farms, Osara, Nigeria) are shown in Table 1. The broiler chicks were exposed to 24-h duration of photoperiod and divided by simple randomization into 4 groups of 15 chicks each: group I, control; group II, fisetin; group III, probiotic; and group IV, combination of fisetin (Sigma Inc., New Orleans, Louisiana, USA) and probiotic (*Saccharomyces cerevisiae*) (Montajat Pharmaceuticals, Bioscience Division, Dammam, Saudi Arabia). Each bird was identified by the leg using a masking tape for proper recordings. Biosecurity was ensured by providing foot wears and protective clothing for all the assistants, and the pen was inaccessible to nonessential persons, animals, or other birds.

Experimental design

The 60 experimental birds were weighed individually once per week by a Mettler Toledo® Digital Precision Weighing Balance, with 0.01 g sensitivity (Model MT-500D, Columbus, Ohio, USA). Group I was administered distilled water *ad libitum*; group II, fisetin at a dose of 5 mg/kg (Khan et al., 2013); group III, probiotic (*Saccharomyces cerevisiae*) at a dose of 4.125 × 10^6 cfu/100 mL (Aluwong et al., 2017), based on the method of competitive exclusion; and group IV was administered fisetin (5 mg/kg) and probiotic (*Saccharomyces cerevisiae*). All administrations were performed orally by 1-mL tuberculin syringe for the first 7 days of life (Aluwong et al., 2017).

Table 1
Composition and proximate analysis of broiler chicken diets

Feed composition	Starter	Finisher
Ingredients (%)		
Crude protein	22.00	19.50
Fat	5.10	3.80
Crude fiber	4.30	3.00
Calcium	1.20	1.20
Available phosphorus	0.45	0.44
Methionine	0.56	0.50
Lysine	1.30	1.20
Metabolizable energy (Kcal/kg)	3000.00	3100.00
Proximate analysis		
Crude protein (%)	22.00	21.00
Fat (%)	7.90	6.80
Crude fiber (%)	4.30	3.00
Calcium (%)	2.00	2.00
Available phosphorus (%)	0.80	0.70
Methionine (%)	0.56	0.50
Lysine (%)	1.20	1.20
Metabolizable energy (Kcal/kg)	2900.00	2980.00

Experimental procedures

Thermal environment data from the study area

The dry-bulb temperature (DBT) and wet-bulb temperature (WBT) were measured at the experimental site by a wet- and dry-bulb thermometer (Brannan® Sapphire Instruments, New Delhi, India). From the DBT and WBT values, the temperature-humidity index (THI) was calculated (Tao and Xin, 2003):

$$\text{THI} = 0.85(\text{Tdb}) + 0.15(\text{Twb}),$$

where THI = temperature-humidity index for broiler chickens, Tdb = dry-bulb temperature, and Twb = wet-bulb temperature. The values were recorded inside the poultry pen on each experimental day.

Measurement of tonic immobility responses

The TI was induced at weeks 3, 4, and 5 (Sinkalu et al., 2016) in 60 broiler chickens (15 per group) at 07:00 h, 13:00 h, and 18:00 h. Briefly, at each hour of measurement, each bird was placed on its back on an improvised cradle, and its breast was pressed gently for 15 s. The cradle, measuring 50 × 40 × 25 cm, was made using Dunlop foam and covered with cloth (Wang et al., 2014). The cradle was scooped in the center to allow each broiler chicken acquire dorsal recumbency. Any broiler chicken that righted itself within 2 s was caught gently again, and the procedure was repeated. This was performed up to 5 times. If TI induction failed after 5 attempts, then TI duration was recorded as 0 s. If the broiler chicken did not acquire standing position after 600 s, which was the permissible maximum duration of TI, then the induction process was interrupted and TI duration was recorded as 600 s (Egbuniwe et al., 2016).

Measurement of vigilance behavior ranking

The vigilance behavior exhibited at self-righting during each TI test was recorded and ranked at weeks 3, 4, and 5 in 60 broiler chickens (15 per group) at 07:00 h, 13:00 h, and 18:00 h (Sinkalu et al., 2016) each day of the recording. Briefly, the ranking was performed on a scale of 1 to 3; with 1 representing fearlessness; 2, slightly fearful; and 3, fearfulness. The vigilance behavior ranking of each broiler chicken at self-righting was carried out as follows:

1 If self-righting process was quick and spontaneous (≤180 s with no vocalization),
2 If the process was slow with a short vigilance period (> 180 s/or < 600 s with peeping and vocalization), and
3 If vigilance process was very slow and with a prolonged period (600 s).

Biochemical assay

Malondialdehyde concentration

Breast muscle tissue (5 g) was collected at week 5 of the experiment. Five broiler chickens, randomly selected per group and fasted overnight, were stunned and slaughtered by jugular venesection. Part of the breast muscle (5 g) of each broiler chicken was severed and homogenized in a test tube. Briefly, in a test tube, 2 mL of 15% trichloroacetic acid, 2 mL of thiobarbituric acid (TBA), and 100 μL of the homogenate of breast muscle tissue were added. After incubating the mixture at 80°C for 30 minutes in a water bath, it was cooled for 10 minutes and centrifuged at 1600 × g for 10 minutes as modified by Atawodi et al. (2011). The absorbance of the supernatant collected was measured at 535 nm using a spectrophotometer (23A Perlong Medical Equipment Co., Ltd., Nanjing, China). The concentration of TBA expressed in nmol/L protein was calculated:

$$\text{Concentration(nmol/L protein)} = \frac{\text{Absorbance of sample}}{1.5 \times 10^{-5} \times \text{protein concentration(mg)}}$$

Superoxide dismutase activity

As mentioned previously, breast muscle tissue was collected at week 5 of the experiment. Five broiler chickens were randomly selected per group and fasted. They were stunned and slaughtered via jugular venesection, and part of the breast muscle tissue (5 g) was placed in a test tube and homogenized. Briefly, SOD activity was determined using 100 μL of the homogenate of the breast muscle tissue. Exactly 2.5 mL of 50 mmol/L carbonate buffer and 0.3 mL of 0.3 mmol/L epinephrine were added to the homogenate. The reference mixture comprised 2.5 mL of 50 mmol/L carbonate buffer, 0.3 mL of 0.3 mmol/L epinephrine, and 0.2 mL of distilled water. The absorbance of the mixtures was measured for 150 seconds at 480 nm using the method modified by Atawodi et al. (2011).

$$\text{Increase in absorbance per minute} = (A_2 - A_1)/2.5$$

$$\text{Percentage (\%) inhibition} = 100 - (\text{increase in absorbance for sample/increase in absorbance of blank}) \times 100$$

Where A_1 = first absorbance in the reaction linear range and A_2 = second absorbance in the reaction linear range.

Statistical analyses

The data obtained were expressed as mean ± standard error of the mean (mean ± SEM). Thermal environment data from the study area were subjected to 1-way analysis of variance, followed by Tukey's multiple comparison post hoc test to compare differences between the means. The behavioral parameters (TI and vigilance behavior ranking) were analyzed using Kruskal-Wallis analysis of variance. The analyses were performed using GraphPad Prism 5.03 for windows (GraphPad Software, San Diego, California, USA). Values of $P < 0.05$ were considered significant.

Results

Thermal environment data from the study area

During the study period, the mean DBT recorded at weeks 3 (30.67 ± 0.67°C), 4 (30.62 ± 0.66°C) and 5 (30.43 ± 0.56°C) did not differ significantly. The overall DBT for the study period ranged from 26.00°C-36.00°C, with a mean of 30.57 ± 0.36°C. The RH during the study period ranged from 49.00%-93.00%, with a mean of 79.22 ± 1.26 %. Week 3 had the least mean RH of 74.67 ± 2.15 %, which was significantly ($P < 0.05$) lower than those for weeks 4 (81.43 ± 2.41 %) and 5 (81.57 ± 1.64 %). The mean THI for the study period was 30.10 ± 0.34 and it ranged from 25.55-35.30. There was no significant ($P > 0.05$) difference in THI between the weeks of the study period (Table 2).

Effects of age, probiotic, and fisetin on diurnal variations in duration of tonic immobility in Arbor Acre broiler chickens

The TI duration in the control group relatively increased as the age of the broiler chickens increased at weeks 3, 4, and 5 (135.70 ± 13.65 seconds, 162.20 ± 20.33 seconds and 171.10 ± 30.41 seconds,

Table 2
Thermal environment data from the study area

Weeks	DBT (°C)	RH (%)	THI
3	30.67 ± 0.67 (26.00–36.00)	74.67 ± 2.15 (49.00–93.00)	30.09 ± 0.64 (25.55–35.10)
4	30.62 ± 0.66 (26.00–36.00)	81.43 ± 2.41 (61.00–93.00)	30.20 ± 0.62 (25.70–35.30)
5	30.43 ± 0.56 (26.00–34.00)	81.57 ± 1.64 (67.00–93.00)	30.01 ± 0.54 (25.70–33.40)
Overall mean ± SEM	30.57 ± 0.36 (26.00–36.00)	79.22 ± 1.26 (49.00–93.00)	30.10 ± 0.34 (25.55–35.30)

RH, relative humidity; THI, temperature-humidity index; DBT, dry-bulb temperature.
n = 15; values in parenthesis are minimum-maximum.

respectively), but the difference in the values was insignificant. At week 3, the longest duration of TI was recorded in the controls, whereas the shortest ($P < 0.05$) duration was recorded in the fisetin + probiotic group (94.27 ± 10.97 seconds). At week 4, the shortest TI duration was recorded in the probiotic-supplemented group (68.62 ± 12.34 seconds), whereas the longest TI (162.20 ± 20.33 seconds) was obtained in the control group. At week 5, the shortest duration was recorded both in the probiotic and fisetin + probiotic groups (67.27 ± 13.97 seconds and 81.86 ± 7.12 seconds, respectively), and the values were lower than that recorded in controls. At 7:00 h, the longest ($P < 0.05$) diurnal TI duration (166.00 ± 15.50 seconds) was recorded in the controls, whereas the shortest ($P < 0.05$) diurnal TI duration (43.89 ± 9.20 seconds) was obtained in the probiotic-administered group. At 13:00 h, the controls had the longest diurnal duration of TI (226.10 ± 20.12 seconds), whereas the shortest ($P < 0.05$) and the least TI (58.34 ± 11.78 seconds) was recorded in the probiotic-administered broiler chickens. At 18:00 h, the longest diurnal TI duration was recorded in the control group of broiler chickens (200.11 ± 5.50 seconds), but the shortest diurnal TI duration (54.33 ± 8.42 s) was obtained in the broiler chickens administered probiotic. The TI duration was generally higher at 07:00 h compared to the value obtained at 13:00 h or 18:00 h, and at week 3, the diurnal difference in TI durations was significant ($P < 0.05$) only in probiotic broiler chickens. At week 4, TI duration was significantly ($P < 0.05$) lower at 07:00 h compared with that of 18:00 h in controls, and in probiotic broiler chickens, TI duration was longer ($P < 0.05$) than the value recorded at either 13:00 h or 18:00 h (Table 3). At week 5, TI duration was lower predominantly at 18:00 h than the value recorded at 13:00 h; an exception occurred in probiotic broiler chickens, where the TI at 07:00 h was also lower ($P < 0.05$) than that obtained at 18:00 h.

Effects of age, probiotic, and fisetin on diurnal fluctuations in vigilance behavior of Arbor Acre broiler chickens

At week 3, the vigilance behavior ranking recorded in the probiotic (1.35 ± 0.04), fisetin + probiotic (1.40 ± 0.06), and fisetin (1.49 ± 0.07) broiler chickens were lower ($P < 0.05$) than that (1.78 ± 0.06) recorded in the control group, which was the highest. At week 4, the highest ($P < 0.05$) vigilance behavior ranking was obtained in the control group (1.94 ± 0.03), whereas the lowest ($P < 0.05$) was recorded in the probiotic group (1.31 ± 0.02). At week 5, the lowest ($P < 0.05$) vigilance behavior ranking (1.15 ± 1.40) was obtained in the probiotic-supplemented group, but the highest ($P < 0.05$) value (2.01 ± 0.19) was recorded in the control group. At 7:00, 13:00, and 18:00 h, there was no significant difference in the vigilance behavior ranking recorded in the control, fisetin, probiotic, and fisetin + probiotic broiler chickens (Table 4).

Changes in malondialdehyde concentration and superoxide dismutase activity in the breast muscle of broiler chickens

The MDA concentrations at week 5 in the breast muscle of broiler chickens treated with probiotic (2.86 ± 0.19 nmol/L, $P < 0.001$), fisetin (6.61 ± 0.06, $P < 0.05$), and fisetin + probiotic (3.67 ± 0.71, $P < 0.05$) were lower than that of the control group (10.92 ± 1.03 nmol/L). In fisetin-administered group, MDA concentration was higher ($P < 0.05$) than the value obtained in the fisetin + probiotic or probiotic group (Figure 1). The highest SOD activity was recorded in fisetin + probiotic group (7.16 ± 0.07 IU/L), and it was higher ($P < 0.05$) compared with that recorded in the control group (4.67 ± 0.47 IU/L). The SOD activities in probiotic (6.28 ± 0.38 IU/L) and fisetin (5.92 ± 0.50 IU/L) groups were significantly ($P < 0.05$) higher when compared with that of the controls (4.67 ± 0.47 IU/L) (Figure 2).

Discussion

The THI revealed the impact of DBT and RH on animals (Tao and Xin, 2003). In the present study, the overall mean THI (30.10 ± 0.34), with a range of 25.55-35.30, demonstrates that the broiler chickens were exposed to heat stress (Tao and Xin, 2003). Sinkalu et al. (2015) show that THI values greater than 20.8 induce heat stress in broiler chickens older than 3 weeks. The heat stress

Table 3
Effects of age, probiotic, and fisetin administration on diurnal variations in tonic immobility duration in Arbor Acre broiler chickens (seconds)

A Age, Wk		Hour of the day, h	Group (s, mean ± SEM; n = 15)			
			Control	Fisetin	Probiotic	Fisetin + probiotic
3	3	7:00	123.80 ± 11.77	118.00 ± 7. 13	88.00 ± 8.90^{1}	98.20 ± 13.21
		13:00	162. 94 ± 24.70^{a}	111.00 ± 13.40^{a}	95.10 ± 9.53^{b}	73.60 ± 14.99^{b}
		18:00	120. 37 ± 11.42	101. 87 ± 11.33	108 .00± 9.42^{2}	111.00 ± 21.06
		Subtotal mean ± SEM	135.70 ± 13.65^{a}	110.30 ± 4.67^{a,i}	97.03 ± 5.85^{b,i}	94.27 ± 10.97^{b}
4	4	7:00	130. 50 ± 12. 38^{1}	134. 60 ± 11.98	93.20 ± 13.78^{1}	115.00 ± 27.09
		13:00	156.00 ± 25.00	130.70 ± 10.89	58.34 ± 11.78^{2}	78.27 ± 10.20
		18:00	200.11 ± 5.50^{a,2}	118.00 ± 27.00^{a}	54.33 ± 8.42^{b,2}	83. 43 ± 7.22^{b}
		Subtotal mean ± SEM	162.20 ± 20.33^{a}	127.80 ± 5.01^{b,ii}	68.62 ± 12.34^{b,ii}	92.21 ± 11.49^{b}
5	5	7:00	166.00 ± 15.50^{1}	134.70 ± 09.50^{1}	43.89 ± 9.20^{1}	79. 50 ± 11.37
		13:00	226.10 ± 20.12^{2}	126.11 ± 11.70	92.21 ± 9.50^{2}	95.20 ± 8.25^{1}
		18:00	121.11 ± 10.24^{3}	109.00 ± 14.21^{2}	65.70 ± 9.31^{3}	70.89 ± 0.90^{2}
		Subtotal mean ± SEM	171.10 ± 30.41^{a}	123.30 ± 7.55^{b,ii}	67.27 ± 13.97^{c,ii}	81.86 ± 7.12^{c}

SEM, standard error of the mean.
^{a,b,c,1,2,3} = Means with different superscript letters (within rows), numbers (within columns), and subtotal mean with superscript; ^{i,ii} (within the columns) are significantly different ($P < 0.05$).

Table 4
Effects of age, probiotic, and fisetin administration on diurnal fluctuations in vigilance behavior of Arbor Acre broiler chickens (mean ± SEM; n = 15)

Age, wk	Hour of the day, h	Group			
		Control	Fisetin	Probiotic	Fisetin + probiotic
3	7:00	1.89 ± 0.30	1.63 ± 0.21	1.42 ± 0.08	1.50 ± 0.11
	13:00	1.69 ± 0.11	1.45 ± 0.16	1.34 ± 0.11	1.30 ± 0.09
	18:00	1.75 ± 0.18	1.40 ± 0.23	1.30 ± 0.20	1.41 ± 0.20
	Subtotal mean ± SEM	1.78 ± 0.06^{a,i}	1.49 ± 0.07^{b}	1.35 ± 0.04^{c}	1.40 ± 0.06^{b,i}
4	7:00	1.95 ± 0.21	1.78 ± 0.14	1.31 ± 0.16	1.45 ± 0.09
	13:00	2.00 ± 0.12	1.49 ± 0.11	1.27 ± 0.08	1.39 ± 0.21
	18:00	1.88 ± 0.13	1.51 ± 0.23	1.34 ± 0.27	1.49 ± 0.31
	Subtotal mean ± SEM	1.94 ± 0.03^{a,ii}	1.59 ± 0.09^{b}	1.31 ± 0.02^{c}	1.44 ± 0.02^{c,i}
5	7:00	2.22 ± 0.31	1.75 ± 0.34	1.23 ± 0.12	1.43 ± 0.13
	13:00	1.93 ± 0.20	1.60 ± 0.12	1.13 ± 0.23	1.22 ± 0.11
	18:00	1.89 ± 0.11^{a}	1.67 ± 0.31^{a}	1.09 ± 0.23^{b}	1.20 ± 0.13^{b}
	Subtotal mean ± SEM	2.01 ± 0.19^{a,ii}	1.67 ± 0.12^{a}	1.15 ± 0.14^{b}	1.28 ± 0.10^{b,ii}

SEM, standard error of the mean.
^{a,b,c} = Means with different superscript letters (within rows) and subtotal mean with superscript; ^{i,ii} (within the columns) are significantly different ($P < 0.05$).

occurring during the early rainy season may adversely affect energy balance and the fitness of birds, which may further result in poor performance, immune suppression, and high mortality. Administration of antioxidants, such as fisetin and probiotic, could be beneficial during the thermally stressful season.

The duration of TI shows that the broiler chickens reared under heat-stressed conditions and administered fisetin and/or probiotic recorded the shortest TI duration when compared with the control group, which recorded the longest TI duration. The results are in agreement with the finding of Sinkalu et al. (2016), who demonstrated that exposure of broiler chickens to heat stress reduces welfare, as evidenced by longer duration of TI. Thus, heat stress by prolonging TI duration increased fear responses in the broiler chickens. The decreased fear responses in broiler chickens supplemented with fisetin and/or probiotic supports the previous finding that antioxidants ameliorate heat stress by scavenging the ROS, generated in excess (Zhang et al., 2018). The result agrees with the finding of Ghareeb et al. (2014) that antioxidant administration is beneficial in alleviating behavioral stress responses, induced by heat stress. The TI indicates fearfulness and its duration is an index of stress level in broiler chickens (Ghareeb et al., 2014). The finding of relatively longer ($P > 0.05$) duration of TI at week 4 than week 3 in the control broiler chickens, apparently, shows that the fear response rose with increase in age in the control group, not treated with antioxidant, and that broiler chicken production during heat-stressed conditions may compromise welfare and health.

Heat generation increases as broiler chickens grow as a result of increase in their metabolic rate, which further increases the heat load on the birds (Sugiharto et al., 2017). The administration of antioxidants, fisetin, and probiotic, apparently, ameliorated the increased heat load imposed on the broiler chickens, and decreased their fear responses. The result shows, for the first time, that fisetin and/or probiotic administration to broiler chickens subjected to heat-stressed conditions reduced TI duration and that both agents exhibited antioxidant activities by scavenging the excess ROS produced in heat stress. The general increase in TI duration during the afternoon hours of the day, especially at weeks 4 (in controls) and 5 (in all the groups) indicates that the broiler chickens were more fearful during the afternoon than morning hours of the day, especially in the controls. Diurnal variation in the behavioral stress responses as evidenced by increase in TI duration was more pronounced in probiotic-treated broiler chickens, indicating that probiotic considerably alleviated the behavioral stress response, compared with any other treatment group. The proximate mechanism underlying the probiotic-induced diurnal fluctuations requires further investigation. However, Egbuniwe et al. (2016) showed that the antioxidants, betaine, and/or ascorbic acid exerted a significant effect on TI responses in broiler chickens exposed to heat stress. Overall, probiotic broiler chickens showed the shortest duration of TI followed by fisetin + probiotic and finally fisetin groups. Thus, probiotic alleviated behavioral stress responses in broiler chickens better than fisetin or its combination with probiotic. This finding suggests that probiotic is a potent agent against behavioral stress responses in broiler chickens exposed to heat stress. The result shows that it is beneficial to pretreat the broiler chickens before exposure to stressful environmental conditions during the early rainy season. Furthermore, the results show that broiler chickens administered probiotic and fisetin + probiotic exhibited swift vigilance behavior as evidenced by a short duration of vigilance, when compared with the values obtained in the control and fisetin-administered broiler chickens. The administration of fisetin and/or probiotic to broiler chickens did not demonstrate diurnal variations in vigilance behavior ranking, which agrees with the finding of Sinkalu et al. (2016) that vigilance behavior does not show diurnal variation in broiler chickens administered with the antioxidant, melatonin.

The findings demonstrated, for the first time, that a probiotic considerably reduced fear, which is a behavioral stressor in broiler chickens. The findings are in agreement with that of Sinkalu et al. (2016), who shows that TI is a stressful and fearful experience that increases the degree of perturbation. Overall, the administration of fisetin and/or probiotic to broiler chickens induced boldness and confidence by evoking alertness and suppressing fear. The result agrees with the finding of Egbuniwe et al. (2016) that antioxidant administration reduces fear in broiler chickens. Skomorucha et al. (2010) reported that high AT and increase in age may compromise the defensive mechanism of broiler chickens exposed to heat stress.

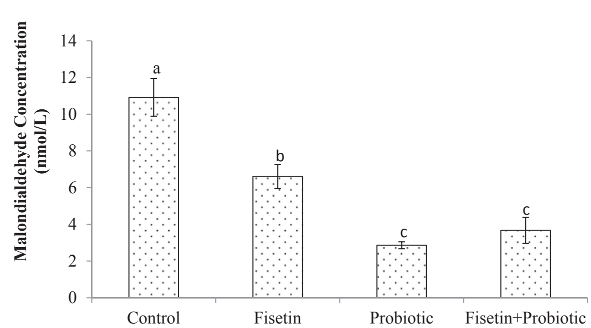

Figure 1. Malondialdehyde concentration in the skeletal muscle of broiler chickens during the study period; ^{a,b,c} = means with different superscript letters are significantly ($P < 0.05$) different, n = 15.

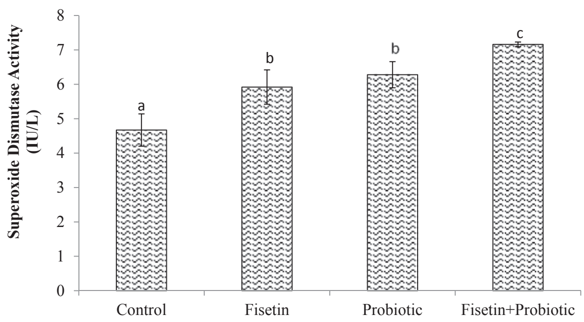

Figure 2. Superoxide dismutase activities in the skeletal muscle of broiler chickens during the study period; [a,b,c] = means with different superscript letters are significantly ($P < 0.05$) different, n = 15.

Thus, the exposure of broiler chickens to high AT may increase TI duration, as recorded during the hot hour of the day (13:00 h) in the present study, especially at week 5 in all the broiler chickens. The increase in metabolic rate associated with heat production in broiler chickens was, apparently, responsible for the increased TI duration as the age of the broiler chickens increased from week 4 to 5. The result shows that fisetin and/or probiotic administration induced calming effect and increased the alertness of the broiler chickens, which may enhance performance and productivity. The decrease in vigilance of broiler chickens recorded in the present study was an evidence that the birds were subjected to stressful conditions. Therefore, the administration of fisetin and/or probiotic may alleviate the risk of adverse effects of behavioral stress responses in broiler chickens and, consequently, improve performance.

The result showed that administration of fisetin and/or probiotic decreased MDA concentration in the broiler chickens. The MDA concentration in the body indirectly reflects the lipid peroxidation, indicating the level of antioxidant protection as ROS generation increases (Ismail et al., 2015; Egbuniwe et al., 2017). The finding further demonstrates that fisetin and/or probiotic administration protected against the negative effects of oxidative stress in heat-stressed broiler chickens. The result was in agreement with the finding of Bai et al. (2016) that the administration of probiotics is beneficial by decreasing the detrimental effects of excess generation of ROS on broiler chickens. The finding that both fisetin and probiotic, apparently, reduced the effects of oxidative damage in the breast muscle of broiler chickens may be due to the ability of the 2 antioxidants to scavenge ROS, generated in excess during heat stress (Bai et al., 2016; Sandeep et al., 2018). Overall, the study shows that fisetin and probiotic were beneficial in reducing oxidative stress in broiler chickens exposed to heat stress. Excessive ROS generation in the body leads to oxidative stress and damage to proteins, nucleic acids, and other biological macromolecules. Therefore, increased MDA concentration is an indication of tissue damage (Delles et al., 2014; Akbarian et al., 2016). The result suggests that lipid peroxidation, occurring in heat-stressed broiler chickens, may be ameliorated by the administration of fisetin and/or probiotic. Furthermore, the result shows that probiotic treatment ameliorated the negative effects of oxidative stress and increased the activity of the antioxidant enzyme, SOD. Thus, probiotic may inhibit efficiently the excessive ROS generation that increases cell damage in broiler chickens exposed to heat stress. The finding of the study is in agreement with that of Bai et al. (2016), who reported that the administration of probiotic to broiler chickens reduces MDA concentration, produced by the liver and, subsequently, increases the activity of SOD in the liver. The results obtained from the present study agree with that of Egbuniwe et al. (2016), who demonstrated that SOD activity is higher in broiler chickens subjected to heat stress and treated with betaine and ascorbic acid than in the control group. The endogenous antioxidant defense system in broiler chickens relies on other external antioxidant sources (Zhang et al., 2018); therefore, fisetin and/or probiotics, which are natural sources of antioxidants, prevented apparently oxidative stress and enhanced the total antioxidant capacity of the heat-stressed broiler chickens.

Conclusion

In conclusion, administration of fisetin, either alone or in combination with probiotic, decreased the duration of TI and vigilance behavior ranking, increased SOD activity, and lowered MDA concentration in heat-stressed broiler chickens.

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Ethical considerations

The ethical approval was granted by the Ethical Committee on Animal Use and Care, Ahmadu Bello University, Zaria; with reference number, ABUCAUC/2018/021.

Conflict of interest

The authors declare no conflict of interest.

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