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The association between avian physiology and meat quality

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The association between avian physiology and meat quality

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Editorial: The association between avian physiology and meat quality

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Editorial on the Research Topic
The association between avian physiology and meat quality

Introduction

World population is projected to reach 9 billion by the year 2050. With restricted availability of natural resources, food shortage, particularly of protein, has become a global concern (FAO, 2009). A variety of food protein alternatives have been recently developed and launched to the market. However, during the next decades, a rise in poultry meat demand has been projected worldwide (USDA, 2023). This is due to its excellent nutritional value, high production efficiency, affordable price for low-income families and lesser greenhouse gas emissions than other livestocks. The poultry meat produced must be of high quality to ensure food security and minimize food waste.

It is widely accepted that meat quality is tightly linked with animal physiology (Joo et al., 2013; Terlouw et al., 2021). Although several studies have previously documented such a fundamental link, several factors such as the dramatic change in climate conditions due to global warming and the occurrence of muscle abnormalities may exert stress to avian species in different ways than previously experienced. On the other hand, the increasing availability of newly-developed farm management tools and feed additives can help to maintain optimal physiological state and meat quality even in such challenging conditions. The ultimate goal of this Research Topic: *The Association between Avian Physiology and Meat Quality* was to provide comprehensive updates on such aspects and extend our understading to aid in assuring a sustainable production of high-quality poultry meat.

As a consequence of selection for productive traits, commercial meat-type chickens and turkeys exhibit rapid growth and relevant accretion of muscle mass, particularly of the breast muscle. In this Research Topic, Lee et al. compared the histological characteristics of myofibers and muscle bundles in *pectoralis major* and *gastrocnemius* muscles of commercial broilers and layers. The clear histological differences between the two breeds suggested that the greater muscle mass of fast-growing meat-type chickens could be a result of both myofiber hyperplasia and hypertrophy. Using male Japanese quails as a model, Kim et al.

demonstrated the role of myostatin (*MSTN*) gene on histological characteristics and composition of breast muscle. A significant increase in body weight and muscle mass was observed in the *MSTN* knock-out quails with no differences in meat quality indices compared to the wild-type. A slight but significant increased proportion of glycolytic fast-twich (type IIB) fibers was observed in the deep region of the breast collected from the knock-out birds without any impacts on *postmortem* pH of the breast meat. This research report suggested the potential application of *MSTN* mutation for enhancing muscle mass of the birds while determining no significant effects on meat quality.

On the other hand, breeding selection focusing mainly on production performance appeared to exert a negative impact on poultry meat quality. The massive muscling, focusing on breast muscles, of fast-growing birds appeared to outgrow their life support systems, particularly vascularization, leading to development of growth-related myopathies. As reported in an opinion article of Malila, growing evidence has indicated an association between growth-related myopathies and *in-vivo* oxidative stress, which in its turn can impair meat quality because of lipids and proteins oxidation. Several feed additives having antioxidant activities have been examined for improving meat quality. Herein, the effects of tannic acid were addressed by Choi et al.. Results showed that the dietary supplementation of tannic acid up to 2 g/kg appeared to negatively affect overall growth performance, feed efficiency, bone health and fat accumulation. However, tannic acid supplementation in starter/grower phases enhanced gut health and nutrient transportation, and increased nutrient digestibility in the finisher phase. These findings not only supported the benefits of tannic acid on gut health but also suggested the dosage and the optimal duration of the supplementation.

In terms of flavor, the meat belonging to fast-growing birds appeared to contain a lower amount of inosine monophosphate (IMP), the most important umami compound in the meat. To define biological pathways associated with IMP deposition in chicken meat, Yu et al. compared the transcriptome profiles of chicken muscles showing relatively high or low IMP content. The authors investigated the effects of muscle tissue (breast vs leg), gender (hen vs rooster), production management (cage vs free-range) and growth rates (fast vs slow). Potential candidate genes regulating IMP muscle deposition were identified for further breeding program.

Interestingly, Zhang et al. reported a connection between knob size, one of the important consumer purchasing criteria in China, and bone protrusion size in Yangzhou geese. Despite no differences in production performance, leg muscle of geese with large knob exhibited a greater insoluble collagen and expressible water content along with a higher growth hormone levels than those of small-knob geese.

A large proportion of the articles in this Research Topic focused on the impact of thermal stress as climate change is one of the most urgent challenges affecting all living organisms. Cartoni Mancinelli et al. investigated the relationships among behavior, physiological conditions, and meat quality of commercial broilers exposed to chronic heat stress. They observed a two-stage behavioral response when the environmental temperature reached 25°C and over 27°C. The modified behaviors were associated with altered blood parameters reflecting an oxidative and inflammatory state that affected breast meat quality. Such findings offered crucial insights for identifing thermal discomfort among broilers as well as to better understand the impact of heat stress on meat quality. Reed et al. examined differential expression patterns of non-coding microRNAs (miRNAs) in turkey muscle stem cells (SCs) to define an in-depth biological response to thermal challenges. Potential target genes of differentially expressed miRNAs were also predicted to underline the potential consequences of the miRNA differential expression. Overall, their findings suggested a significant impact of thermal challenges on SCs proliferation and differentiation among the fast-growing birds. The crucial roles of SCs in muscle growth, development, repair and subsequent meat quality of poultry are further emphasized in an opinion article by Velleman. It was suggested that the assement of SCs biological activity upon thermal challenges should be included in the poultry selection strategies.

Nutritional strategies for heat stress alleviation were also extensively investigated. Herein, Brugaletta et al. studied the response of commercial broilers to arginine supplementation upon an exposure to cyclic thermal stress. Although arginine supplementation at the tested dosage did not significantly enhanced the productive performance of heat-stressed broilers, the metabolomic analysis unveiled the potential role of arginine in counterbalancing the adverse effects of such stressor on energy homeostasis mechanisms through increasing creatine levels and regulating AMP levels. An increase in digestion and absorption of dietary amino acids was also hypothesized, suggesting the additional benefits of arginine supplementation on improving intestinal health and function under heat stress. Moreover, Señas-Cuesta et al. addressed the effects of providing *Lippia origanoides* essential oils containing herbal betaine to commercial broilers subjected to cyclic thermal stress. The dietary inclusion of such oils had some beneficial effects on body weight gain, intestinal conditions and bone quality compared to heat-stressed chickens.

Overall, the articles published in this Research Topic provide insightful updates and extend our comprehension regarding the link between avian physiology and meat quality to ensure the future production of high-quality poultry meat.

Author contributions

YM: Conceptualization, Writing–original draft, Writing–review and editing. MZ: Conceptualization, Writing–original draft, Writing–review and editing. FS: Conceptualization, Writing–original draft, Writing–review and editing. CO: Conceptualization, Writing–original draft, Writing–review and editing. SV: Conceptualization, Writing–original draft, Writing–review and editing.

Conflict of interest

The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

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Effects of tannic acid supplementation on growth performance, gut health, and meat production and quality of broiler chickens raised in floor pens for 42 days

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A study was conducted to investigate the effects of tannic acid (TA) supplementation on growth performance, gut health, antioxidant capacity, gut microbiota, and meat yield and quality in broilers raised for 42 days. A total of 700 one-day-old male broiler chickens (Cobb500) were allocated into 5 treatments with 7 replicates of 20 birds per pen. There were five treatments: 1) tannic acid 0 (TA0: basal diet without TA); 2) tannic acid 0.25 (TA0.25: basal diet+0.25 g/kg TA); 3) tannic acid 0.5 (TA0.5: basal diet+0.5 g/kg TA); 4) tannic acid 1 (TA1: basal diet+1 g/kg TA); and 5) tannic acid 2 (TA2: basal diet+2 g/kg TA). The dietary phases included starter (D 0 to 18; crumble feed), grower (D 18 to 28; pellet feed), and finisher (D 28 to 42; pellet feed). On D 18, the supplementation of TA linearly reduced body weight (BW) and average daily feed intake (ADFI) ($p < 0.05$), and on D 28, the supplementation of TA linearly reduced BW, average daily gain (ADG), and feed conversion ratio (FCR) ($p < 0.05$). Relative mRNA expression of genes related to mucin production (*MUC2*), tight junction proteins (*CLDN2* and *JAM2*), and nutrient transporters (*B0AT1* and *SGLT1*) was linearly increased by the supplementation of TA ($p < 0.05$). The supplementation of TA tended to linearly increase the relative abundance of the family Enterobacteriaceae ($p = 0.08$) and quadratically increased the relative abundance of the families Lachnospiraceae and Ruminococcaceae in the cecal microbial communities ($p < 0.05$). On D 36, the ratio of the phyla Firmicutes and Bacteroidetes was quadratically reduced by the supplementation of TA ($p < 0.05$). On D 42, bone mineral density and the lean to fat ratio were linearly decreased by the supplementation of TA ($p < 0.05$). On D 43, total chilled carcass weight was linearly reduced ($p < 0.05$), and proportion of leg weight was increased by supplementation of TA ($p < 0.05$). The supplementation of TA linearly reduced pH of the breast meat ($p < 0.05$) and linearly increased redness (a*) ($p < 0.05$). Although the supplementation of TA positively influenced gut health and gut microbiota in the starter/grower phases, it negatively affected overall growth performance, bone health, and meat production in broilers on D 42.

KEYWORDS

tannic acid, gut microbiota, gut health, floor pen, pelleting process, meat production, and fat accumulation

1 Introduction

In the past, antibiotic growth promoters (AGP) have been supplemented to broiler diets to enhance growth performance and gut health and to prevent diseases in broilers (Caly et al., 2015). Due to the public concerns about the spread of antibiotic resistant bacteria and their genes, there is a global movement to implement antibiotic-free production in the poultry industry (Haque et al., 2020). However, withdrawal of AGP without appropriate alternative strategies against bacterial infections could result in reduced production efficiency and broiler health and welfare issues by inducing severe microbial infection in broilers (Cervantes, 2015). It has been essential for the poultry industry to find appropriate bioactive compounds that can improve growth performance and gut health in antibiotic-free production. Diverse bioactive compounds, such as essential oils (Yang et al., 2021), amino acids (Teng et al., 2021), organic acids (Adil et al., 2010), plant extracts (Mogire et al., 2021; Yadav et al., 2022), and exogenous enzymes (Lu et al., 2020), have been studied and used as AGP alternatives in poultry production. Alternatives for AGP should be able to enhance growth performance, gut health, and meat production and quality of broilers and should be safe to the public and eco-friendly and cost-effective in broiler production (Yang et al., 2015).

Tannins, polyphenol compounds that can precipitate proteins, are considered as AGP alternatives in broiler production due to their effective antimicrobial, antioxidative, and anti-inflammatory effects in chickens (Choi and Kim, 2020). Tannic acid (TA), which is composed of 7–8 gallic acids molecules and one glucose molecule as a central core, is a standard of hydrolysable tannins and present in woods such as oak, chestnut, and acacia (Romani et al., 2006). Traditionally, TA was considered as an anti-nutritional factor due to its protein precipitation capacity, which can result in reduced nutrient digestibility and proteolytic activity in the liver of chickens (Marzo et al., 2002; Redondo et al., 2014). Many recent studies showed that the supplementation of TA at appropriate dosages (up to 2 g/kg) improved growth performance, gut health, immune system, and gut microbiota in broilers under non-challenge conditions and diverse challenging conditions (*Eimeria* spp., *Salmonella* spp., etc.) (Diaz Carrasco et al., 2018; Tonda et al., 2018; Ramah et al., 2020). Our previous study (Choi et al., 2022d) demonstrated that 0.5 g/kg TA increased activities of endogenous antioxidant enzymes, whereas higher than 1 g/kg TA exhibited antinutritional effects in broilers on D 21. However, it is still uncertain whether the supplementation of TA (up to 2 g/kg) would beneficially or negatively influence growth performance, gut health, antioxidant capacity, gut microbiota, and meat production and quality in broilers on D 42 (slaughter age). Therefore, this study was aimed to investigate the effects of TA supplementation (up to 2 g/kg TA) on growth performance, gut health, antioxidant capacity, gut microbiota, and meat yield and quality in broilers on D 42.

2 Materials and methods

2.1 Animals, diets, experimental design, and growth performance

The current study was reviewed and approved by the Institutional Animal Care and Use Committee at the University of Georgia, Athens, GA. A total of 700 one-day-old Cobb 500 male broiler chickens were randomly allotted to 5 treatments with 7 replicates of 20 birds per pen in a completely randomized design. The five treatments included 1) tannic acid 0 (TA0; basal diet without TA); 2) tannic acid 0.25 (TA0.25; basal diet + 0.25 g/kg TA); 3) tannic acid 0.5 (TA0.5; basal diet + 0.5 g/kg TA); 4) tannic acid 1 (TA1; basal diet + 1 g/kg TA); and 5) tannic acid 2 (TA2; basal diet + 2 g/kg TA). The TA (> 99% purity; Chinese natural gall nuts) was purchased from Sigma-Aldrich Co. (St Louis, MO) and was included in the entire experimental period. Before TA was added to the basal diets, TA was premixed with 10 kg basal diets. The experiment period was divided into starter (D 0 to 18; crumble feed), grower (D 18 to D 28; pellet feed), and finisher (D 28 to 42; pellet feed) phases, and the diets were formulated to meet or exceed recommendation levels according to the Cobb 500 nutrient requirement guide (2018) (Table 1). Conditioning temperature was 80°C for the feed pelleting process. On D 28 to 35, all diets included 0.3% titanium dioxide (Acros Organics, Morris Plains, NJ) as an inert marker to determine nutrient digestibility. Birds were raised in floor pens (width: 1.52 m, length: 1.22 m, height: 0.61 m) equipped with one feeder and three drinker nipples per pen, and birds had free access to water and feed. Temperature and light were controlled in accordance with the recommendation of the Cobb 500 broiler management guide (2018). Body weight (BW) and feed disappearance were measured on D 18, 28, and 42 to calculate average daily gain (ADG), average daily feed intake (ADFI), and feed conversion ratio (FCR).

2.2 Sampling, dual-energy X-ray absorptiometry, litter ammonia, and foot pad lesion

On D 18 and 36, one bird per pen was randomly selected and euthanized *via* cervical dislocation to collect samples of

TABLE 1 Ingredients and nutrient compositions of basal diets (As-fed basis).

Items	D 0 to 18	D 18 to 28	D 28 to 42
Feed form	Crumble	Pellet	Pellet
Ingredients (kg/ton)			
Corn	614.01	672.67	690.87
Soybean meal (480 g crude protein/kg)	323.935	263.16	239.22
Dicalcium phosphate	15.164	12.622	12.82
Filler[a]	10	10	10
Soybean oil	12.433	18.58	24
limestone	11.564	10.571	10.655
DL-Methionine 99%	2.912	2.656	2.486
L-Lysine HCl 78%	1.974	2.003	2.144
Vitamin Premix[b]	2.5	2.5	2.5
Common Salt	3.417	3.455	3.47
L-threonine	0.791	0.475	0.538
Mineral Premix[c]	0.8	0.8	0.8
Coccidiostats[d]	0.5	0.5	0.5
Total	1,000	1,000	1,000
Calculated energy and nutrient value, %			
Metabolizable energy, kcal/kg	3,000	3,100	3,150
Crude protein	20.5	18	17
SID[d] Methionine	0.598	0.54	0.51
SID[d] Total sulfur amino acids	0.88	0.8	0.76
SID[d] Lysine	1.17	1.02	0.97
SID[d] Threonine	0.78	0.66	0.63
Total calcium	0.87	0.76	0.76
Available phosphate	0.435	0.38	0.38

[a]Sand and tannic acid were included to obtain wanted tannic acid dosages in the feed as follows: Tannic acid 0 (TA0): sand 10 g/kg + tannic acid 0 g/kg; Tannic acid 0.25 (TA0.25): sand 9.75 g/kg + tannic acid 0.25 g/kg; Tannic acid 0.5 (TA0.5): sand 9.5 g/kg + tannic acid 0.5 g/kg; Tannic acid 1 (TA1): sand 9 g/kg + tannic acid 1 g/kg; and Tannic acid 2 (TA2): sand 8 g/kg + tannic acid 2 g/kg. Tannic acid was purchased from Sigma–Aldrich (St. Louis, MO). In the finisher phase, titanium dioxide 3 g/kg (Acros Organics, Morris Plains, NJ) was included in the sand part.

[b]Vitamin mix provided the following in mg/100 g diet: thiamine-HCl, 1.5; riboflavin 1.5; nicotinic acid amide 15; folic acid 7.5; pyridoxine-HCl, 1.2; d-biotin 3; vitamin B-12 (source concentration, 0.1%) 2; d-calcium pantothenate 4; menadione sodium bisulfite, 1.98; α-tocopherol acetate (source 500,000 IU/g), 22.8; cholecalciferol (source 5000,000 IU/g) 0.09; retinyl palmitate (source 500,000 IU/g), 2.8; ethoxyquin, 13.34; I-inositol, 2.5; dextrose, 762.2.

[c]Mineral mix provided the following in g/100 g diet: Ca $(H_2PO_4)_2 \cdot H_2O$, 3.62; $CaCO_3$, 1.48; KH_2PO_4, 1.00; Na_2SeO_4, 0.0002; $MnSO_4 \cdot H_2O$, 0.035; $FeSO_4 \cdot 7H_2O$, 0.05; $MgSO_4 \cdot 7H_2O$, 0.62; KIO_3, 0.001; NaCl, 0.60; $CuSO_4 \cdot 5H_2O$, 0.008; $ZnCO_3$, 0.015; $CoCl_2 \cdot 6H_2O$, 0.00032; $NaMoO_4 \cdot 2H_2O$, 0.0011; KCl, 0.10; dextrose, 0.40.

[d]Coban 90, Elanco Animal Health, Greenfield, IN.

[e]SID: standard ileal digestible amino acid.

liver, intestinal tissue (mid-duodenum, mid-jejunum, and mid-ileum), and cecal content. All tissue samples were washed with PBS to remove remaining digesta and blood. Samples of liver, mid-jejunum tissue, and cecal content were snap-frozen and stored at −80°C for further analyses. For intestinal morphology, mid-duodenum, mid-jejunum, and mid-ileum samples were fixed in a 10% formaldehyde solution. On D 36, randomly selected four birds were euthanized, and digesta samples from 10 cm below Meckel's diverticulum to the upper 10 cm of the ileo-cecal-colic junction were collected and oven-dried at 75°C until constant weight was achieved. On D 42, randomly selected one bird per pen was euthanized *via* cervical dislocation and scanned using dual-energy X-ray absorptiometry (DEXA, GE Healthcare, Madison, WI) to determine total tissue weight (g), bone mineral content (BMC; g), bone mineral density (BMD; g/cm^2), lean weight (g), fat weight (g), body fat percentage (%), and lean:fat (g/g). On D 42, severity of foot pad dermatitis (FPD) was measured from all birds in each pen according to Eichner et al. (2007): score 0: no lesion; score 1: FPD covers less than 25% of the food pad; score 2: FPD covers 25%–50% of the food pad; and score 3: FPD covers more than 50% of the food pad. Both foot pads were checked in birds, and scores from both foot pads were averaged, and FPD incidence (%) was also calculated. Ammonia level (mg/kg) on the litter was measured using a Chillgard® RT Refrigerant Monitor (MSA, Cranberry Township, PA) connected to a HOBO® monitoring station (Onset, Bourne, MA) according to Aston et al. (2019).

TABLE 2 Primers used in the study.

Genes	Sequence, 5′–3′	Amplicon	Accession number
GAPDH	F: GCT AAG GCT GTG GGG AAA GT	161	NM_204305.2
	R: TCA GCA GCA GCC TTC ACT AC		
Beta actin	F: CAA CAC AGT GCT GTC TGG TGG TA	205	NM_205518.2
	R: ATC GTA CTC CTG CTT GCT GAT CC		
ZO2	F: ATC CAA GAA GGC ACC TCA GC	100	NM_204918.1
	R: CAT CCT CCC GAA CAA TGC		
CLDN2	F: CCT GCT CAC CCT CAT TGG AG	145	NM_001277622.1
	R: GCT GAA CTC ACT CTT GGG CT		
MUC2	F: ATG CGA TGT TAA CAC AGG ACT C	110	JX284122.1
	R: GTG GAG CAC AGC AGA CTT TG		
B0AT1	F: GGG TTT TGT GTT GGC TTA GGA A	60	XM_419056.6
	R: TCC ATG GCT CTG GCA GAG AT		
PepT1	F: CCC CTG AGG AGG ATC ACT GTT	66	NM_204365.2
	R: CAA AAG AGC AGC AGC AAC GA		
SGLT1	F: GCC ATG GCC AGG GCT TA	71	NM_001293240.1
	R: CAA TAA CCT GAT CTG TGC ACC AGT A		
EAAT3	F: TGC TGC TTT GGA TTC CAG TGT	79	XM_424930.6
	R: AGC AAT GAC TGT AGT GCA GAA GTA ATA TAT G		

1 *GAPDH*, glyceraldehyde 3-phosphate dehydrogenase; *ZO2*, zonula occludens 2; *CLDN2*, claudin 2; *MUC2*, mucin 2; *B0AT1*, sodium-dependent neutral amino acid transporter 1; *PepT1*, peptide transporter 1; *SGLT1*, sodium glucose transporter 1; and *EAAT3*, excitatory amino acid transporter 3.

2.3 Apparent ileal digestibility of dry matter, organic matter, ash, crude protein, and crude fat

Oven-dried feed (75°C till constant weight; 0.5 g) and ileal digesta (0.3 g) samples were ashed at 600°C overnight, and concentrations of titanium dioxide were determined according to Short et al. (1996). The concentration of crude protein (CP) was analyzed using nitrogen combustion analyses according to AOAC international (2000) analytical method 990.03. The crude fat (CF) was determined according to AOAC international (2000) analytical method 942.05. Apparent ileal digestibility (AID) of dry matter (DM), organic matter (OM), ash, CP, and CF was calculated according to Lin and Olukosi (2021).

2.4 Intestinal morphology

After 72 h of fixation in 10% formalin solution, the fixed intestine samples were embedded in paraffin and cut into 4 μm, and the samples were stained with hematoxylin and eosin (H&E). The images of H&E-stained slides were taken using a microscope (BZ-X810; Keyence, Osaka, Japan). Five well-shaped villus and their corresponding crypts were selected per slide, and villus height (VH) and crypt depth (CD) were measured by using ImageJ (National Institutes of Health, Bethesda, MD). The VH to CD ratio (VH:CD) was calculated for each villi and crypt.

2.5 Jejunal brush border digestive enzyme activities and serum alkaline phosphatase

Around 100 mg of mid-jejunum (whole tissue) samples were homogenized in 1.8 ml PBS using a bead beater (Biospec Products, Bartlesville, OK). Afterwards, the samples were centrifuged at 12,000 × *g* for 15 min at 4°C, and the protein concentrations of the supernatants was determined using Pierce BCA protein assay kits according to the manufacturer's instructions (Thermo Fisher Scientific, Waltham, MA). Activities of maltase and sucrase in the supernatants were analyzed according to the method of Lackeyram (2012). Briefly, 100 μl supernatants were mixed with 400 μl maltose (75 mM) and sucrose solution (75 mM), separately and incubated at 41°C for 30 min. Afterwards, the concentrations of glucose were determined using a Glucose Oxidase Reagent Set

TABLE 3 Growth performance parameters including body weight (BW, g), average daily gain (ADG, g/d), average daily feed intake (ADFI, g/d), and feed conversion ratio (FCR, g/g) in broilers fed diets supplemented with tannic acid on D 42[a].

								Polynomial contrast[c]	
Items	TA0	TA0.25	TA0.5	TA1	TA2	SEM	*p*-value[b]	Linear	Quadratic
Initial BW, g	45.96	45.94	45.94	45.96	45.94	0.09	0.986		
Starter (D 0 to 18)									
BW	890.24[a]	860.97[a,b]	866.14[a,b]	853.03[a,b]	822.86[b]	38.63	0.045	0.003	0.787
ADG	46.90[a]	45.28[a,b]	45.57[a,b]	44.84[a,b]	43.16[b]	2.14	0.044	0.003	0.787
ADFI	59.79	58.09	58.39	57.27	56.64	2.47	0.187	0.030	0.411
FCR	1.28	1.28	1.28	1.28	1.31	0.04	0.469	0.108	0.479
Grower (D 18 to 28)									
BW	2020.4[a]	1959.23[a,b]	1943.7[a,b]	1973.35[a,b]	1892.55[b]	56.15	0.004	0.001	0.911
ADG	113.19[a]	109.65[a,b,c]	107.76[b,c]	112.03[a,b]	106.97[c]	2.89	0.001	0.007	0.948
ADFI	173.42	170.73	168.97	170.98	173.66	4.64	0.305	0.445	0.089
FCR	1.53[b,c]	1.56[b,c]	1.57[b]	1.53[c]	1.62[a]	0.03	< 0.001	<0.001	0.005
Finisher (D 28 to 42)									
BW	3,772.68	3,671.24	3,630.39	3,711.05	3,634.49	116.86	0.154	0.155	0.528
ADG	125.16	122.29	120.48	124.12	124.42	6.35	0.648	0.709	0.447
ADFI	214.79	212.52	214.21	216.75	218.59	10.86	0.857	0.323	0.927
FCR	1.72	1.74	1.78	1.75	1.76	0.07	0.616	0.507	0.413
Whole (D 0 to 42)									
ADG	88.77	86.28	85.34	87.26	85.44	2.77	0.143	0.152	0.514
ADFI	138.51	136.38	136.66	137.5	138.49	4.42	0.839	0.642	0.481
FCR	1.48[b]	1.50[a,b]	1.52[a,b]	1.49[a,b]	1.53[a]	0.03	0.023	0.008	0.713

[a]TA0 (tannic acid 0; basal diet without TA); TA0.25 (tannic acid 0.25; basal diet + 0.25 g/kg TA); TA0.5 (tannic acid 0.5; basal diet + 0.5 g/kg TA); TA1 (tannic acid 1; basal diet + 1 g/kg TA); and TA2 (tannic acid 2; basal diet + 2 g/kg TA).

[b]Treatment groups (7 replicates per treatment) were compared using PROC MIXED, followed by the Tukey's individual comparison test. Different letters in the same row means significant differences ($p < 0.05$) among the treatments.

[c]Orthogonal polynomial contrasts were conducted to assess the significance of linear or quadratic effects of the supplementation of TA, in broilers.

(Pointe Scientific, Canton, MI) according to the manufacturer's protocol. To determine activities of lipase in the supernatants, the 10 times diluted supernatants (60 µl) were incubated with 1 mg/ml *p*-nitrophenyl palmitate solution (Sigma-Aldrich Co., St Louis, MO; 140 µl) at 41°C for 30 min according to the method of Elgharbawy et al. (2018). The activities of leucine aminopeptidase (LAP) were assayed by incubating 100 µl supernatant with 100 µl 1 mg/ml L-leucine-*p*-nitroanilide solution (Sigma-Aldrich Co., St Louis, MO) at 41°C for 30 min according to Maroux et al. (1973). To determine activities of alkaline phosphatase, the 20 µl supernatant (2 times dilution) and serum (10 times diluted) were incubated with 180 µl 10 mM *p*-nitrophenyl phosphate solution at 41°C for 60 min according to Lackeyram et al. (2010). The absorbance of the end products (*p*-nitrophenyl and *p*-nitroanilide) was determined at 400 nm by using a spectrophotometer (VICTOR Nivo, Perkin Elmer, Pontyclun, United Kingdom) and quantify using a prepared standard curve. The activities of the enzymes except alkaline phosphatase in the serum were expressed as their values per mg protein per min. The activities of serum alkaline phosphatase were expressed as their values per mL serum per min.

2.6 RNA extraction and real-time reverse transcription-PCR analysis

Approximately 100 mg of mid-jejunum (whole tissue) samples were homogenized using a bead beater (Biospec Products, Bartlesville, OK) in QIAzol lysis reagents (Qiagen, Valencia, CA). Afterwards, RNA was extracted according to the manufacturer's protocol. RNA quantity and quality were measured by a NanoDrop 2000 spectrophotometer (Thermo Fisher Scientific). One microgram of RNA was used to synthesize the first-strand cDNA by using high-capacity cDNA synthesis kits (Applied Biosystems, Foster City, CA) according to the manufacturer's instructions. The 20 µl cDNA was diluted with 80 µl water. Primers used in the study are listed in Table 2. Real-time reverse transcript (RT)-PCR was conducted using SYBR Green Master Mix with a Step Onethermocycler (Applied Biosystem). The final volume for PCR mixture was 10 µl which included 5 µl of SYBR Green Master Mix (Applied Biosystems), 1.5 µl of cDNA, 0.5 µl of forward and reverse primers (10 µM each), and 2.5 µl of water. The thermal cycle condition for all genes was 95°C denature for 10 min, 40 cycles at 95°C for 15 s and 60°C for 1 min, 95°C for 15 s, 60°C for 1 min,

TABLE 4 Duodenal, jejunal, and ileal morphology parameters including villus height (VH, μm), crypt depth (CD, μm), and VH:CD in broilers fed diets supplemented with tannic acid on D 18 and 36[a].

								Polynomial contrast[3]	
Items	TA0	TA0.25	TA0.5	TA1	TA2	SEM	*p*-value[b]	Linear	Quadratic
D 18									
Duodenal VH	2,239.4	2,282.5	2,337.6	2,256.7	2,269.6	260.8	0.964	0.979	0.760
Duodenal CD	212	221.4	215.9	214.0	191	39.71	0.664	0.195	0.478
Duodenal VH:CD	11.23	11.18	11.08	10.69	12.21	1.86	0.637	0.305	0.268
Jejunal VH	1,240.7	1,151.5	1,218.1	1,205.7	1,198.8	194.83	0.937	0.926	0.890
Jejunal CD	286.36[a]	219.76[b]	203.72[b]	223.19[b]	200.78[b]	36.08	< 0.001	0.003	0.027
Jejunal VH:CD	4.55[b]	5.52[a,b]	6.29[a]	5.59[a,b]	6.26[a]	1.09	0.036	0.031	0.209
Ileal VH	916.94	805.3	910.39	856.22	791.58	103.75	0.096	0.075	0.745
Ileal CD	222.44[a]	173.9[b]	185.65[a,b]	184.37[a,b]	193.79[a,b]	30.29	0.057	0.517	0.046
Ileal VH:CD	4.26	4.88	5.10	4.85	4.26	0.815	0.212	0.451	0.046
D 36									
Duodenal VH	2,596.9	2,718.2	2,582.5	2,608.2	2,849.4	424.4	0.737	0.311	0.522
Duodenal CD	206.66	201.69	172.23	198.67	204.99	33.85	0.325	0.731	0.224
Duodenal VH:CD	13.09	14.62	15.91	13.70	14.56	2.93	0.459	0.768	0.536
Jejunal VH	1,543.5	1781.9	1,410.2	1795.8	1758	248.96	0.042	0.109	0.796
Jejunal CD	179.9	186.74	163.79	175.59	193.21	28.03	0.366	0.342	0.201
Jejunal VH:CD	9.11	10	8.89	11.24	9.4	1.88	0.165	0.661	0.113
Ileal VH	1,050.5	912.5	958.5	1,010.1	1,016.7	147.93	0.454	0.657	0.496
Ileal CD	157.47	155.29	149.88	149.77	157.91	24.36	0.943	0.912	0.412
Ileal VH:CD	6.96	6.32	6.78	7.04	6.68	1.28	0.849	0.971	0.795

[a]TA0 (tannic acid 0; basal diet without TA); TA0.25 (tannic acid 0.25; basal diet + 0.25 g/kg TA); TA0.5 (tannic acid 0.5; basal diet + 0.5 g/kg TA); TA1 (tannic acid 1; basal diet + 1 g/kg TA); and TA2 (tannic acid 2; basal diet + 2 g/kg TA).

[b]Treatment groups (7 replicates per treatment) were compared using PROC MIXED, followed by the Tukey's individual comparison test. Different letters in the same row means significant differences ($p < 0.05$) among the treatments.

[c]Orthogonal polynomial contrasts were conducted to assess the significance of linear or quadratic effects of the supplementation of TA in broilers.

and 95°C for 15 s. After the PCR amplification, melting curve analysis and product size verification by gel electrophoresis were conducted to check the specificity of the PCR reactions. The geometric mean of Ct values of glyceraldehyde 3-phosphate dehydrogenase (*GAPDH*) and beta-actin were used as reference values to normalize all target genes' mRNA abundance (Vandesompele et al., 2002). Relative mRNA abundance of target genes was calculated using the $2^{-\Delta\Delta CT}$ method, and the TA0 group was set as the control group (Livak and Schmittgen, 2001). Each sample was analyzed in duplicate, and the negative control, containing water instead of cDNA, was included in each run.

2.7 Liver total antioxidant capacity, concentrations of glutathione and oxidized glutathione, and activities of superoxide dismutase

Approximately 100 mg of liver samples were homogenized using a bead beater (Biospec Products, Bartlesville, OK) in selected solution for each assay. Afterwards, the samples were centrifuged at 12,000 × *g* for 15 min at 4°C, and protein concentration of the supernatants were analyzed using Pierce BCA protein assay kits (Thermo Fisher Scientific) after 20-time sample dilution. The total antioxidant capacity (TAC) of the collected supernatant was analyzed using a commercial kit (QuantiCromAntioxidant Assay Kit, BioAssay Systems, Hayward, CA) after 2-time sample dilution. Concentrations of glutathione (GSH) and oxidized glutathione (GSSG) in the supernatant were analyzed using Caymans GSH assay kits (Cayman Chemical, Ann Arbor, MI) with 20- and 2-time sample dilutions, respectively. The activities of superoxide dismutase (SOD) in the supernatants were determined Caymans SOD assay kits (Cayman Chemical) after 400-time sample dilution. The TAC, concentrations of GSH and GSSG, and SOD activities were expressed as values per mg protein.

2.8 DNA extraction and microbiome analysis

DNA was extracted from the cecal contents using QIAamp® DNA stool mini kits (Qiagen GmbH, Hilden, Germany) according to manufacturer's protocol. After quality and quantity of extracted DNA were checked using a NanoDrop

TABLE 5 Activities of jejunal brush border digestive enzymes including maltase (nmol glucose released/mg protein/min), sucrase (nmol glucose released/mg protein/min), leucine aminopeptidase (LAP; nmol p-nitroaniline liberated/mg protein/min), intestinal alkaline phosphatase (IAP; μmol p-nitrophenol liberated/mg protein/min), lipase (mmol p-nitrophenyl phosphate liberated/mg protein/min), and serum alkaline phosphatase (SAP; μmol p-nitrophenol liberated/ml serum/min) in broilers fed diets supplemented with tannic acid on D 18 and 36[a].

								Polynomial contrast[c]	
Items	TA0	TA0.25	TA0.5	TA1	TA2	SEM	*p*-value[b]	Linear	Quadratic
D 18									
Maltase	0.218	0.247	0.227	0.233	0.232	0.066	0.951	0.906	0.826
Sucrase	0.162	0.277	0.334	0.315	0.243	0.136	0.170	0.634	0.027
LAP	25.41	25.88	20.55	25.84	22.27	6.23	0.388	0.452	0.968
Lipase	1.612	1.138	0.751	0.861	1.212	0.623	0.113	0.533	0.016
IAP	0.206	0.241	0.196	0.213	0.219	0.053	0.596	0.912	0.775
SAP	0.23	0.26	0.25	0.23	0.26	0.06	0.844	0.634	0.791
D 36									
Maltase	2.221	1.809	1.876	1.750	1.815	0.681	0.713	0.421	0.353
Sucrase	0.453	0.497	0.426	0.326	0.408	0.209	0.639	0.417	0.350
LAP	12.77	13.67	11.37	11.64	12.71	2.65	0.495	0.775	0.236
Lipase	1.250	1.148	1.115	0.996	1.244	0.249	0.316	0.930	0.038
IAP	0.254	0.238	0.235	0.256	0.271	0.044	0.544	0.199	0.492
SAP	0.21	0.20	0.20	0.18	0.2	0.04	0.680	0.442	0.226

[a]TA0 (tannic acid 0; basal diet without TA); TA0.25 (tannic acid 0.25; basal diet + 0.25 g/kg TA); TA0.5 (tannic acid 0.5; basal diet + 0.5 g/kg TA); TA1 (tannic acid 1; basal diet + 1 g/kg TA); and TA2 (tannic acid 2; basal diet + 2 g/kg TA).
[b]Treatment groups (7 replicates per treatment) were compared using PROC MIXED, followed by the Tukey's individual comparison test.
[c]Orthogonal polynomial contrasts were conducted to assess the significance of linear or quadratic effects of the supplementation of TA in broilers.

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TABLE 6 Apparent ileal digestibility (%) of dry matter (DM), organic matter (DM), organic matter (OM), ash, crude protein (CP), and crude fat (CF) in broilers fed diets supplemented with tannic acid on D 18 and 36[a].

								Polynomial contrast[c]	
Items	TA0	TA0.25	TA0.5	TA1	TA2	SEM	*p*-value[b]	Linear	Quadratic
DM	74.37[b]	81.00[a]	79.26[a]	79.24[a]	80.68[a]	2.39	<0.01	0.003	0.033
OM	76.10[b]	82.65[a]	80.83[a]	80.71[a]	82.12[a]	2.22	<0.01	0.003	0.03
Ash	40.39[b]	49.57[a,b]	48.86[a,b]	50.60[a,b]	52.79[a]	6.75	0.019	0.008	0.115
CP	80.57[b]	87.35[a]	85.08[a]	85.19[a]	86.62[a]	2.09	<0.01	0.002	0.028
CF	84.62	90.41	85.82	85.98	87.01	4.52	0.185	0.962	0.948

[a]TA0 (tannic acid 0; basal diet without TA); TA0.25 (tannic acid 0.25; basal diet + 0.25 g/kg TA); TA0.5 (tannic acid 0.5; basal diet + 0.5 g/kg TA); TA1 (tannic acid 1; basal diet + 1 g/kg TA); and TA2 (tannic acid 2; basal diet + 2 g/kg TA).
[b]Treatment groups (7 replicates per treatment) were compared using PROC MIXED, followed by the Tukey's individual comparison test. Different letters in the same row means significant differences ($p < 0.05$) among the treatments.
[c]Orthogonal polynomial contrasts were conducted to assess the significance of linear or quadratic effects of the supplementation of TA in broilers.

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TABLE 7 Relative mRNA expression of gene associated with tight junction proteins and nutrients transporters in broilers fed diets supplemented with tannic acid on D 18 and 36[a].

Items	TA0	TA0.25	TA0.5	TA1	TA2	SEM	*p*-value[b]	Polynomial contrast[c]	
								Linear	Quadratic
D 18									
Z O 2	1.11[b]	1.11[b]	1.14[b]	1.93[a]	1.31[a,b]	0.51	0.021	0.148	0.022
CLDN2	1.08	0.87	1.34	1.59	2.09	0.86	0.103	0.009	0.94
JAM2	1.42	0.84	0.72	1.03	4.6	3.23	0.16	0.035	0.171
MUC2	1.16[a,b]	0.76[b]	1.01[a,b]	1.47[a,b]	1.79[a]	0.56	0.017	0.003	0.67
B0AT1	1.06[a,b]	0.54[b]	0.91[a,b]	1.84[a]	1.38[a,b]	0.69	0.017	0.039	0.195
PepT1	1.12	0.9	1.29	2.03	1.32	0.71	0.061	0.203	0.038
SGLT1	1.14[a,b]	0.77[b]	0.93[a,b]	1.65[a]	1.76[a]	0.54	0.005	0.001	0.894
EAAT3	1.11[b]	1.11[b]	1.14[b]	1.93[a]	1.31[a,b]	0.51	0.022	0.148	0.022
D 36									
Z O 2	1.14	2.03	1.49	1.7	1.48	1.04	0.606	0.95	0.451
CLDN2	1.84	1.55	1.26	0.98	1.51	1.84	0.926	0.762	0.385
JAM2	1.59	1.44	5.44	2.76	2.67	3.18	0.158	0.692	0.187
MUC2	1.02	1.12	1.56	1.83	1.43	0.53	0.064	0.116	0.015
B0AT1	1.09	0.97	1.07	1.54	1.18	0.71	0.233	0.313	0.17
PepT1	1.59	0.8	1.78	1.38	1.45	1.4	0.748	0.885	0.979
SGLT1	1.1	1.21	1.49	1.69	1.55	0.59	0.331	0.130	0.146
EAAT3	2.3	1.8	1.93	1.02	1.19	2	0.741	0.262	0.524

[a]TA0 (tannic acid 0; basal diet without TA); TA0.25 (tannic acid 0.25; basal diet + 0.25 g/kg TA); TA0.5 (tannic acid 0.5; basal diet + 0.5 g/kg TA); TA1 (tannic acid 1; basal diet + 1 g/kg TA); and TA2 (tannic acid 2; basal diet + 2 g/kg TA). *Z O 2*, zonula occludens 2; *CLDN2*, claudin 2; *MUC2*, mucin 2; *B0AT1*, sodium-dependent neutral amino acid transporter 1; *PepT1*, peptide transporter 1; *SGLT1*, sodium glucose transporter 1; and *EAAT3*, excitatory amino acid transporter 3.

[b]Treatment groups (7 replicates per treatment) were compared using PROC MIXED, followed by the Tukey's individual comparison test. Different letters in the same row means significant differences ($p < 0.05$) among the treatments.

[c]Orthogonal polynomial contrasts were conducted to assess the significance of linear or quadratic effects of the supplementation of TA in broilers.

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TABLE 8 Total antioxidant capacity (TAC; μM Trolox Equivalents/mg protein), concentrations of glutathione (GSH; μM/mg protein) and oxidized glutathione (GSSG; μM/mg protein), and activities of superoxide dismutase (SOD; U/mg protein) in broilers fed diets supplemented with tannic acid on D 18 and 36[a].

Items	TA0	TA0.25	TA0.5	TA1	TA2	SEM	*p*-value[b]	Polynomial contrast[c]	
								Linear	Quadratic
D 18									
TAC	78.90	80.51	76.48	80.28	78.23	4.29	0.923	0.898	0.929
GSH	15.36	17.17	14.14	13.69	15.16	3.06	0.271	0.491	0.210
GSSG	2.07	2.25	1.8	1.86	1.96	0.51	0.509	0.494	0.337
Reduced GSH	11.22	12.67	10.54	9.97	11.24	2.17	0.219	0.517	0.188
Reduced GSH: GSSG	5.64	5.67	6.05	5.37	5.93	0.92	0.676	0.753	0.650
SOD	23.04	22.27	20.91	23.7	22.7	6.93	0.958	0.874	0.964
D 36									
TAC	90.31	80.65	77.94	81.93	79.12	8.38	0.073	0.114	0.146
GSH	17.11	15.83	14.37	16.20	15.96	4.3	0.828	0.896	0.543
GSSG	1.87	1.84	1.59	1.79	1.80	0.59	0.931	0.954	0.636
Reduced GSH	12.91	12.14	11.21	12.61	11.58	3.33	0.891	0.667	0.881
Reduced GSH: GSSG	7.01	7.02	7.16	7.25	6.54	1.33	0.902	0.512	0.461
SOD	24.34	25.57	25.51	25.24	25.5	8.29	0.998	0.879	0.883

[a]TA0 (tannic acid 0; basal diet without TA); TA0.25 (tannic acid 0.25; basal diet + 0.25 g/kg TA); TA0.5 (tannic acid 0.5; basal diet + 0.5 g/kg TA); TA1 (tannic acid 1; basal diet + 1 g/kg TA); and TA2 (tannic acid 2; basal diet + 2 g/kg TA).

[b]Treatment groups (7 replicates per treatment) were compared using PROC MIXED, followed by the Tukey's individual comparison test. Different letters in the same row means significant differences ($p < 0.05$) among the treatments.

[c]Orthogonal polynomial contrasts were conducted to assess the significance of linear or quadratic effects of the supplementation of TA in broilers.

2000 spectrophotometer (Thermo Fisher Scientific), the samples were sent to LC sciences (Houston, TX) for 16 s rRNA gene sequencing (Choi et al., 2022d). Qimme2 (version 2022.02) was used to process and analyze 16s rRNA gene sequences (Bolyen et al., 2019). According to Choi and Kim (2022), 16s rRNA sequences were processed. The sampling depth for both D 18 and 36 time points was set as 45,000. By using Qiime2's built-in functions, alpha diversity, beta diversity, and phylum and family level composition were analyzed and presented.

2.9 Slaughter, carcass processing, and breast myopathy evaluation

On D 42, three birds per pen were randomly selected from each pen for processing, and feed was removed from the pen for 12 h (Wang J. et al., 2020). On D 43, the selected birds were individually weighed and transferred to the processing plant at the University of Georgia. Birds were shackled, electrically stunned, bled, scalded, and defeathered. Following head and feet removal, the carcasses were eviscerated. Weights of the hot carcass and abdominal fat collected from fat around cloaca, bursa of Fabricius, gizzard, and proventriculus (Castro et al., 2019) were recorded. The carcasses were rinsed and chilled in ice-cold water at 1°C for 4 h. Legs, breast muscle, tender, wings, and skeleton were separated by trained personnel, and their weights were recorded. Breast myopathies and quality defects including white striping [score 0 (normal), 1, 2, and 3 (severe)], woody breast [score 1 (normal), 2, and 3 (severe)], spaghetti meat [score 0 (normal), 1, and 2 (severe)], and petechial hemorrhagic lesions [score 0 (normal), 1, 2, and 3 (severe)] were assessed by a trained expert according to published criteria (Kuttappan et al., 2017; Pang et al., 2020; Baldi et al., 2021; Prisco et al., 2021).

2.10 Breast meat quality measurements

Breast muscles from two birds per pen were stored at 1°C overnight for further meat quality analyses. Color and pH of the breast muscles were analyzed according to Brambila et al. (2018) with the modification. Color indicators including lightness (L*), redness (a*), and yellowness (b*) were determined in duplicate on the dorsal surface of breast meat by a Minolta Spectrophotometer CM-700 days (Konica Minolta Inc., Ramsey, NJ). Meat pH was analyzed (one measurement per fillet) by using a Thermo Scientific Orion Star™ A221 portable pH meter with a spear tipped probe (Thermal Scientific Orion 8163BNWP) (Thermo Fisher Scientific, Waltham, MA 02451, United States) that penetrated the cranial end of the intact breast muscle. Drip loss was analyzed by using a EZ-driploss method (Kaić et al., 2021). One cylindrical muscle core (2.5 cm diameter) was removed from the cranial side of the breast meat and trimmed to a similar height. The cores were weighed and placed in individual EZ containers (Danish Meat Research Institute, Taastrup, Denmark). The sealed containers were then stored in a refrigerator at 4°C. The

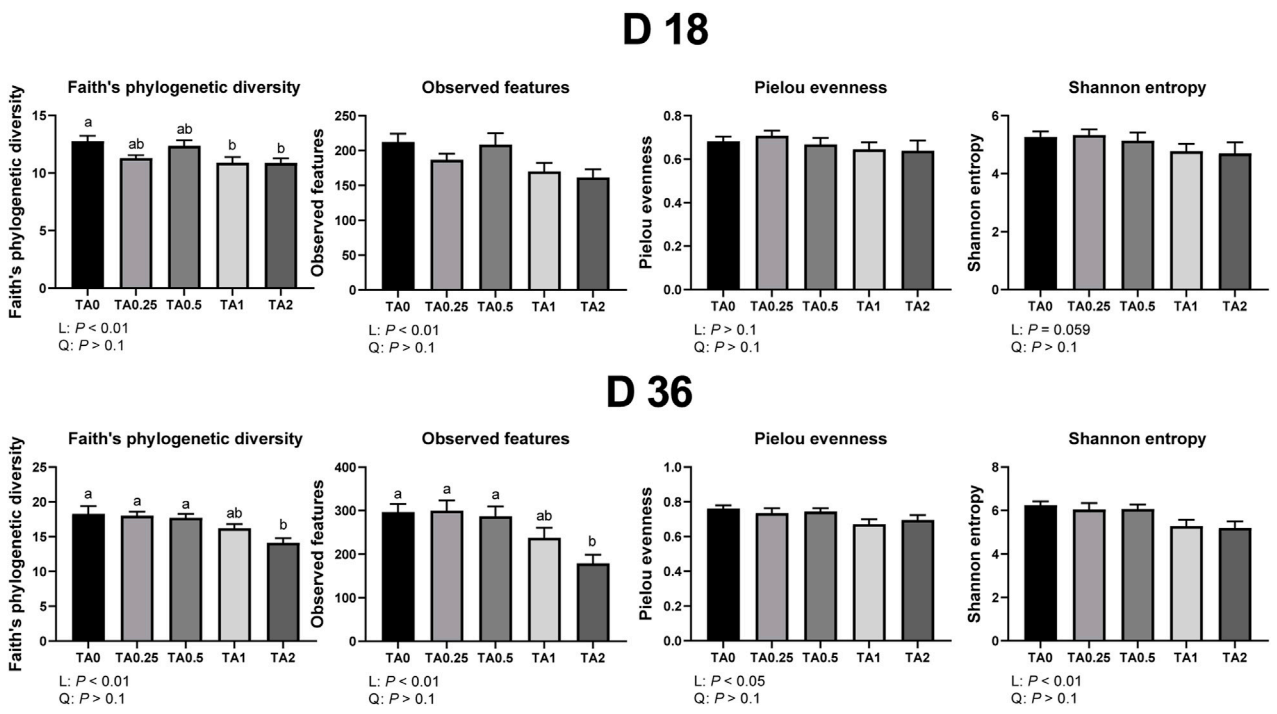

FIGURE 1
Alpha diversity parameters of the cecal microbial communities in the TA0 (tannic acid 0; basal diet without TA), TA0.25 (tannic acid 0.25; basal diet + 0.25 g/kg TA), TA0.5 (tannic acid 0.5; basal diet + 0.5 g/kg TA), TA1 (tannic acid 1; basal diet + 1 g/kg TA), and TA2 (tannic acid 2; basal diet + 2 g/kg TA) groups on D 18 and 36. All treatment groups (7 replicates per treatment) were compared using PROC MIXED followed by the Tukey's individual comparison test. Different letters in the same row means significant differences ($p < 0.05$) among the treatments. Orthogonal polynomial contrasts were used to evaluate the significance of linear or quadratic effects of the supplementation of TA in broilers.

samples were reweighted (approximately 7 g–8 g) after 48 h to determine drip loss (%). For thawing loss (%), intact breast samples were weighed and individually sealed in cooking bags before frozen at −20°C. The frozen samples were stored for 2 weeks at −20°C and were thawed at 4°C overnight and weighed again after liquid was removed. Cooking was performed by using a Henny Penny MCS-6 combi oven (Henny Penny Corp. Eaton, OH) on the Tender Steam setting at 84°C. Fillets were cooked ventral side up in stainless steel oven pans to an endpoint temperature of 74°C in the thickest part of the fillet (Brambila et al., 2018). A thermocouple system with hypodermic needle microprobes (Physitemp Instruments, Inc., Clifton, NJ) was used to monitor temperature. The samples were reweighed after the liquid was removed.

2.11 Statistical analyses

SAS (version 9.4; SAS Inst. Inc., Cary, NC) and GraphPad Prism (Version 9.1.0; GraphPad Software, San Diego, CA) were used for statistical analyses and graph construction. Treatment groups were compared using PROC MIXED followed by the Tukey's individual comparison test. Orthogonal polynomial contrasts were conducted to assess the significance of linear or quadratic effects of the supplementation of TA in broilers. Pen was considered as the experimental unit, and values of individual birds in the same pen were averaged for meat analyses. Breast meat myopathy score and FPD score and incidence were analyzed using the Kruskal–Wallis test followed by the Dwass–Steel–Critchlow–Fligner *post hoc* test. Significance level was set at $p < 0.05$, and tendencies were also presented at $0.05 < p \leq 0.10$ (Choi et al., 2021).

3 Results

3.1 Growth performance

Results of the growth performance are presented in Table 3. In the starter phase, the TA2 group had significantly lower BW and ADG compared to the TA0 group, and the supplementation of TA linearly decreased BW and ADG in broiler chickens ($p < 0.01$). The ADFI was also linearly reduced by the supplementation of TA

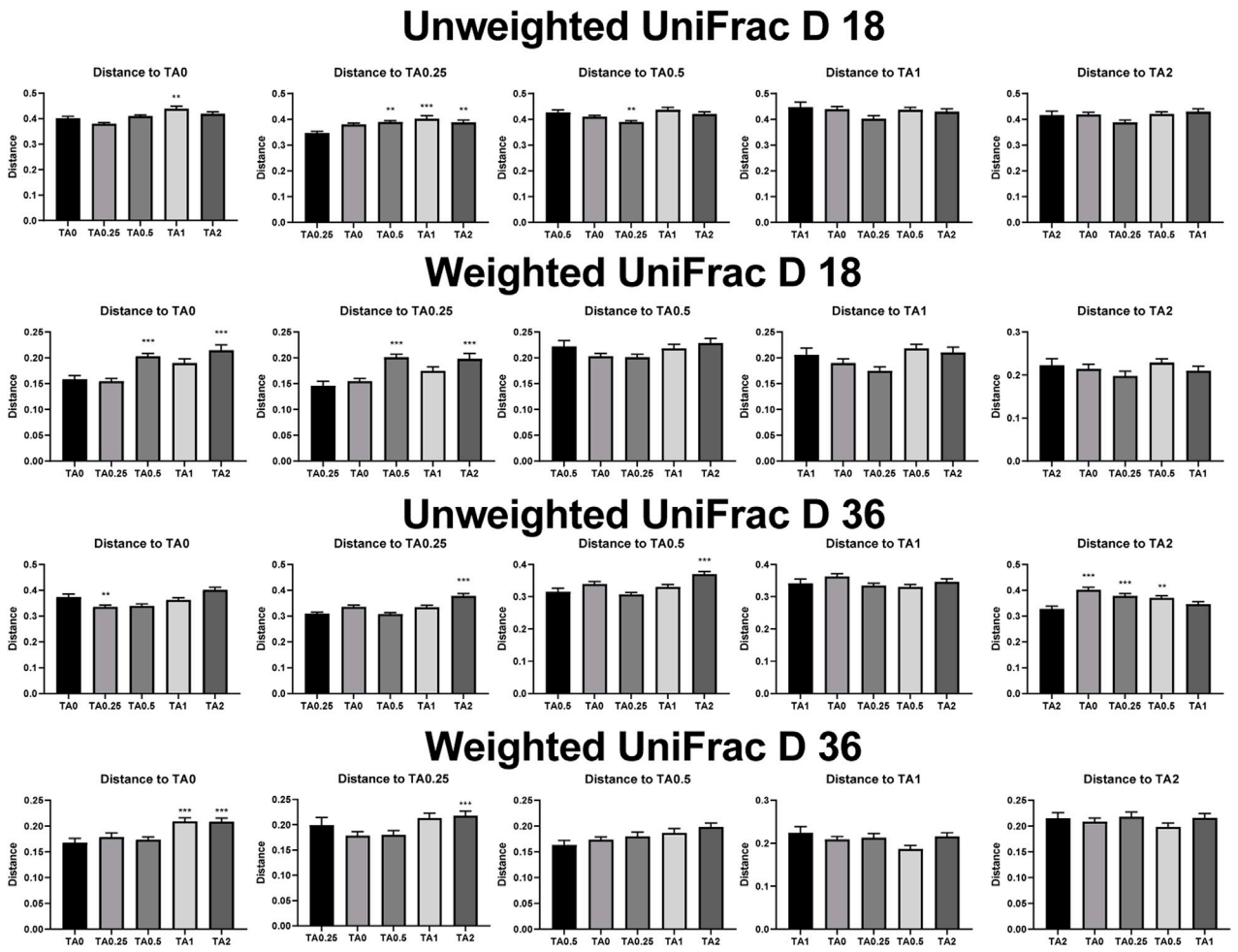

FIGURE 2
Beta diversity indices including unweighted and weighted unifrac of the cecal microbial communities in the TA0 (tannic acid 0; basal diet without TA), TA0.25 (tannic acid 0.25; basal diet + 0.25 g/kg TA), TA0.5 (tannic acid 0.5; basal diet + 0.5 g/kg TA), TA1 (tannic acid 1; basal diet + 1 g/kg TA), and TA2 (tannic acid 2; basal diet + 2 g/kg TA) groups on D 18 and 36. All treatment groups (7 replicates per treatment) were compared using PROC MIXED followed by the Tukey's individual comparison test. Orthogonal polynomial contrasts were used to evaluate the significance of linear or quadratic effects of the supplementation of TA in broilers.

in broilers in the starter phase ($p < 0.05$). In the grower phase, the supplementation of TA linearly reduced BW and ADG and linearly and quadratically increased FCR of broilers ($p < 0.01$). The TA2 group had significantly lower BW compared to the TA0 group. The TA2 group had significantly lower ADG compared to the TA0 and TA1 groups ($p < 0.05$). The TA0.5 group had a significantly lower ADG compared to the TA0 ($p < 0.05$). The TA2 group had the highest FCR ($p < 0.05$) among the treatment groups, and the TA1 group had significantly lower FCR compared to the TA0.5 group. No statistical differences were observed in the growth performance parameters of the finisher phase ($p > 0.1$). In the whole phase, the supplementation of TA linearly increased FCR ($p < 0.01$), and the TA2 group had significantly higher FCR compared to the TA0 group.

3.2 Intestinal morphology

As shown in Table 4, the TA0 group had significantly higher jejunal CD compared to the TA supplemented groups, and the supplementation of TA linearly ($p < 0.01$) and quadratically ($p < 0.05$) reduced jejunal CD on D 18. The TA0.5 and TA2 groups had greater jejunal VH:CD compared to the TA0 group ($p < 0.05$), and the supplementation of TA linearly increased jejunal VH:CD. The supplementation of TA tended to linearly decrease ileal VH ($p = 0.075$). The TA0.25 group tended to have lower ileal CD compared to the TA0 group ($p = 0.057$). The supplementation of TA quadratically decreased ileal CD and increased ileal VH:CD ($p < 0.05$). There were no statistical differences in the intestinal morphology on D 36 ($p > 0.1$).

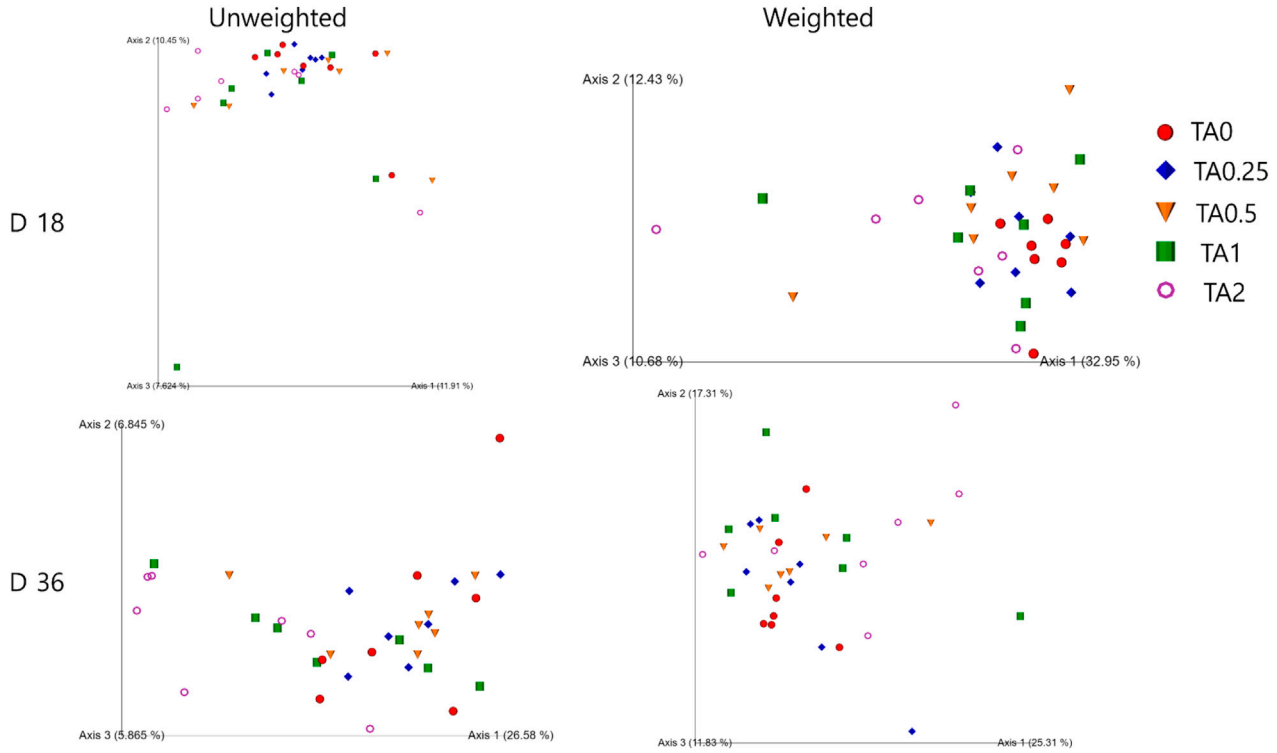

FIGURE 3
Visualized beta diversity parameters including unweighted and weighted unifrac of the cecal microbial communities in the TA0 (tannic acid 0; basal diet without TA), TA0.25 (tannic acid 0.25; basal diet + 0.25 g/kg TA), TA0.5 (tannic acid 0.5; basal diet + 0.5 g/kg TA), TA1 (tannic acid 1; basal diet + 1 g/kg TA), and TA2 (tannic acid 2; basal diet + 2 g/kg TA) groups on D 18 and 36. All treatment groups (7 replicates per treatment) were compared using PROC MIXED followed by the Tukey's individual comparison test. Orthogonal polynomial contrasts were used to evaluate the significance of linear or quadratic effects of the supplementation of TA in broilers.

3.3 Activities of jejunal brush border digestive enzymes and serum alkaline phosphatase

As shown in Table 5, the supplementation of TA quadratically increased sucrase activities ($p < 0.05$) and quadratically decreased lipase activities in the jejunum tissue ($p < 0.05$). On D 36, jejunal lipase activities were quadratically decreased by the supplementation of TA ($p < 0.05$). However, no differences were observed in the activities of jejunal sucrase, LAP, intestinal alkaline phosphatase (IAP), and serum alkaline phosphatase (SAP) ($p > 0.1$).

3.4 Apparent ileal digestibility of dry matter, organic matter, ash, crude protein, and crude fat

As shown in Table 6, the supplementation of TA linearly ($p < 0.01$) and quadratically ($p < 0.05$) increased AID of DM, OM, and CP on D 36. The TA0.25, TA0.5, TA1, and TA2 groups had significantly higher AID of DM compared to the TA0 group ($p < 0.01$). The AID of OM was significantly lower in the TA0 group compared to the TA0.25, TA0.5, TA1, and TA2 groups ($p < 0.05$). The TA2 group had significantly AID of ash compared to the TA0 group, and the supplementation of TA linearly increased AID of ash ($p < 0.01$). The TA0 group had the lowest AID of CP among the treatments ($p < 0.05$). No differences were observed in the AID of CF among the treatments ($p > 0.1$).

3.5 Relative mRNA expression of genes related tight junction proteins and nutrient transporters in the jejunum

Relative mRNA expression of genes related to tight junction proteins and nutrient transporters in the jejunum is presented in Table 7. On D 18, the TA1 group had significantly higher relative mRNA expression of zonula occludens 2 (*Z O 2*) compared to the TA0, TA0.25 and TA0.5 groups, and the supplementation of TA quadratically

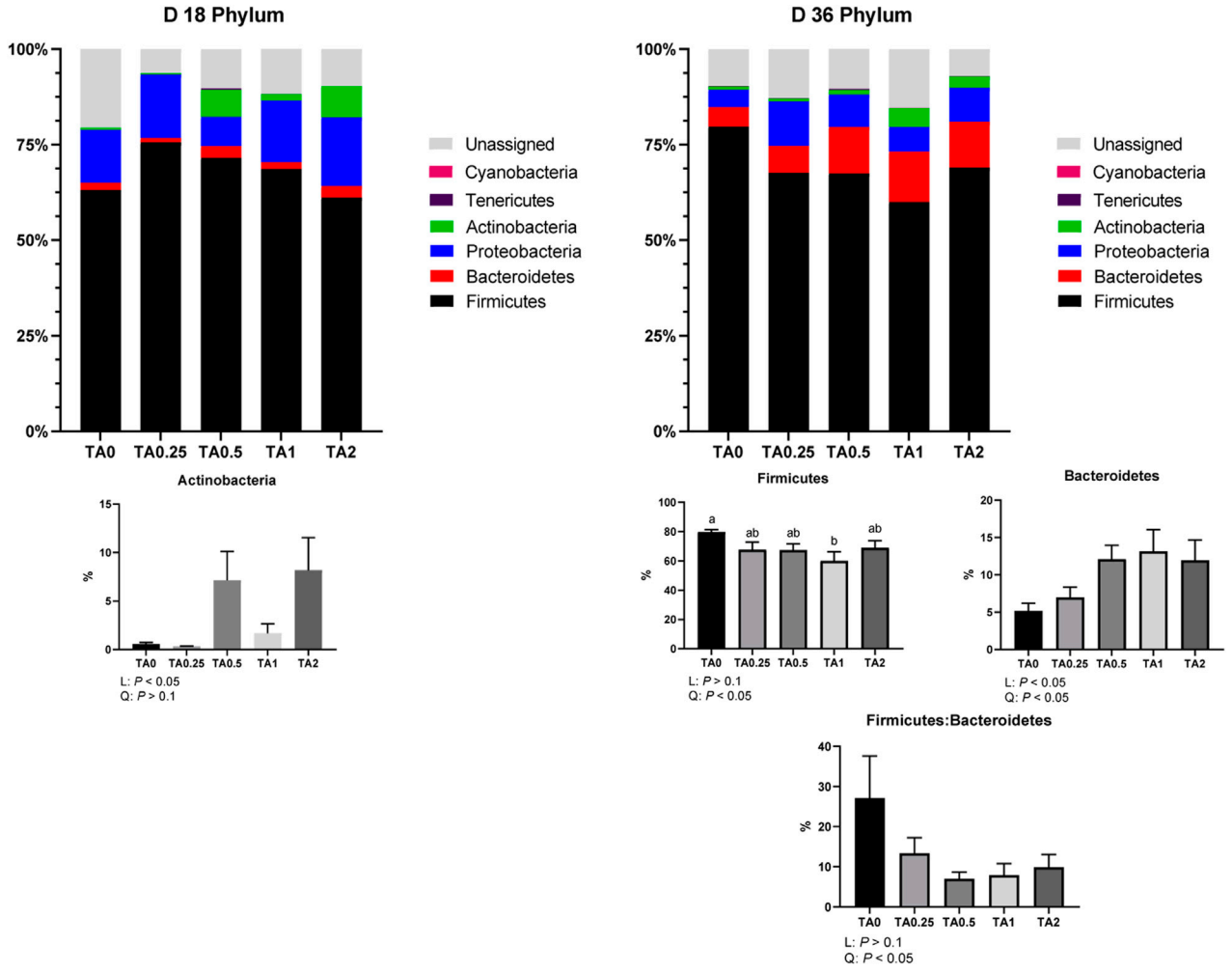

FIGURE 4
Phylum-level composition of the cecal microbial communities in the TA0 (tannic acid 0; basal diet without TA), TA0.25 (tannic acid 0.25; basal diet + 0.25 g/kg TA), TA0.5 (tannic acid 0.5; basal diet + 0.5 g/kg TA), TA1 (tannic acid 1; basal diet + 1 g/kg TA), and TA2 (tannic acid 2; basal diet + 2 g/kg TA) groups on D 18 and 36. All treatment groups (7 replicates per treatment) were compared using PROC MIXED followed by the Tukey's individual comparison test. Orthogonal polynomial contrasts were used to evaluate the significance of linear or quadratic effects of the supplementation of TA in broilers.

increased relative mRNA expression of *Z O 2* ($p < 0.05$). The supplementation of TA linearly increased relative mRNA expression of claudin 2 (*CLDN2*; $p < 0.01$) and junctional adhesion molecule 2 (*JAM2*; $p < 0.05$). The TA2 group had significantly higher relative mRNA expression of mucin 2 (*MUC2*) compared to the TA0.25 group ($p < 0.05$), and the supplementation of TA linearly increase relative mRNA expression of *MUC2* ($p < 0.05$). The TA1 group had significantly higher relative mRNA expression of sodium-dependent neutral amino acid transporter (*B0AT1*) compared to the TA0.25 group ($p < 0.05$), and the supplementation of TA linearly increase relative mRNA expression of *B0AT1* ($p < 0.05$). The supplementation of TA tended to modulate ($p = 0.061$) and quadratically increased relative mRNA expression of peptide transporter 1 (*PepT1*) ($p < 0.05$). The TA1 and TA2 groups had significantly higher relative mRNA expression of sodium glucose cotransporter 1 (*SGLT1*) compared to the TA0.25 group, and the supplementation of TA linearly increased relative mRNA expression of *SGLT1* ($p < 0.01$). The TA1 group had significantly higher relative mRNA expression of excitatory amino acid transporter 3 (*EAAT3*) compared to the TA0, TA0.25, and TA0.5 groups, and the supplementation of TA quadratically increased relative mRNA expression of *EAAT3* ($p < 0.05$). The supplementation of TA tended to modulate ($p = 0.064$) and quadratically increased relative mRNA expression of mucin 2 (*MUC2*) on D 36 ($p < 0.05$). However, no differences in relative mRNA expression of tight junction proteins and nutrient transporters were observed among the treatments on D 36 ($p > 0.1$).

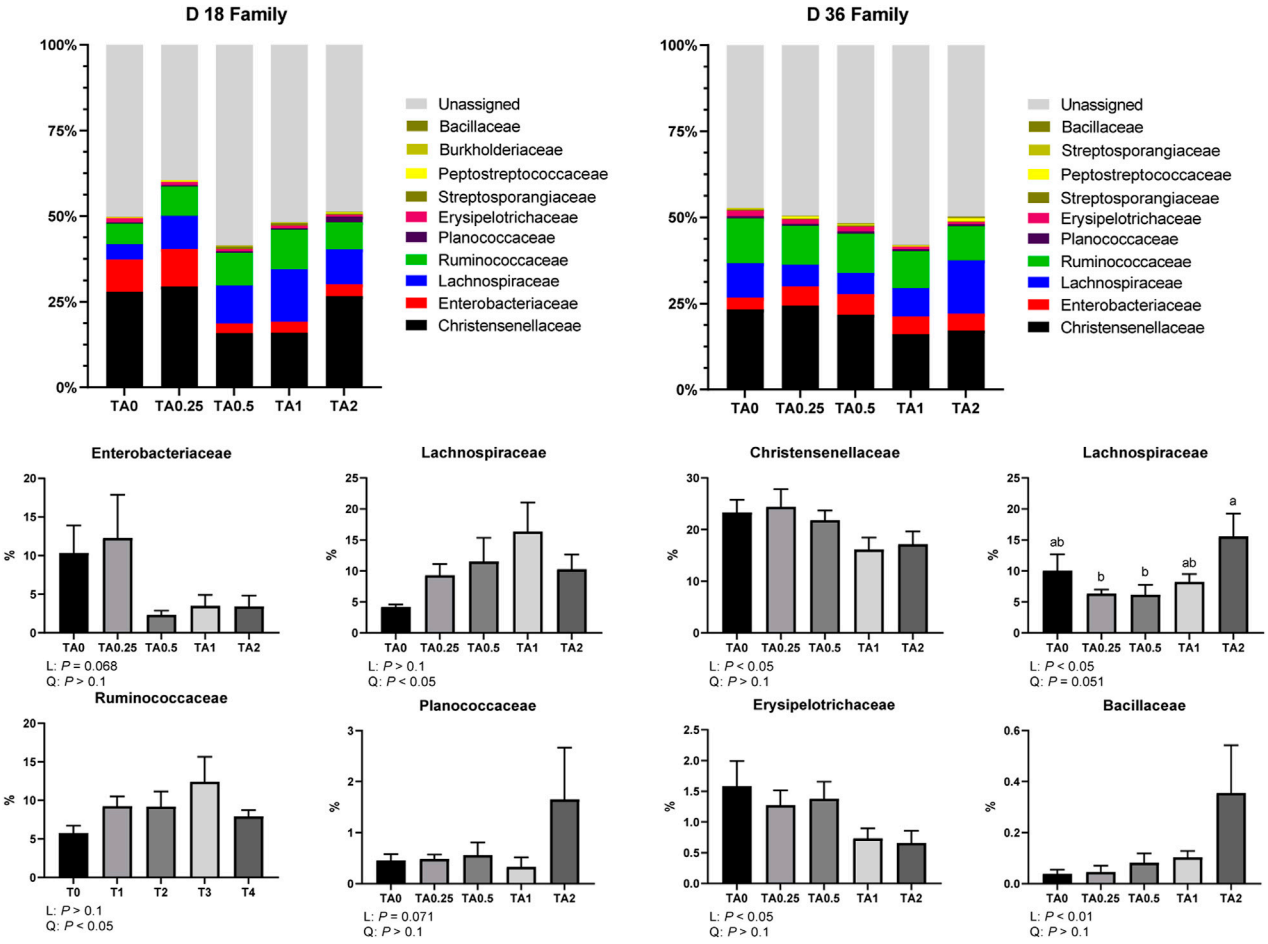

FIGURE 5
Family-level composition of the cecal microbial communities in the TA0 (tannic acid 0; basal diet without TA), TA0.25 (tannic acid 0.25; basal diet + 0.25 g/kg TA), TA0.5 (tannic acid 0.5; basal diet + 0.5 g/kg TA), TA1 (tannic acid 1; basal diet + 1 g/kg TA), and TA2 (tannic acid 2; basal diet + 2 g/kg TA) groups on D 18 and 36. All treatment groups (7 replicates per treatment) were compared using PROC MIXED followed by the Tukey's individual comparison test. Orthogonal polynomial contrasts were used to evaluate the significance of linear or quadratic effects of the supplementation of TA in broilers.

3.6 Liver total antioxidant capacity, concentrations of glutathione and oxidized glutathione, and activities of superoxide dismutase

The supplementation of TA tended to modulate TAC in the liver on D 36 (p = 0.073). No differences were observed in concentrations of GSH and GSSG and activities of SOD in the liver on D 18 and 36 (Table 8; p > 0.1).

3.7 Alpha diversity in the cecal bacterial communities

Alpha diversity indices including faith's phylogenetic diversity, observed features, pielou evenness, and shannon entropy in the cecal bacterial communities on D 18 and 36 are shown in Figure 1. On D 18, the TA1 and TA2 groups had significantly lower faith's phylogenetic diversity (communities' evolutionary distance) compared to the TA0 group (p < 0.05), and the supplementation of TA linearly reduced faith's phylogenetic diversity and observed features (richnesss) in the cecal bacterial communities (p < 0.05). The supplementation of TA tended to linearly reduce shannon entropy (richness and evenness) in the cecal bacterial communities (p = 0.059). On D 36, the TA2 group had lower faith's phylogenetic diversity and observed features compared to TA0, TA0.25, and TA0.5 groups. The supplementation of TA linearly reduced faith's phylogenetic diversity (p < 0.01), observed features (p < 0.01), pielou evenness (evenness; p < 0.05), and shannon entropy (p < 0.01) in the cecal bacterial communities.

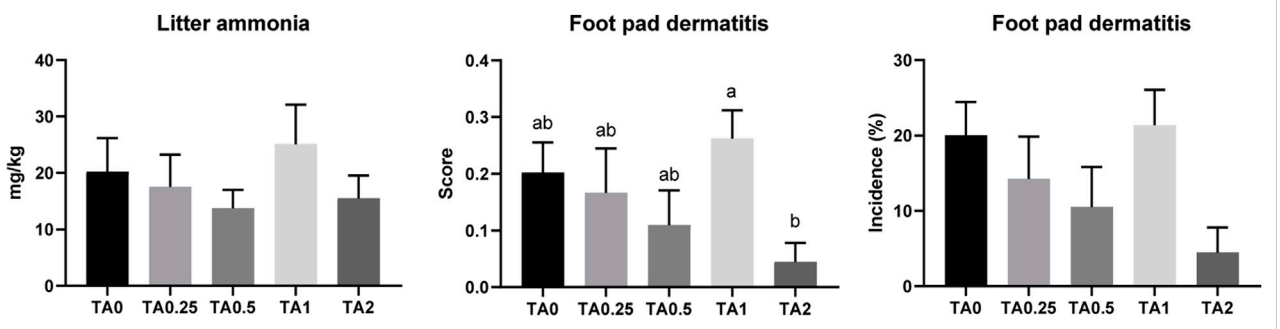

FIGURE 6
Litter ammonia concentration (mg/kg) and foot pad dermatitis score and incidence (%) in the TA0 (tannic acid 0; basal diet without TA), TA0.25 (tannic acid 0.25; basal diet + 0.25 g/kg TA), TA0.5 (tannic acid 0.5; basal diet + 0.5 g/kg TA), TA1 (tannic acid 1; basal diet + 1 g/kg TA), and TA2 (tannic acid 2; basal diet + 2 g/kg TA) groups on D 42. For litter ammonia concentration, all treatment groups (7 replicates per treatment) were compared using PROC MIXED followed by the Tukey's individual comparison test, and different letters in the same row means significant differences ($p < 0.05$) among the treatments. Orthogonal polynomial contrasts were used to evaluate the significance of linear or quadratic effects of the supplementation of TA. Foot pad dermatitis score were analyzed using the Kruskal–Wallis test followed by the Dwass–Steel–Critchlow–Fligner *post hoc* test.

3.8 Beta diversity in the cecal bacterial communities

As shown in Figure 2, the TA1 group had significantly greater unweighted unifrac distance (the sum of the branch length without considering bacterial abundance) compared to the TA0 group on D 18. The TA0.5, TA1, and TA2 groups had significantly greater unweighted unifrac distance compared to the TA0.25 group. The TA0.25 group had significantly greater unweighted unifrac distance compared to the TA0.5 group ($p < 0.05$). The TA0.5 and TA2 groups had significantly greater weighted unifrac distance (the sum of the branch length with considering bacterial abundance) compared to the TA0 group. The TA0.5 and TA2 groups had significantly greater weighted unifrac distance compared to the TA0.25 group. On D 36, TA0.25 had significantly lower unweighted unifrac distance compared to the TA0 group. The TA2 group had significantly greater unweighted unifrac distance compared to the TA0.25 and TA0.5 groups. The TA0, TA0.25, and TA0.5 groups had significantly higher unweighted unifrac distance compared to the TA2 group. The TA1 and TA2 groups had significantly greater weighted unifrac distance compared to the TA0 group. The TA2 group had significantly higher weighted unifrac distance compared to the TA0.25 group. However, no visual differences were observed in the beta diversity indices including weighted and unweighted emperor on D 18 and 36 (Figure 3).

3.9 Bacterial composition in the cecal bacterial communities

As shown in Figure 4, the relative abundance of the phylum Actinobacteria was linearly increased by the supplementation of TA on D 18. On D 36, the relative abundance of the phylum Firmicutes was quadratically reduced by the supplementation of TA, and the TA1 group had significantly lower relative abundance of the phylum Firmicutes compared to the TA0 group. The relative abundance of the phylum Bacteroidetes was linearly ($p < 0.05$) and quadratically ($p < 0.05$) increased by the supplementation of TA. The supplementation of TA quadratically decreased the ratio of the phyla Firmicutes and Bacteroidetes ($p < 0.05$).

As shown in Figure 5, the supplementation of TA tended to linearly reduce the relative abundance of the family Enterobacteriaceae ($p = 0.068$) and tended to linearly increase the relative abundance of the family Planococcaceae ($p = 0.071$) on D 18. The relative abundance of the families Lachnospiraceae and Ruminococcaceae was quadratically increased by the supplementation of TA. On D 36, the supplementation of TA linearly decreased the relative abundance of the families Christensenellaceae and Erysipelotrichaceae ($p < 0.05$). The supplementation of TA linearly increased the relative abundance of the family Bacillaceae ($p < 0.01$). The supplementation of TA linearly increased the relative abundance of the family Lachnospiraceae ($p < 0.05$) and tended to quadratically increased the relative abundance of the family Lachnospiraceae ($p = 0.051$). The TA2 group had significantly higher relative abundance of the family Lachnospiraceae compared to the TA0.25 and TA0.5 groups.

3.10 Litter ammonia concentration and foot pad dermatitis

There were no differences in litter ammonia concentrations among the treatments on D 42 (Figure 6). The TA1 group had a significantly higher FPD score compared to the TA2 group on D

TABLE 9 Bone health parameters including bone mineral content (BMC; g), bone mineral density (BMD; g/cm^2) and body composition parameters including tissue weight (g), lean weight (g), fat weight (g), body fat percentage (%), and lean:fat (g/g) in broilers fed diets supplemented with tannic acid on D 42.

								Polynomial contrast[c]	
Items	TA0	TA0.25	TA0.5	TA1	TA2	SEM	*p*-value[b]	Linear	Quadratic
BMC	585.71[a]	546.57[a,b]	508.29[a,b]	525.43[a,b]	464.86[b]	59.76	0.011	0.001	0.516
BMD	216.14[a]	204[a,b]	206.43[a,b]	205.71[a,b]	199.71[b]	9.97	0.051	0.017	0.428
Tissue weight	3,668.44	3,444.03	3,388.12	3,462.08	3,304.22	359.7	0.430	0.141	0.596
Fat	668.38	644.74	677.21	712.4	686.23	84.33	0.663	0.401	0.435
Fat percentage	18.39	18.81	19.93	20.79	20.73	2.02	0.113	0.022	0.155
Lean weight	2,980.71	2,799.29	2,710.91	2,749.55	2,616.1	310.87	0.285	0.065	0.465
Lean:Fat	4.5	4.38	4.02	3.9	3.84	0.55	0.115	0.021	0.194

[a]TA0 (tannic acid 0; basal diet without TA); TA0.25 (tannic acid 0.25; basal diet + 0.25 g/kg TA); TA0.5 (tannic acid 0.5; basal diet + 0.5 g/kg TA); TA1 (tannic acid 1; basal diet + 1 g/kg TA); and TA2 (tannic acid 2; basal diet + 2 g/kg TA).
[b]Treatment groups (7 replicates per treatment) were compared using PROC MIXED, followed by the Tukey's individual comparison test. Different letters in the same row means significant differences ($p < 0.05$) among the treatments.
[c]Orthogonal polynomial contrasts were conducted to assess the significance of linear or quadratic effects of the supplementation of TA in broilers.

TABLE 10 Hot weight, abdominal fat (g and %) weight and meat yield in broilers fed diets supplemented with tannic acid on D 43[a].

								Polynomial contrast[c]	
Items	TA0	TA0.25	TA0.5	TA1	TA2	SEM	*p*-value[b]	Linear	Quadratic
Hot weight (g)	2,890.9	2,873.5	2,798.0	2,841.7	2,792.6	124	0.488	0.174	0.632
Abdominal fat (g)	42.10	40.48	42.67	40.05	48.29	7.21	0.237	0.077	0.197
Abdominal fat (%)	1.45	1.41	1.52	1.41	1.73	0.23	0.072	0.018	0.189
Total chilled weight	2,931.1	2,899.3	2,828.1	2,865.0	2,817.5	129.5	0.449	0.142	0.539
Legs (%)	26.92[b]	27.36[a,b]	27.95[a,b]	27.52[a,b]	28.40[a]	0.92	0.053	0.010	0.736
Breast (%)	27.22	26.29	26.33	26.15	26.00	1.30	0.457	0.168	0.350
Tender (%)	5.10	4.91	4.78	4.95	4.91	0.37	0.633	0.635	0.407
Wings (%)	9.73	9.80	10.05	9.89	9.96	0.37	0.525	0.349	0.400
Skeleton (%)	31.04	31.64	30.89	31.49	30.74	1.48	0.751	0.557	0.541

[a]TA0 (tannic acid 0; basal diet without TA); TA0.25 (tannic acid 0.25; basal diet + 0.25 g/kg TA); TA0.5 (tannic acid 0.5; basal diet + 0.5 g/kg TA); TA1 (tannic acid 1; basal diet + 1 g/kg TA); and TA2 (tannic acid 2; basal diet + 2 g/kg TA).
[b]Treatment groups (7 replicates per treatment) were compared using PROC MIXED, followed by the Tukey's individual comparison test.
[c]Orthogonal polynomial contrasts were conducted to assess the significance of linear or quadratic effects of the supplementation of TA in broilers.

42. However, no differences were observed in the incidence of FPD among the treatments on D 42 ($p > 0.1$).

3.11 Bone health parameters and body composition

The supplementation of TA linearly reduced BMD ($p < 0.01$) and BMC ($p < 0.05$), and the TA2 group tended to have lower BMD ($p = 0.051$) and had significantly lower BMC ($p < 0.05$) compared to the TA0 group ($p < 0.05$) on D 42 (Table 9). The body fat percentage was linearly increased by the supplementation of TA ($p < 0.05$), and the supplementation of TA tended to reduce lean weight ($p = 0.065$). The lean:fat was linearly reduced by the supplementation of TA ($p < 0.05$).

3.12 Hot weight, abdominal fat, chilled weight, and meat yield

On D 43, hot weight was linearly decreased by the supplementation of TA ($p < 0.05$) (Table 10). The

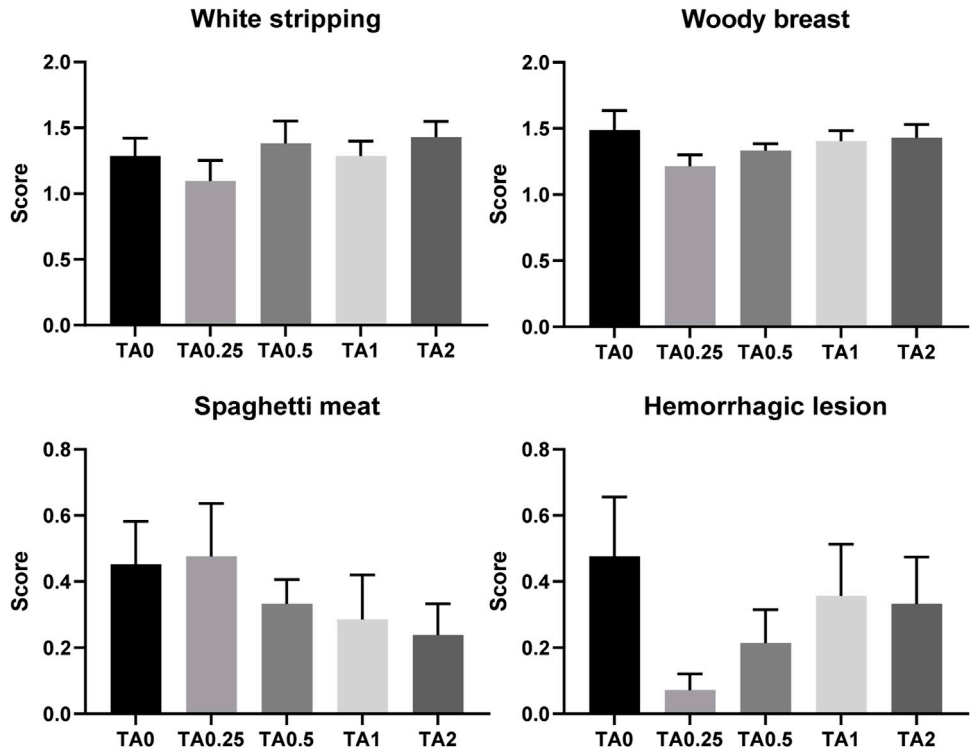

FIGURE 7
Breast muscle myopathies including white striping, woody breast, spaghetti meat, and hemorrhagic lesion in the TA0 (tannic acid 0; basal diet without TA), TA0.25 (tannic acid 0.25; basal diet + 0.25 g/kg TA), TA0.5 (tannic acid 0.5; basal diet + 0.5 g/kg TA), TA1 (tannic acid 1; basal diet + 1 g/kg TA), and TA2 (tannic acid 2; basal diet + 2 g/kg TA) groups on D 42. There were 21 replicates per treatment and core for each parameter was analyzed using the Kruskal–Wallis test followed by the Dwass–Steel–Critchlow–Fligner *post hoc* test.

supplementation of TA tended to linearly increase abdominal fat weight ($p = 0.077$) and linearly increased abdominal fat percentage ($p < 0.05$). Proportion of leg weight was increased by the supplementation of TA ($p < 0.05$).

3.13 Breast muscle myopathies and meat color (L*, a*, b*), pH, drip loss, thawing loss, and cooking loss in the breast meat

No differences were observed in average breast muscle myopathy scores for white striping, woody breast, spaghetti meat, or hemorrhagic lesions as shown in Figure 7 ($p > 0.1$). The supplementation of TA linearly reduced pH of the breast meat ($p < 0.05$; Table 11) and linearly increased redness (a*) ($p < 0.01$). The TA2 group had significantly greater redness value compared to the TA0 group. The yellowness (b*) tended to be increased due to the supplementation of TA ($p = 0.086$). The supplementation of TA quadratically modulated cooking loss ($p < 0.05$) in the breast meat, and the TA2 group tended to have lower cooking loss compared to the TA1 group ($p = 0.054$).

4 Discussion

Our previous study showed that higher than 1 g/kg TA exhibited antinutritional effects to reduce growth performance, whereas 0.5 g/kg TA increased antioxidant capacity in broilers on D 21 (Choi et al., 2022d). The supplementation of TA (0.5 g/kg–2.75 g/kg) improved gut barrier integrity and decreased oocyst shedding in broilers infected with *Eimeria maxima* (Choi et al., 2022c). The supplementation of TA (1 g/kg–2 g/kg) enhanced growth performance and gut health *via* antimicrobial and immunostimulatory effects in broilers infected with *Salmonella* Typhimurium (Choi et al., 2022a). Based on our previous studies, we aimed to evaluate the efficacy of the supplementation of TA in broilers raised for 42 days in floor pens in the current study to simulate actual conditions of broiler production. Sampling points were determined at D 18 and D 36 to represent starter/grower phase and finisher phase, respectively, in the current study. Therefore, the purpose of the study was to investigate the effects of the TA supplementation (up to 2 g/kg TA) on growth performance, intestinal morphology, activities of brush border digestive enzymes, AID of nutrients, relative mRNA

TABLE 11 The pH, meat color (L*, a*, b*), drip loss (%), thawing loss (%), and cooking loss (%) in the breast meat of broilers fed diets supplemented with tannic acid[a].

Items	TA0	TA0.25	TA0.5	TA1	TA2	SEM	*p*-value[b]	Polynomial contrast[c]	
								Linear	Quadratic
pH	6.02	6.06	6.02	5.99	5.96	0.08	0.225	0.037	0.944
Lightness (L*)	58.21	57.60	57.64	59.24	57.94	1.6	0.319	0.701	0.319
Redness (a*)	0.616[b]	1.055[a,b]	0.755[b]	1.041[a,b]	1.765[a]	0.57	0.008	0.001	0.471
Yellowness (b*)	13.53	13.81	13.05	14.27	14.40	1.18	0.220	0.086	0.933
Drip loss	2.72	2.93	2.81	2.48	2.80	0.90	0.911	0.865	0.626
Thawing loss	2.83	2.77	2.72	3.26	2.43	0.51	0.651	0.574	0.292
Cooking loss	29.97[a,b]	30.66[a,b]	30.85[a,b]	32.72[a]	27.99[b]	2.78	0.054	0.168	0.011

[a]TA0 (tannic acid 0; basal diet without TA); TA0.25 (tannic acid 0.25; basal diet + 0.25 g/kg TA); TA0.5 (tannic acid 0.5; basal diet + 0.5 g/kg TA); TA1 (tannic acid 1; basal diet + 1 g/kg TA); and TA2 (tannic acid 2; basal diet + 2 g/kg TA).

[b]Treatment groups (21 replicate per treatment) were compared using PROC MIXED, followed by the Tukey's individual comparison test. Different letters in the same row means significant differences ($p < 0.05$) among the treatments.

[c]Orthogonal polynomial contrasts were conducted to assess the significance of linear or quadratic effects of the supplementation of TA in broilers.

expression of tight junction proteins and nutrient transporters, liver antioxidant capacity, bone health, body composition, and meat yield and quality in broilers on D 42.

In the current study, diets were crumbled and pelleted in the starter and grower/finisher phases, respectively. Almost all commercial broiler feeds are crumbled or pelleted in current broiler production (Brickett et al., 2007). The pelleting processing includes steaming (e.g., conditioning) with high temperature and pelleting (e.g., agglomeration) to produce large particles from small particles (Abdollahi et al., 2013). In these harsh conditions, stability and molecular or physical traits of feed additives can be altered (Choi et al., 2020a). Kim et al. (2010) reported that thermal process (e.g., autoclave heat) improved antioxidant capacity and antimicrobial effects of TA, and our unpublished data showed that pelleting temperature (80°C) improved antimicrobial effects of TA against *S.* Typhimurium in *vitro* conditions. However, steaming and agglomeration during feed processing may induce interactions of TA and nutrients (e.g., proteins, polysaccharides, etc.), which can decrease nutrient utilization in the gastrointestinal tract of chickens.

In the current study, supplementation of TA linearly reduced feed intake of broiler chickens in the starter phase while supplementation of TA did not influence feed intake in the grower and finisher phases. Tannins are known to induce astringent taste by forming complexes with salivary proline-rich proteins, which can decrease feed palatability and feed intake in animals (Treviño et al., 1992; Lee et al., 2010). In the starter and grower phases, BW and feed efficiency were linearly reduced, but no statistical differences were observed in the finisher phase in broilers fed dietary TA in the current study. These results is in consistent with our previous study suggested that young broilers are less tolerant to the intake of TA (Choi et al., 2022b). These results had different trends from our previous studies as follows. Choi et al. (2022d) reported that higher than 1 g/kg TA started to linearly reduce BW of broilers on D 21. However, growth retardation effects of TA were exhibited from 0.25 g/kg TA in the current study. Potentially, the pelleting process may have induced the interaction between TA and dietary nutrients (e.g., proteins). Although AID was not measured on D 18 in the current study, nutrient digestibility would have been severely decreased by the supplementation of TA. However, the supplementation of TA improved AID of DM, OM, ash, and CP on D 36 potentially as compensation effects and did not alter growth performance in the finisher phase of the current study, indicating that older birds have more tolerance to the TA supplementation. Potentially, mature gastrointestinal tracts (e.g., lower pH and higher pancreatic enzymes) may have hydrolyzed TA-nutrient complex in broilers on D 36 (Adamczyk et al., 2011). In contrast, a previous study by Tonda et al. (2018) reported that the supplementation of TA extract improved BWG and feed efficiency in cocci-vaccinated (live vaccine) broilers fed pelleted feed on D 0 to 21. This would be because live vaccines are known to spread coccidiosis in a flock and can decrease growth performance, which can provide challenging conditions to chickens, and this indicates the supplementation of TA extract could be effective in challenging conditions (Greif, 2000). However, the basal diets included a coccidiostat (monensin sodium; 500 mg/kg) to exclude the anti-coccidial effects of TA and was conducted in a hygienic laboratory scale facility, and therefore there would be limited gap to improve growth performance of broilers, which potentially explains reduced or maintained growth performance in broilers supplemented with TA in the current study.

On D 18, the TA supplementation linearly increased jejunal VH:CD and quadratically decreased ileal VH:CD along with reduced CD in the current study. Increased VH:CD indicates

augmented nutrient digestion and absorption in chickens (Abd El-Hack et al., 2020). However, if increased VH:CD was accompanied with decreased CD, it cannot be considered as beneficial effects because deeper CD suggests more proliferation and differentiation of stems cells, which would move to the tip of the villus (Liu et al., 2020). Potentially, the TA supplementation caused an impairment in the development of intestinal morphology by decreasing nutrient utilization *via* forming complex with nutrients (e.g., proteins) (Shinde et al., 2015).

Activities of sucrase, a brush border digestive enzyme, in the jejunum tissue were quadratically increased by the TA supplementation on D 18 in the current study, which suggests that appropriate dosages of TA can still improve gut development in broilers (Yang et al., 2008). Moreover, relative mRNA expression of *MUC2* and nutrients transporters including *B0AT1*, *SGLT1*, *PepT1*, and *EAAT3* were linearly and quadratically increased by the TA supplementation. These data indicate that nutrient utilization capacity of the jejunum could be enhanced by the TA supplementation, but limited availability of nutrients due to interactions between TA and nutrients would be the main factor to decrease growth performance of broilers in the starter and grower phases. Otherwise, reduced availability of nutrients for intestinal absorption due to the formation of TA-nutrient complexes in the luminal side may have increased mRNA expression of nutrients as a resistant reaction to increase nutrient absorption in the gastrointestinal tract (Pinheiro et al., 2013).

Tight junction proteins and *MUC2* are closely associated with gut barrier integrity of broilers (Choi et al., 2020b). In the present study, the TA supplementation linearly and quadratically increased relative mRNA expression of genes related to gut barrier integrity including *ZO2*, *CLDN2*, *JAM2*, and *MUC2* in the jejunum. According to our previous study, the TA supplementation decreased gut permeability in broilers infected with *E. maxima* (Choi et al., 2022c). A previous study by Yu et al. (2020) reported that the supplementation of TA improved gut barrier integrity in weaned piglets. These results suggest that the supplementation of TA has potential to increase gut barrier integrity in broilers.

There were no differences in TAC, concentrations of GSH and GSSG, activities of SOD in the liver on D 18 and 36 among the treatments in the present study. Many *in vitro* studies showed that TA, a polyphonic compound, has strong antioxidant capacity (Andrade Jr et al., 2005; Gülçin et al., 2010). However, direct antioxidant effects of TA in the chickens were in question. This is because TA should stay inside of the chicken body for a sufficient time by maintaining appropriate forms to exhibit antioxidant capacity (Karakaya, 2004). Deposition of TA in the internal organs (e.g., liver) in broiler chickens should be further investigated. Choi et al. (2022d) showed that the supplementation of TA at 0.5 g/kg indirectly improved antioxidant system by enhancing activities of SOD in the liver. The differences would be originated from the pelleting process, which may reduce bioavailability of TA by forming TA-nutrient complexes. However, under the heat stress condition, the supplementation of TA (10 g/kg) showed potential to improve antioxidant capacity in broilers (Ebrahim et al., 2015).

In the present study, the supplementation of TA linearly decreased alpha diversity indices including faith's phylogenetic diversity (communities' evolutionary distance; D 18 and 36), observed features (richness; D 18 and 36), pielou evenness (evenness; D 36), and shannon entropy (richness and evenness; D 36). While it is still controversial, lower alpha diversity may indicate less stable and unmature microbial communities in the gastrointestinal tract of animals (Ebrahim et al., 2015). Moreover, beta diversity indices (unweighted and weighted unifrac) showed that different dosages of TA could modulate cecal microbial communities in broilers.

The relative abundance of the phylum Actinobacteria was linearly increased by the supplementation of TA on D 18 in the current study. The phylum Actinobacteria includes *Bifidobacteria* spp., which can improve gut barrier integrity and immune system of animals and is considered as a beneficial phylum in animals (Binda et al., 2018). On D 18, the supplementation of TA linearly reduced relative abundance of the family Enterobacteriaceae, which includes diverse pathogens such as *Salmonella* spp., *Shigella*, *Escherichia coli*, etc. Consistently, our previous study reported that the TA supplementation reduced cecal *Salmonella* Typhimurium load in the starter phase of broilers (Choi et al., 2022a). Moreover, the TA supplementation quadratically increased the relative abundance of the families Lachnospiraceae and Ruminococcaceae, which have an important role in maintain gut homeostasis by producing volatile fatty acid *via* fiber degradation (Biddle et al., 2013). Consistently, Koo and Nyachoti (2019) reported that the TA supplementation enhanced cecal volatile fatty acid production in pigs. However, on D 36, a ratio of the phyla Firmicutes and Bacteroidetes was quadratically reduced by the supplementation of TA in the current study. The lower ratio of the phyla Firmicutes and Bacteroidetes suggests a lower capacity of fiber degradation and production of short chain fatty acids, important energy sources for the host animals (Singh et al., 2012). In the current study, the TA supplementation linearly decreased the relative abundance of the families Christesenellaceae and Erysipelotrichaceae, which have an important role in fiber degradation to produce short chain fatty acids (Wasti et al., 2021). However, the TA2 group significantly increased the relative abundance of the family Lachnospiraceae compared to the TA0.25 and TA0.5 groups, and the TA supplementation linearly increased the relative abundance of the family Bacillaceae, which are positively correlated with growth performance and feed efficiency (Moula et al., 2018). While the supplementation of TA reduced the relative abundance of

the families Christensenellaceae and Erysipelotrichaceae, the TA supplementation still increased the relative abundance of the families Lachnospiraceae and Bacillaceae in the current study.

Ammonia (NH_3) in poultry houses can negatively affect the health of chickens and humans as well as harm the environment (Naseem and King, 2018). Chickens synthesize uric acid as the end product of purine and protein metabolism, and uric acid is converted into ammonia *via* microbial fermentation in the ceca or in the litter (Kim and Patterson, 2003a; b; Naseem and King, 2018). In the current study, we hypothesized that litter ammonia concentration could be reduced by the supplementation of TA because the TA supplementation increased AID of CP on D 36. Crude protein digestibility is closely associated with litter ammonia concentration (Brink et al., 2022). Moreover, Arzola-Alavarez et al. (2020) reported that the addition of pine bark tannin in the litter reduced ammonia accumulation in the poultry litter. Unabsorbed TA could be excreted to the litter and potentially modulate ammonia concentration in the litter. However, no differences were observed in the litter ammonia on D 42. The TA1 group had a significantly higher FPD score compared to the TA2 group, which implies that the supplementation of TA can modulate FPD score in broilers. Litter ammonia and FPD are closely associated (Youssef et al., 2011), and our current study also showed that litter ammonia and FPD score had similar trends. Otherwise, BW could simply affect litter ammonia because bigger birds would excrete more manure in the litter. The TA1 group had numerically close BW compared to the TA0 group, whereas the TA2 group had the numerically lowest BW on D 42 in the current study. These results may explain the trends of litter ammonia and FPD score of broilers fed diets supplemented with TA in the current study.

Our previous study showed that there was only a linear tendency ($0.05 < p \leq 0.10$) to reduce bone health parameters including BMD and BMC in broilers on D 21, but no statistical differences were observed between the groups fed 0 g/kg TA and 2.5 g/kg TA (Choi et al., 2022d). However, in the present study, BMD and BMC were linearly reduced by the supplementation of TA in broilers, and 2 g/kg TA supplementation significantly reduced BMD and BMC compared to the control group on D 42. Possibly, the TA supplementation might have reduced utilization of calcium, phosphorous, and iron, which are important minerals for bone formation in broilers (Hassan et al., 2003; Afsana et al., 2004; Katsumata et al., 2009; Shang et al., 2015). Moreover, the pelleting process may have induced more formation of TA-mineral complexes, which dramatically reduced BMD and BMC in broilers. A previous study by Tomaszewska et al. (2018) also reported that inclusion of low-tannin faba bean (condensed tannins) negatively affected tibia traits (weight, reduction of the cross section area, and wall thickness) in broilers.

In the present study, the fat percentage measured by DEXA was linearly increased and the ratio of lean to fat decreased in broilers on D 42. This result is in stark contrast to our previous results reporting that the supplementation of TA increased the ratio of lean to fat in broilers on D 21 (Choi et al., 2022d). Discrepancies between the findings of these studies could have originated from differences in the supplementation period and age of birds (D 42 vs. D 21) and feed form (pelleted vs. mash). In our previous study (Choi et al., 2022d), fat accumulation was reduced in broilers D 21 due to less production of cecal volatile fatty acids, which potentially resulted in an imbalance of energy homeostasis. However, in the current study, the pelleting process may have decreased nutrient utilization by inducing the formation of TA-nutrient complexes, and young broilers, which did not have mature enough gastrointestinal tract to hydrolyze TA-nutrient complexes, may have decreased digestibility of energy and nutrients. To compensate limited growth rate at young stage due to reduced nutrient and energy utilization by the supplementation of TA, the broilers may have altered their body metabolism to increase the accumulation of fat (Kobylińska et al., 2022). Consistently, a previous study by Starčević et al. (2015) also showed that the supplementation of TA (5 g/kg) increased fat accumulation in the breast and thigh meat in broilers on D 35.

In the current study, absolute and relative weight of abdominal fat were increased by the supplementation of TA, which is consistent with DEXA results. The abdominal fat weight is a dependable parameter to represent body fat content because abdominal fat is the main and largest area (up to 4% of BW) of fat accumulation in broilers (Thomas et al., 1983; Fouad and El-Senousey, 2014). Furthermore, the supplementation of TA resulted in linearly increased leg meat and linearly decreased breast meat yield ($p = 0.168$), while statistical differences were not observed. Leg meat had higher fat content compared to breast meat (Pikul et al., 1985). Potentially, increased fat metabolism in broiler body by the supplementation of TA resulted in increased leg meat yield and decreased breast meat yield to increase fat accumulation in chickens. Fatty broiler meat and low yield of breast meat are not preferred in modern broiler production (Fouad and El-Senousey, 2014). Low pH of the breast meat results in higher lightness, lower redness, and higher yellowness by decreasing water binding capacity (Allen et al., 1997; Qiao et al., 2001). Our current study showed that the supplementation of TA decreased pH, increased redness and yellowness, and reduced cooking loss in the breast meat. This would be due to immatureness of breast meat and low growth performance caused by the TA supplementation. According to Bianchi et al. (2007), smaller broilers had lower pH and higher redness when compared to the bigger broilers. However, the lightness, an important factor to indicate pale, soft, and exudative (PSE)-like condition in poultry meat (Petracci et al., 2004), was not modulated due to the supplementation of TA in the current

study. Moreover, the supplementation of TA did not dramatically altered those meat quality parameters, and the values were in still normal range (Hertanto et al., 2018). No differences were observed in the breast muscle myopathies such as white striping, woody breast, spaghetti meat, and hemorrhagic lesion in the current study. However, numerical reductions in the scores of spaghetti meat and hemorrhagic lesion in the breast meat would be associated with retarded growth rate and immatureness of breast meat in broilers fed dietary TA because breast meat myopathies are frequently observed in the fast-growing broilers (Caldas-Cueva and Owens, 2020). Therefore, the supplementation of TA did not significantly alter meat quality of broiler chickens.

In the current study, discrepant results from our previous studies and negative effects of the TA supplementation would be mainly attributed to the pelleting process on diets, which induced the formation of TA-nutrient complexes. In order to minimize or inhibit the interaction of TA and dietary nutrients during pelleting process, the encapsulation of TA can be a potential strategy (Choi et al., 2020a). Encapsulation process can provide protection for TA and release TA in the target site of the gastrointestinal tract, where many pathogens propagate (e.g., lower gut) (Choi et al., 2020a). A previous study by Wang M. et al. (2020) reported that encapsulated TA showed beneficial effects on gut health and microbiota of weaned piglets. Future studies should include: 1) investigation of appropriate methods to encapsulate TA and its stability during pelleting process and in the gastrointestinal tract; and 2) investigation into the effects of encapsulated TA on growth performance and gut health in broilers on D 42.

5 Conclusion

The TA supplementation up to 2 g/kg in pelleted diets positively affected gut microbiota, enhanced brush border digestive enzyme activities, upregulated genes related to gut barrier integrity and nutrient transportation in the starter/grower phases, and improved nutrient digestibility in the finisher phase. However, the supplementation of TA decreased overall growth performance and feed efficiency, increased fat accumulation, and negatively affected gut microbiota, bone health, and meat production in broilers on D 42. Therefore, further processing should be applied on TA to enhance their potential beneficial effects on broilers.

Data availability statement

The authors acknowledge that the data presented in this study must be deposited and made publicly available in an acceptable repository, prior to publication. Frontiers cannot accept a manuscript that does not adhere to our open data policies.

Ethics statement

The animal study was reviewed and approved by the Institutional Animal Care and Use Committee at the University of Georgia, Athens, GA.

Author contributions

JC and WK conceived and designed the study. JC, GL, DG, JW, BB, and ZH conducted the experiment. JC analyzed the data. BB and ZH advised and helped meat analyses. JC wrote the manuscript, and all authors critically review the paper. WK supervised all processes for this manuscript.

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Conflict of interest

The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

Publisher's note

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Effects of a myostatin mutation in Japanese quail (*Coturnix japonica*) on the physicochemical and histochemical characteristics of the *pectoralis major* muscle

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The aim of this study was to compare the carcass, meat quality, and histochemical characteristics of *pectoralis major* (PM) muscle between wild type (WT) and myostatin (Mstn) homozygous mutant (HO) quail lines. The HO quail line exhibited significantly heavier body weight (HO vs. WT, 115.7 g vs. 106.2 g, approximately 110%) and PM muscle weight (HO vs. WT, 18.0 g vs. 15.2 g, approximately 120%) compared to the WT ($p < 0.001$). However, the two groups had similar traits (pH, redness, yellowness, and drip loss) for meat quality, although slightly higher lightness and cooking loss were observed in the mutant quail (103% and 141%, respectively, $p < 0.05$). For histochemical traits of PM muscle, Mstn mutant quail exhibited lower type IIA and higher type IIB percentage in the deep region than WT quail ($p < 0.05$), indicating a fiber conversion from the type IIA to IIB. However, the two quail lines had comparable histochemical traits in the superficial region ($p > 0.05$). These data suggest that Mstn mutation greatly increases muscle mass without significantly affecting meat quality.

KEYWORDS

myostatin mutation, meat quality, muscle fiber conversion, pectoralis major muscle, quail

1 Introduction

In the past several decades, the consumption of poultry meat and development of related industries has been steadily growing due to increasing consumer preference for poultry meat (Soglia et al., 2016). This progressive growth is primarily related to the perception of an improved nutritional profile of poultry *versus* meat from other livestock species, such as the low-fat content and the high quantity of high quality protein found in lean poultry meat (Soglia et al., 2016). Meat-type poultry including chicken and turkey have been selected for heavy body and breast weights over multiple generations, and generally exhibit increased muscle mass and faster growth rate when compared with previous generations (Nestor et al., 2008; Putman et al., 2017). However, with the fast-growing performance of modern poultry, excessive fat deposition is one of the biggest concerns for producers and processors, which can lead to decreased consumer acceptability and economic loss (Fouad and El-Senousey, 2014). Thus, decreasing fat accumulation with high rates of lean growth would be a goal in the poultry industry. (Fouad and El-Senousey, 2014).

Myostatin (Mstn) is a well-studied gene that regulates muscle mass and fat deposition. In fact, mutation or knock-out for this gene has been documented to increase muscle mass and decrease fat content in different animal species, such as pigs, cattle, and mice (McPherron and Lee, 2002; Fiems, 2012; Cai et al., 2017). Our previous study also reported that the Mstn knock-out quail line exhibited approximately 30% lower body fat content and approximately 20% heavier muscle mass with increased muscle fiber numbers compared to the wild type (WT) quail line (Lee et al., 2020). Thus, the Mstn gene can be considered as an economically important gene, and genetic selection or manipulation of this gene can contribute to developing leaner lines of poultry that could increase consumers and producers satisfaction.

It has been shown that muscle fiber composition can influence meat quality due to differences in contractile and metabolic traits of muscle fiber types, especially in pigs and poultry that exhibit a rapid rate of *postmortem* metabolism (Choi and Kim, 2009). As the proportion of large diameter glycolytic muscle fibers increases, there is less space between the muscle fibers particularly when compared with small diameter oxidative muscle fibers (Petracci et al., 2017). After exsanguination, limiting the space available to the capillaries that normally remove lactate from the muscle leads to accumulation of lactic acid, consequently causing a more rapid pH decline in muscle, and thus poor meat quality (Petracci et al., 2017). Disruption of Mstn can switch fiber type from slow-to fast-twitch fibers in mature resting muscles in various species of animals, including mice, pigs, and cattle (Stavaux et al., 1994; Hennebry et al., 2009; Qian et al., 2015). However, to our knowledge, there are no studies that have reported both the muscle fiber type composition and meat quality characteristics in any poultry species. Although weights of chicken breast muscle having only type IIB myofibers were not significantly increased by Mstn mutation (Kim et al., 2022), weights of quail breast muscle containing both type IIA and IIB (Choi et al., 2014) were increased by Mstn mutation (Lee et al., 2020). Therefore, Mstn mutant quail can serve as a proper avian model to investigate effects of Mstn mutation on myofiber types and meat quality in poultry. In the current study, we compared histochemical and meat quality characteristics of *pectoralis major* (PM) muscles between WT and Mstn mutant quail.

2 Materials and methods

2.1 Animals care

Japanese quail (*Coturnix japonica*) with a Mstn mutation were produced in our previous study (Lee et al., 2020). All animals used in this study were raised at the poultry facility at the Ohio State University (OSU) in Columbus, Ohio with the same environmental conditions such as consistent room temperature, the same brooder dimensions, and with free access to feed and water after hatch. All experimental procedures and animal care protocols were approved by the Institutional Animal Care and Use Committee (IACUC) of OSU (Protocol 2019A00000024).

2.2 Generation of Mstn mutant quail

As a previously reported (Lee et al., 2020), to analyze genotypes, the genomic DNA was extracted from feather germs and then targeted region in the *Mstn* gene was amplified by PCR with a specific primer set (F: 5′-GCATGGACGAGCTGTACAAGTA, R: 5′-CCCTGCTAATGT TAGGTGCTT) at the condition followed by 35 cycles of 95°C for 40 s, 53°C for 40 s, 68°C for 30 s. The PCR product was sequenced at The Ohio State University Comprehensive Cancer Center.

2.3 Collection of muscle samples

To sample PM muscles, the male quail from the Mstn homozygous mutant (HO, n = 10) and WT (n = 12) lines were euthanized at 2 months of age by CO_2 inhalation according to the IACUC protocol. Body weight (BW) and PM muscle weight (PMW) were measured, and breast percentage was calculated. After measurement of weights of whole PM, cross-sectional area (CSA) of the left PM muscle was measured in an area cut from the lower left to the upper right at the 1/2 point of the muscle (Scheuermann et al., 2004; Choi et al., 2014). Simultaneously, muscle samples (0.5 × 0.5 × 1.0 cm) from the left PM muscle were immediately frozen in liquid nitrogen and stored at −80°C for histochemical analysis. At 15 min *postmortem*, muscle pH value ($pH_{15\ min}$) was measured on each right muscle, and muscle samples were then immediately cooled with an ice-water slurry and stored at 4°C until meat quality analysis. After 24 h *postmortem*, meat quality characteristics, including $pH_{24\ h}$, meat color, drip loss, and cooking loss, were measured using the remaining left-side and entire right-side breasts.

2.4 Meat quality characteristics

Muscle pH values ($pH_{15\ min}$ and $pH_{24\ h}$) at the cranial region of the PM for each sample were measured using a Testo 206-pH2 (Testo AG, Lenzkirch, Germany) with a penetration probe. After 30 min of blooming time at 4°C, surface color of muscle samples at 24 h *postmortem* was determined using a spectrophotometer (CM-700d, Konica Minolta Inc., Ramsey, NJ). Color values, including lightness (L^*), redness (a^*), and yellowness (b^*), were assessed according to the recommendations of the Commission Internationale de l'Eclairage (1978). Drip loss was determined using a meat extract collector tube (Sarstedt Inc., Newton, NC), and percentages of drip loss were calculated with the difference in sample weight before and after 48 h at 4°C. For cooking loss, samples were weighed and put into a polyethylene bag, and then heated in a temperature-controlled water bath at 80°C until the core internal temperature reached 71°C (Honikel, 1998). Cooked samples were cooled in an ice-slurry until equilibration, and cooking loss percentage was calculated by weighing the samples before and after cooking.

2.5 Histochemical analysis

Serial muscle cross-sections (10 μm thickness) were obtained using a cryostat (CM1510S, Leica, Wetzlar, Germany) set

TABLE 1 Comparison of body weight and carcass traits between the wild type (WT) and myostatin homozygous mutant (HO) quail lines at 2-month of age.

	WT (n = 12)	HO (n = 10)	Level of significance
Body weight (g)	106.2 (1.39)[a]	115.7 (1.53)	***
PM muscle weight (g)	15.2 (0.34)	18.0 (0.38)	***
Breast percentage (%)	14.3 (0.22)	15.6 (0.23)	**
CSA of PM muscle (mm^2)	236.0 (5.35)	295.7 (7.19)	***

Levels of significance: NS, no significant; **$p < 0.01$; ***$p < 0.001$.
[a]Standard error of least-square means.
Abbreviations: PM, *pectoralis major*; CSA, cross-sectional area.

at −25°C. To measure fiber characteristics, muscle sections were stained using the myosin ATPase staining kit (KTATP, StatLab, McKinney, TX) following the manufacturer's instructions. All stained samples were analyzed using Image-Pro Plus software (Meida Cybernetics, Silver Spring, MD). In deep and superficial regions, more than 600 fibers in each region were used for statistical analysis of histochemical characteristics, such as percentages of the fiber type. The number percentage of each fiber type was calculated as the proportion of each of the fiber type numbers measured divided by the total fiber numbers measured.

2.6 Statistical analysis

BW, carcass, meat quality, and muscle fiber characteristics between the WT and HO quail lines were analyzed using a general linear mixed model procedure (SAS Institute, Cary, NC). Significant differences of the investigated parameters between the lines were evaluated using the probability difference by setting the significance level at 5% ($p < 0.05$). All data are presented as the least-squares means with standard errors.

3 Results

3.1 Increased body weight and breast muscle weight by myostatin mutation

The HO male quail exhibited a greater BW compared to the WT male quail at 2 months of age (115.7 g vs. 106.2 g, $p < 0.001$, Table 1). PMW, percentages of PMW, and CSA of PM muscle were approximately 18% (18.0 g vs. 15.2 g, $p < 0.001$), 9% (15.6% vs. 14.3%, $p < 0.01$), and 38% (251.4 mm^2 vs. 182.0 mm^2, $p < 0.05$) greater in the HO line than in the WT line, respectively (Table 1).

3.2 Effect of Mstn mutation on meat quality characteristics

As an indicator of glycolytic rate, the early *postmortem* muscle pH and ultimate pH values were not different between the HO and WT groups (Table 2). However, lightness values were greater in the HO quail line compared to the WT (54.3 vs. 52.6, $p < 0.05$); whereas no differences were observed in redness and yellowness values between the two groups (Table 2). The WT and Mstn mutant quail lines exhibited comparable percentage of drip loss (0.93% vs. 0.83%, $p > 0.05$). However, a higher cooking loss was observed in the Mstn mutant quail compared to the WT quail (8.56% vs. 6.06%, $p < 0.05$).

TABLE 2 Comparison of meat quality characteristics between the wild type (WT) and myostatin homozygous mutant (HO) quail lines at 2-month of age.

	WT	HO	Level of significance
$pH_{15\ min}$	6.34 (0.06)[a]	6.34 (0.07)	NS
$pH_{24\ h}$	5.84 (0.04)	5.91 (0.05)	NS
Lightness (L*)	52.6 (0.44)	54.3 (0.48)	*
Redness (a*)	6.70 (0.51)	6.43 (0.56)	NS
Yellowness (b*)	11.6 (1.99)	10.8 (2.18)	NS
Drip loss (%)	0.83 (0.07)	0.93 (0.07)	NS
Cooking loss (%)	6.06 (0.62)	8.56 (0.68)	*

Levels of significance: NS, no significant; *$p < 0.05$.
[a]Standard error of least-square means.

3.3 Increased type IIB myofibers in the deep region of breast muscle by Mstn mutation

There were no significant differences in the percentages of type IIA (67.4% vs. 65.3%, $p > 0.05$, Figure 1A) and type IIB (32.6% vs. 34.7%, $p > 0.05$, Figure 1A) muscle fibers and CSA of the two types of muscle fibers (226.86 μm^2 vs. 255.25 μm^2, $p > 0.05$ for type IIA, and 729.25 μm^2 vs. 814.43 μm^2, $p > 0.05$ for type IIB, Figure 1B) in the superficial region between the WT and HO groups. However, the deep region in the HO group had lower percentages of numbers of type IIA muscle fiber compared to those in the WT group (92.2% vs. 95.6%, $p < 0.01$, Figure 1A). Conversely, higher percentages of type IIB muscle fiber numbers in deep regions were observed in the HO line than in the WT line (7.78% vs. 4.45%, $p < 0.01$, Figure 1A). For the CSA in the deep region, although there were no significant differences in the type IIA 251.29 μm^2 vs. 278.27 μm^2, $p > 0.05$, Figure 1B) between the WT and HO groups, the significance was shown in the type IIB (586.16 μm^2 vs. 864.21 μm^2, $p < 0.05$, Figure 1B) in the two groups. There was a difference in the composition of both muscle fiber types within the deep regions but not in the superficial regions between the WT and HO quail

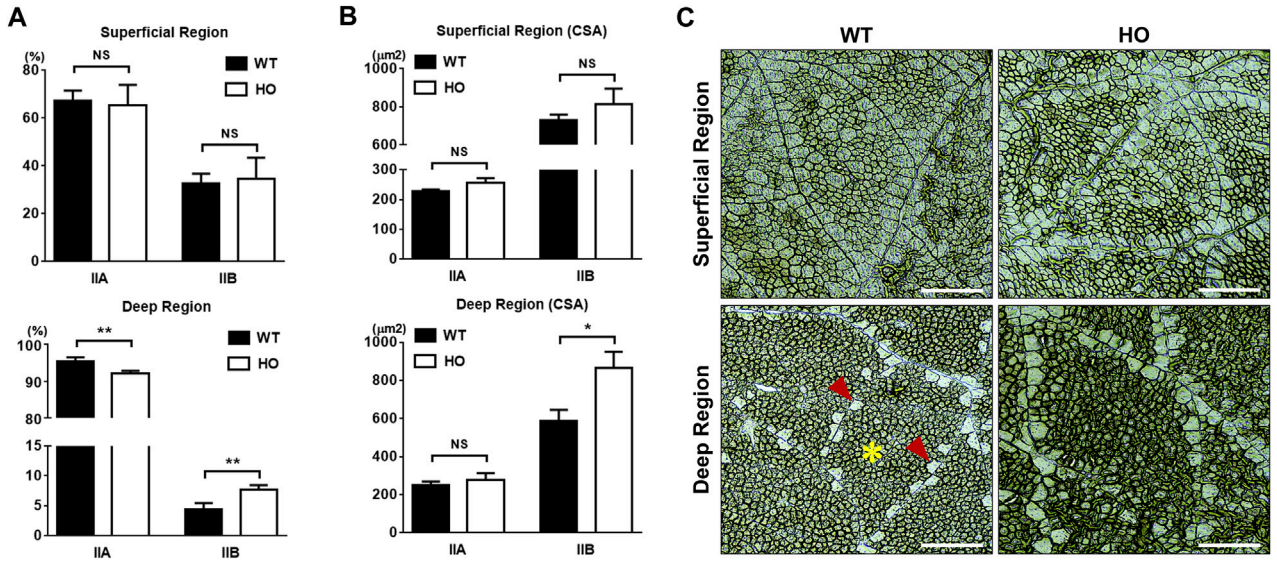

FIGURE 1
Comparison of muscle fiber composition and muscle fiber cross-sections in *pectoralis major* muscle between the wild type (WT) and myostatin homozygous mutant (HO) quail lines at 2-month of age. **(A)**. The percentages of type IIA and IIB muscle fibers in superficial or deep regions. **(B)**. The cross-sectional areas (CSA) of type IIA and IIB muscle fibers in superficial or deep regions. **(C)**. The representative images of the myofiber types of PM. Muscle fibers were stained using the myosin ATPase staining kit according to the manufacturer's instructions, and type IIA muscle fibers stained darker than type IIB muscle fibers. Bars in graphs indicate standard errors. Level of significance: NS, no significant; *, $p < 0.05$; **, $p < 0.01$. Scale bars: 100 μm. Asterisk indicates type IIA myofibers and arrowheads are type IIB myofibers.

lines. The representative images of the myofiber types of PM are shown in Figure 1C.

4 Discussion

Mstn is a negative regulator in muscle growth and development, and is expressed almost exclusively in mature skeletal muscle (McPherron et al., 1997). The inhibitory role of Mstn in muscle development was further confirmed in numerous species of domesticated animals with double muscled phenotypes, including sheep, chicken, mice, cattle, and pigs (McPherron et al., 1997; McPherron and Lee, 1997; Kijas et al., 2007; Stinckens et al., 2008; Kim et al., 2022). Similar to our previous study (Lee et al., 2020), Mstn knock-out quail exhibited significantly heavier BW and greater breast muscle mass than WT quail, suggesting conserved function of Mstn in regulation of muscle growth between mammals and avian species.

Excessively increased muscle mass can develop into muscular abnormalities due to disrupted structure and functions of muscles which affect the rate and extent of *postmortem* metabolism and meat quality variation (Petracci et al., 2017). Pale, soft, and exudative (PSE)-like features in breast muscle have been an issue in fast-growing broilers (Barbut et al., 2008; Petracci et al., 2017). Generally, PSE-like conditions are characterized by low pH (<5.7) and high lightness values (>53) at 24 h *postmortem* in poultry species (Carvalho et al., 2014; Lee and Choi, 2021). In the present study, there were no significant differences in most of the meat quality indexes including *postmortem* pH, redness, yellowness, and drip loss of PM, except for a subtle decrease (1.5%) in cooking loss and an increased lightness value (1.7) in the mutant quail. However, the meat quality indexes were not confirmed with sensory testing since consumption of meat products from genome-edited animals has not yet been approved. It is still questionable whether the minor changes in meat quality characteristics in Mstn mutant quail can affect consumer satisfaction of meat products.

The architecture of the PM muscle from volant species has characteristics of locomotory muscles most specialized to produce power, and demonstrates an increasing proportion of slow-twitch muscle fibers along a ventral to dorsal area gradient (Rosser et al., 1987). In small birds, including quail, the deep regions composed primarily of type IIA muscle fibers are frequently activated for isometric function and sustained locomotory activity associated with flapping and flight (Rosser et al., 1987). The superficial regions with more glycolytic capacity show bursts of maximum power output through a series of very rapid and powerful contractions compared with the deeper areas (Rosser et al., 1987). In our previous study, breast muscles of quail, a volant species, consist mainly of type IIA and IIB muscle fibers due to their flight behavior, and type IIB muscle fibers were more abundantly found in the superficial regions of PM muscles compared to the deep regions (Choi et al., 2014). Fast-twitch muscle fibers, especially type IIB muscle fibers, are faster contracting muscle fibers with higher glycolytic capacity compared to slow-twitch muscle fibers (type I and IIA fibers) (Choi and Kim, 2009). It was reported that muscles lacking Mstn have faster and more glycolytic characteristics due to the myogenic transition from slow-twitch to fast-twitch muscle fibers (Qian et al., 2015; Baati et al., 2017). In chickens having only

type IIB fibers in PM, Mstn mutation did not affect breast muscle weight, but increased leg muscle containing various fiber types (Kim et al., 2020). However, Mstn mutation in quail increased weights of breast muscle containing type IIA and IIB myofibers. This suggests degrees of muscle growth in response to Mstn mutation could vary depending on myofiber composition in muscle in avian species.

In general, muscles having a higher amount of type IIB muscle fibers can show higher glycolytic potentials with lower pH during the *postmortem* period compared to muscles having a lower amount of type IIB muscle fibers, leading to deterioration in meat quality of chicken (Lee and Choi, 2021). Mstn mutation in quail did not affect fiber types in superficial regions of PM, but slightly increased (approximately 3.3%) type IIB fibers in the deep region, possibly resulting in no difference in *postmortem* pH between the WT and Mstn mutant quail lines. These findings suggest that Mstn can be a candidate gene for increasing meat production without affecting meat quality in the poultry species.

Data availability statement

The raw data supporting the conclusion of this article will be made available by the authors, without undue reservation.

Ethics statement

The animal study was reviewed and approved by the Institutional Animal Care and Use Committee (IACUC) of OSU (Protocol 2019A00000024).

Author contributions

Conceptualization, D-HK, YC, and KL; Methodology, D-HK, BL, JL, BB, YC, and KL; Validation, D-HK, BL, YC, and KL; Investigation, D-HK, YC, and JL; Resources, BB and KL; Data curation, D-HK, BL, and YC; Writing—original draft preparation, D-HK and BL; Writing—review and editing, D-HK, BL, JL, BB, YC, and KL; Visualization, D-HK, BL, and YC; Supervision, YC and KL; Project administration, KL; Funding acquisition, KL All authors have read and agreed to the published version of the manuscript.

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Conflict of interest

The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

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Satellite cell-mediated breast muscle growth and repair: The impact of thermal stress

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Poultry exposed to prolonged periods of thermal stress exhibit negative effects on breast meat quality through altered muscle structure, increased fat deposition, and altered protein levels. Birds are homotherms and maintain their body temperature in a narrow range (Yahav, 2000; 2015). Newly hatched poults and chicks are unable to maintain a consistent internal body temperature especially when challenged with external hot or cold temperatures (Dunnington and Siegel, 1984; Modrey and Nichelmann, 1992; Shinder et al., 2007). With more temperature extremes anticipated due to climate change, it is expected that the immediate posthatch period will result in newly hatched birds being more thermally challenged. The pectoralis major (*p. major*) muscle (breast muscle) has been shown to be sensitive to temperature extremes during the immediate posthatch timeframe with permanent effects on the morphological structure of the muscle including fat content and its overall development and growth (Velleman et al., 2014; Piestun et al., 2017; Patael et al., 2019; Halevy, 2020). Furthermore, chronic heat stress in chicks has been shown to increase collagen deposition (Halevy, 2020; Patael et al., 2019) and decrease circulatory supply in the *p. major* muscle (Hadad et al., 2014; Joiner et al., 2014). Proximity to the circulatory supply is required for activity of the adult myoblast (satellite cell) population of cells (Christov et al., 2007; Rhoads et al., 2009).

Satellite cells are responsible for posthatch muscle growth, repair and regeneration of the muscle, and are associated with the quality of poultry breast meat. Satellite cells were first reported by Mauro (1961) being localized between the basal lamina and sarcolemma of a muscle fiber. Muscle fiber formation is complete prior to hatch (Smith, 1963). After hatch, further muscle fiber growth occurs through the process of hypertrophy. Hypertrophy is dependent on satellite cell proliferation, differentiation, and fusion with existing muscle fibers donating their nuclei to increase protein synthesis potential (Moss and Leblond, 1971; Cardasis and Cooper, 1975). The first week posthatch is a period of maximal mitotic activity (Mozdziak et al., 1994; Halevy et al., 2000). During this period, satellite cells are sensitive to both cold and hot temperatures in terms of their proliferation (Xu et al., 2021), differentiation (Xu et al., 2021), cellular fate (Xu et al., 2022a), and ultimately will be a primary determinant of *in vivo* muscle fiber formation which will impact meat quality. For example, the proliferation (Halevy et al., 2001; Clark et al., 2016; Harding et al., 2016; Xu et al., 2021; Xu and Velleman, 2023) and differentiation (Halevy et al., 2001; Clark et al., 2016; Harding et al., 2016; Xu et al., 2021; Xu and Velleman, 2023) of satellite cells is affected by thermal stress both cold and hot. Cold temperatures inhibit both the proliferation and differentiation of satellite cells which will result in smaller myofibers and limit muscle mass accretion especially when satellite cells have high mitotic activity during the first wk posthatch and are responsive to temperature. Clark et al. (2016) in turkeys showed a linear increase in proliferation with temperature increasing from 33°C to 43°C in a stepwise manner. Similar results in chickens were reported by Harding et al. (2016). Thermal stress especially hot temperatures can increase the conversion of satellite cells to an adipogenic fate

(Xu et al., 2022a) which will be associated with altered breast meat quality through increased fat content. Thus, the cellular biology of breast muscle satellite cells is central to both the morphological structure of muscle through their regulation of muscle mass accretion and meat quality. Furthermore, the repair and regeneration of myofibers back to their original state is under the control of satellite cells. If satellite cell activation back into the cell cycle from a quiescent state is suppressed or the proliferation and/or differentiation is impaired, the repair of existing muscle fibers to their original state will be negatively impacted. Interestingly, as shown by Xu et al. (2021) in turkeys that satellite cell proliferation and differentiation has increased with selection for growth whereas the opposite has occurred in broilers (Xu and Velleman, 2023). Thus, meat-type turkeys have a greater potential to repair and regenerate myofiber damage.

Satellite cells are not a homogeneous population of cells. Schultz and Lipton (1982) were the first to report satellite cell heterogeneity with proliferation being age dependent. Satellite cell heterogeneity can take many different forms with satellite cells from different muscle fiber types expressing the genes specific to the fiber type it originated from (Feldman and Stockdale, 1991; Lagord et al., 1998). Heterogeneity of satellite cells exists in a single fiber-type muscle like the turkey and chicken *p. major* muscle that contains homogenous Type IIb fibers. McFarland et al. (1995) and Yun et al. (1997) used single cell cloning to determine that satellite cells isolated from the *p. major* muscle of one turkey exhibit different rates of proliferation and differentiation and different growth factor responsiveness.

Commercial meat type poultry, chickens and turkeys, have been selected, in part, for rapid growth and heavy weight of the breast muscle. How selection for growth has affected the growth properties of satellite cells and their responsiveness to temperature is important to the morphological structure of the *p. major* muscle and breast meat quality. Faster growing poultry in general have a higher metabolic rate and produce more heat (Chwalibog et al., 2007; Buzala et al., 2015). To further complicate thermal regulation, growth-selected poultry tend to have reduced circulatory supply in the *p. major* muscle and thus reduced dissipation of heat (Yahav, 2000; Havenstein et al., 2003; Yahav et al., 2005). In general, faster growing heavy weight meat-type broilers have reduced thermal tolerance compared to slower growing lines (Yahav, 2000).

How selection for increased muscle mass accretion of the *p. major* muscle has impacted the biological activity of the satellite cells and response to thermal stress will affect muscle growth and development including the response to myofiber damage. Xu et al. (2021) reported results from a study comparing *p. major* satellite cell proliferation, differentiation, and response to hot and cold temperature in a historical turkey line representing commercial turkeys of the late 1960s (Nestor et al., 1969) to a modern-day commercial meat-type turkey line. It was found that both hot and cold thermal stress *in vitro* affected the proliferation and differentiation of the satellite cells isolated from both the historical and the current commercial turkey *p. major* muscle. Both proliferation and differentiation were increased by heat stress and reduced with cold. The cells during proliferation were more sensitive to temperature than during differentiation with temperature during proliferation having more of an effect on myotube formation. Myotube formation is the precursor to myofiber development and thus altering the proliferation process could have long-term effects on the morphological structure of the breast muscle in turkeys. Furthermore, the growth-selected faster growing modern commercial turkey satellite cells were significantly more sensitive to hot temperatures during both proliferation and differentiation. *In vivo*, this would result in the formation of larger diameter muscle fibers, giant fibers, reducing available connective tissue spacing between muscle fibers and bundles. In an anerobic muscle like the *p. major* muscle reducing the amount of available connective tissue spacing limits the area needed for capillary supply to support satellite cell activity and remove the by-products of anaerobic respiration. As shown by Wilson et al. (1990) and Velleman et al. (2003) as muscle begins to lose spacing between muscle fiber bundles and individual myofibers, degradation of the muscle fiber structure commences. Once the fibers are damaged, the fibers must be regenerated to their original state through the activation of satellite cells. If myofiber structure is not appropriately regenerated, the fiber structure of the muscle will be replaced with connective tissue and fat through fibrosis. Since the modern commercial turkey has increased proliferation and forms larger fibers with hot temperatures, it is likely to be more prone to myofiber degenerative myopathies.

Interestingly, *p. major* muscle necrotic/fibrotic myopathies are primarily observed in heavy weight fast growing chickens and not in meat type turkeys. This raises the issue of differences between satellite cell biological activity to regenerate muscle in chickens and turkeys, and if satellite cells between chickens and turkeys have a similar response to thermal stress. In a recent study, Xu et al. (2023) compared the current commercial meat-type chicken *p. major* muscle satellite cells to cell lines isolated in the 1990s. The modern commercial chicken satellite cells had decreased proliferation and differentiation and were less responsive to both hot and cold thermal stress compared to the 1990s *p. major* muscle satellite cells. This is completely opposite to that of the modern commercial meat-type turkey which has increased proliferation and differentiation compared to older slower-growing turkeys (Xu et al., 2021). Turkey derived *p. major* muscle satellite cells from current birds are also responsive to both hot and cold thermal stress during proliferation and differentiation through both the mechanistic target of rapamycin (mTOR) (Xu et al., 2022b) and Frizzled 7 (Fzd7)-mediated wingless-type mouse mammary tumor virus integration site family/planar cell polarity (Wnt/PCP) pathway (Xu et al., 2022a). The mTOR pathway stimulates protein synthesis and enhances myofiber hypertrophy (Bodine et al., 2001; Wang and Proud, 2006), whereas the Wnt/PCP is a regulator of satellite cell migration (Fortier et al., 2008; Wang et al., 2018). The modern-day commercial meat-type chicken satellite cells are less sensitive to temperature and during proliferation are more responsive to mTOR and Fzd7 expression. Taken together, the results of Xu et al. (2023) are suggestive of the modern-day broiler satellite cells having reduced regeneration potential of damaged muscle due to decreased proliferation and differentiation and temperature sensitivity of the satellite cells.

Reduced regeneration potential of broiler *p. major* satellite cells would result in current commercial broilers being more susceptible to the negative effects of necrotic/fibrotic myopathies like Wooden breast which are not observed in turkeys. In support of the negative impact of reduced regeneration potential of broiler satellite cells, Wooden breast affected muscle is composed of a high percentage of

smaller diameter myofibers with disorganized contractile sarcomeres (Clark and Velleman, 2017; Velleman et al., 2018). Based on the reduced biological activity of modern-day broiler satellite cells, the ability to regenerate damaged myofibers is reduced leading to the necrosis and subsequent fibrosis. Furthermore, it is evident that selection for accretion of breast muscle mass in both turkeys and chickens has changed the satellite cell populations from those in slower growing or historic lines with changes in proliferation and differentiation (Xu et al., 2021; Xu et al., 2023) and in the case of turkeys documented changes in key cell surface receptors affecting satellite cell function and cellular fate (Xu et al., 2023).

In summary, both thermal stress and the biological activity of satellite cells pose multidimensional threats to the growth, development, and subsequent meat quality of the poultry breast muscle. Satellite cells have their peak mitotic activity, the first week posthatch, and during this time are responsive to extrinsic stimuli including temperature which can alter cellular fate, and proliferation and differentiation. In addition, satellite cells are not a homogenous population of cells and have been altered by selection for growth. In the case of broiler chickens, satellite cell proliferation, differentiation, and the potential to regenerate myofiber damage has declined likely being associated with the onset of fibrotic/necrotic myopathies (Xu and Velleman, 2023). Also, the satellite cell response to temperature extremes has changed in both turkeys and chickens. Selection strategies used by the poultry industry as it continues to move forward should include assessments of satellite cell biological activity and responsiveness to both hot and cold temperature extremes.

Author contributions

The author confirms being the sole contributor of this work and has approved it for publication.

Conflict of interest

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Metabolic and microbiota response to arginine supplementation and cyclic heat stress in broiler chickens

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Little attention has been paid to the biological role of arginine and its dietary supplementation in broilers under heat stress (HS) conditions. Therefore, the main aim of this study was to assess the response of broilers to arginine supplementation and cyclic HS, with a focus on liver, pectoral muscle, and blood metabolic profiles and the cecal microbiota. Day-old male Ross 308 broilers (n = 240) were placed in 2 rooms with 12 pens each for a 44-day trial. Pens were assigned to one of two groups (6 pens/group/room): the control group (CON) was given a basal diet in mash form and the treated group (ARG) was fed CON diet supplemented with crystalline *L*-arginine. The total arginine:lysine ratio of CON diet ranged between 1.02 and 1.07, while that of ARG diet was 1.20. One room was constantly kept at thermoneutral (TN) conditions, while the birds in the other room were kept at TN conditions until D34 and subjected to cyclic HS from D35 onwards (~34°C; 9:00 A.M.–6:00 P.M.). Blood, liver, *Pectoralis major* muscle, and cecal content were taken from 2 birds per pen (12 birds/group/room) for metabolomics and microbiota analysis. Growth performance data were also collected on a pen basis. Arginine supplementation failed to reduce the adverse effects of HS on growth performance. Supplemented birds showed increased levels of arginine and creatine in plasma, liver, and *P. major* and methionine in liver, and reduced levels of glutamine in plasma, liver, and *P. major*. HS altered bioenergetic processes (increased levels of AMP and reduced levels of fumarate, succinate, and UDP), protein metabolism (increased protein breakdown to supply the liver with amino acids for energy production), and promoted the accumulation of antioxidant and protective molecules (histidine-containing dipeptides, beta-alanine, and choline), especially in *P. major*. Arginine supplementation may have partially counterbalanced the effects of HS on energy homeostasis by increasing creatine levels and attenuating the increase in AMP levels, particularly in *P. major*. It also significantly reduced cecal observed diversity,

Abbreviations: [1]H-NMR, proton nuclear magnetic resonance; AA, amino acid; AMPK, AMP-activated kinase; ARG, arginine-supplemented group; BW, body weight; CON, control group; DFI, daily feed intake; DWG, daily weight gain; FCR, feed conversion ratio; FI, feed intake; GI, gastrointestinal; HS, heat stress; RH, relative humidity; TN, thermoneutral.

while HS increased alpha diversity indices and affected beta diversity. Results of taxonomic analysis at the phylum and family level are also provided.

KEYWORDS

broiler chicken, arginine, heat stress, metabolism, plasma, liver, breast muscle, microbiota

1 Introduction

Global warming is one of the knottiest problems the animal-food industry is and will be facing (Nardone et al., 2010; Bezner Kerr et al., 2022). Rising environmental temperatures have a great impact on the sustainability of chicken meat production, affecting the performance and heath of birds (Renaudeau et al., 2012; Rostagno, 2020) and deteriorating product quality (Song and King, 2015; Wang et al., 2017; Zaboli et al., 2019). The risk of suffering from heat stress (**HS**) and its multifaceted and serious physiological consequences is extremely high for broilers, especially for fast-growing lines (Brugaletta et al., 2022). Looking for strategies intended to prevent or mitigate the detrimental effects of HS is therefore imperative and has become a major research topic in poultry science. Many interventions have been tested so far to help broilers cope with HS, as documented in comprehensive review articles (Gous and Morris, 2005; Lin et al., 2006; Naga Raja Kumari and Narendra Nath, 2018; Saeed et al., 2019; Wasti et al., 2020; Goel, 2021; Nawaz et al., 2021; Vandana et al., 2021).

A specific dietary approach has caught our attention, namely formulating broiler diets with levels of arginine, an essential amino acid (**AA**) for chickens (Arnold et al., 1936; Klose et al., 1938; Tamir and Ratner, 1963), above those recommended by the NRC (National Research Council, 1994) or the breeding companies [e.g., Aviagen (2022)]. The reasons for testing arginine supplementation as a potential nutritional strategy for HS alleviation are as follows. First, it has been demonstrated that diet composition and environmental conditions considerably influence arginine requirement of broilers (Khajali and Wideman, 2010; Hassan et al., 2021). In this regard, precise and consistent arginine requirements for broilers under HS are not readily available in the literature. Alterations in feed intake, physiology, metabolism, and gut health and function induced by HS contribute to complicating the calculation of arginine or other nutrient needs for heat-stressed broilers (Teyssier et al., 2022). For example, considering the "ideal protein" concept widely adopted in poultry nutrition (Wiseman and Garnsworthy, 1999), Balnave and Brake (2002) attributed the increased arginine to lysine ratios in broilers undergoing HS to a likely reduction in intestinal absorption of arginine. Second, arginine has been shown to be involved—either directly or through its derivatives—in countless biochemical pathways and body functions, such as modulation of immune, inflammatory, and oxidative responses, regulation of gene expression, protein synthesis, and secretion of anabolic hormones, and contribution to skeletal muscle development, as well as maintenance of gut health, homeostasis, and eubiosis, as recently discussed elsewhere (Brugaletta et al., 2023). Considering these properties, it is worth trying to determine whether high dietary arginine levels produce a reduction in the severity of the effects of HS normally observed in broilers, such as immunodeficiency (Renaudeau et al., 2012; Farag and Alagawany, 2018; Chauhan et al., 2021), inflammation and oxidative stress (Lambert, 2009; Tan et al., 2010; Akbarian et al., 2016; Goel et al., 2021), protein turnover modification indicating catabolic states (Zuo et al., 2015; Lu et al., 2018; Ma et al., 2021), gut health degradation (Rostagno, 2020; Ruff et al., 2020), and perturbation in the gastrointestinal (**GI**) microbiota (Suzuki et al., 1983; Burkholder et al., 2008; Song et al., 2014; He et al., 2021; Liu et al., 2022). Third, the significant arginine-mediated improvements in performance found in broiler studies conducted under thermoneutral (**TN**) settings (Basoo et al., 2012; Xu et al., 2018; Zampiga et al., 2018; Liu et al., 2019; Sirathonpong et al., 2019; Brugaletta et al., 2023) make arginine supplementation a promising tool for minimizing performance losses caused by HS. Nevertheless, and this is the fourth and final reason, very little attention has been paid to the biological role of arginine and its dietary supplementation in heat-stressed broilers (Figure 1), suggesting that there is much room for further progress in these intriguing research areas. An essential step to take is to clarify the role of arginine in metabolism and intestinal health of broilers, which are profoundly affected by HS, as mentioned above.

Therefore, the main aim of this study was to assess the response of broilers to arginine supplementation and HS, with a focus on liver, pectoral muscle, and blood metabolic profiles and the cecal microbiota. Specifically, control and supplemented birds were subjected to a cyclic HS period to simulate field conditions typical of the summer in temperate areas of the world, that is a succession of diurnal hot temperatures and cooler nights. Growth performance data were also collected and analyzed to get as complete a picture as possible.

2 Materials and methods

2.1 Experimental design, housing, and husbandry conditions

In this study, approved by the Ethical Committee of the University of Bologna (ID: 4387), birds were reared, monitored, and slaughtered in compliance with EU legislation (i.e., Dir. 2007/43/EC, Reg. 2009/1099/EC, and Dir. 2010/63/EU) and had *ad libitum* access to feed and water.

One-day-old male Ross 308 broilers (n = 240) were supplied by a commercial hatchery and vaccinated against infectious bronchitis, Marek's, Newcastle and Gumboro diseases, and coccidiosis. Chicks were randomly placed in 2 identical environmental chambers (Bologna, Italy), hereafter referred to as rooms. Each room was divided into 12 equally sized floor pens

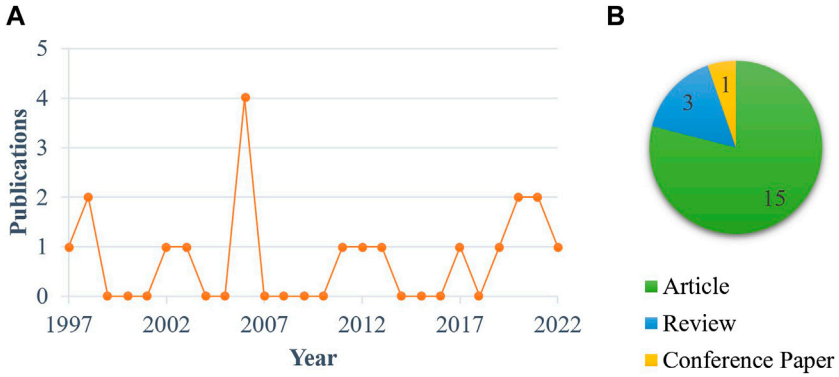

FIGURE 1
Publication trend **(A)** and type **(B)** of papers mentioning or focusing on arginine in heat-stressed broilers. Note: The literature search was carried out by searching the Scopus database on September 12, 2022 entering the following query string: {TITLE [(chicken OR broiler) AND (arginine OR arg) AND heat AND stress AND NOT (arginine AND vasopressin) AND NOT plant AND NOT polymorphism] OR ABS [(chicken OR broiler) AND (arginine OR arg) AND heat AND stress AND NOT (arginine AND vasopressin) AND NOT plant AND NOT polymorphism]}. No restriction on the date and type of publication was set.

equipped with ~3 kg/m^2 of chopped straw as bedding material (litter of ~7 cm in depth), a bell feeder (minimum 4 cm of feeder front space per bird), and 1 nipple drinker for every 3 birds. Pens were randomly assigned to one of two experimental groups (i.e., 6 replicate pens/group/room) and were arranged in a block design. The control group (**CON**) was given a commercial antibiotic-free basal diet in mash form, which was formulated to meet the nutrition specifications released by the breeding company (Aviagen, 2022). The treated group (**ARG**) was fed the same basal diet supplemented on top with crystalline *L*-arginine (~1.5 g/kg feed; purity of 98%; BESTAMINO™, CJ BIO, Seoul, Korea). The basal diet formula and composition according to the three-phase feeding program used (i.e., starter, 0–14 days; grower, 15–27 days; finisher, 28–44 days) are shown in Table 1. Analysis of AA concentration of the experimental diets was outsourced to Evonik Industries AG labs (Hanau, Germany). The total arginine level of the basal diet was 1.50, 1.38, and 1.23%, while that of the supplemented diet was 1.74, 1.56, and 1.37% in the starter, grower, and finisher phase, respectively. The total arginine to lysine ratio of the control diet ranged between 1.02 and 1.07 and was consistent with the breeding company's specifications (Aviagen, 2022), whereas that of the supplemented diet was 1.20 in all feeding phases. The artificial photoperiod was 23 L:1D during the first 7 and last 3 days, while 18 L:6D (i.e., light phase 6:00 A.M.–10:00 P.M., dark phase 10:00 P.M.–02:00 A.M., light phase 02:00 AM–04:00 AM, and dark phase 04:00 AM–06:00 AM) for the remainder days following EU legislation (i.e., Dir. 2007/43/EC) and the breeding company's guidelines for lightning and pre-processing management (Aviagen, 2018). Environmental temperature and relative humidity (**RH**) were recorded with climate data loggers (Trotec GmbH, Heinsberg, Germany) located at animal level (3 data loggers/room having a recording time of 900 s). As for the temperature program, one room was constantly kept at TN conditions following the instructions of the breeding company (Aviagen, 2018), while the birds in the other room were kept at TN conditions until D34 and subjected to cyclic HS from D35 to D43, with the temperature increased daily to ~34°C from 9:00 A.M. to 6:00 P.M. (Figure 2). RH was adjusted by means of humidifiers (Trotec GmbH) and it ranged between 40% and 70% in both rooms during the HS period.

2.2 Data and sample collection

Growth performance data were collected on a replicate basis. The number and body weight (**BW**) of birds were recorded at placement (D0), at every feeding phase switch (D15/28), and at slaughter (D44), while feed intake (**FI**) was measured for each feeding phase. Daily weight gain (**DWG**), daily feed intake (**DFI**), and feed conversion ratio (**FCR**) were calculated for the feeding phases separately and cumulative FI and cumulative FCR were computed for the period consisting of a feeding phase and its previous one/ones (i.e., starter + grower; starter + grower + finisher). The number and BW of dead or culled birds were daily recorded daily to compute the mortality rate and correct the performance data for mortality.

Three birds per pen (i.e., 18 birds/group/room) were randomly chosen and labeled to measure the rectal temperature with a veterinary thermometer (Scala Electronic GmbH, Stahnsdorf, Germany). The rectal temperature was taken on the first and eighth day of the HS period (i.e., D35 and D42, respectively) at two time points, namely 9:00 A.M. and 6:00 P.M.

At slaughter in a commercial abattoir (D44), biological samples were collected from two of the three birds previously labeled in each pen (i.e., 12 birds sampled/group/room). Blood was taken from the wing vein and kept at RT before being centrifuged to get plasma, which was subsequently stored at −80°C until metabolomics analysis through proton nuclear magnetic resonance (**^{1}H-NMR**). Hepatic tissue (~1 cm^3) was dissected from the right caudal lobe of the liver,

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TABLE 1 Basal diet formula and composition according to the 3-phase feeding program.

Ingredient (g/100 g feed)	Feeding phase		
	Starter (0–14 days)	Grower (15–27 days)	Finisher (28–44 days)
Corn	29.87	32.49	15.02
White corn	0.00	0.00	6.00
Wheat	24.97	24.97	39.95
Pea	3.00	3.00	3.00
Soybean meal	15.89	15.17	8.68
Roasted soybean	9.99	14.99	16.99
Potato protein meal	3.51	0.00	0.00
Sunflower meal	3.00	3.00	3.00
Corn gluten meal	3.00	0.00	0.00
Soybean oil	2.27	2.83	4.47
Calcium carbonate	0.19	0.56	0.87
Dicalcium phosphate	1.54	0.48	0.00
Sodium bicarbonate	0.05	0.00	0.05
Sodium chloride	0.31	0.33	0.27
Choline chloride	0.10	0.10	0.06
Lysine sulphate	0.67	0.63	0.54
DL-Methionine	0.30	0.33	0.29
L-Threonine	0.14	0.18	0.14
Amino acid mix (arginine, valine, isoleucine)	0.35	0.24	0.17
Non-starch polysaccharides-degrading enzyme	0.05	0.05	0.05
Phytase	0.20	0.20	0.20
Mycotoxin binder	0.10	0.00	0.00
Vitamin and mineral premix[a]	0.50	0.45	0.25
Composition			
Dry matter[b] (%)	88.24	88.28	88.52
Crude protein[b] (%)	22.88	20.06	18.36
Total lipid[b] (%)	5.89	7.38	9.18
Crude fiber[b] (%)	3.05	3.18	3.11
Ash[b] (%)	5.00	4.47	4.06
Total lysine[b] (%)	1.47	1.31	1.15
Total arginine[b] (%)	1.50	1.38	1.23
Total arginine: lysine[b]	1.05	1.07	1.09
Total methionine + cysteine[b] (%)	1.08	0.99	0.88
Calcium (%)	0.78	0.65	0.59
Phosphorus (%)	0.63	0.46	0.36
Metabolizable energy (kcal/kg)	3,072	3,146	3,296

[a]The premix provides the following per kg of feed: Vitamin A (retinyl acetate), 12,500 IU; vitamin D3, 5,000 IU (i.e., cholecalciferol, 3,500 IU + 25-OH, D3, 1,500 IU); Vitamin E (*DL*-α-tocopheryl acetate), 125 mg; Vitamin K (menadione sodium bisulfite), 6.75 mg; riboflavin, 9.0 mg; Pantothenic acid, 22.0 mg; niacin, 75 mg; Pyridoxine, 5 mg; folic acid, 3.0 mg; Biotin, 0.35 mg; Thiamine, 4.0 mg; Vitamin B_{12}, 50 μg; Mn, 100 mg; Zn, 102 mg; Fe, 30 mg; Cu, 15 mg; I, 2.0 mg; Se, 0.35 mg.
[b]Analyzed values.

frozen in liquid N_2, and stored at −80°C until ^{1}H-NMR analysis. Breast muscle (~1 cm^3) was taken from the left cranial portion of the *Pectoralis major*, frozen in liquid N_2, and stored at −80°C until ^{1}H-NMR analysis. Lastly, the content of both ceca was collected, frozen in liquid N_2, and stored at −80°C until DNA extraction for shotgun metagenomic sequencing.

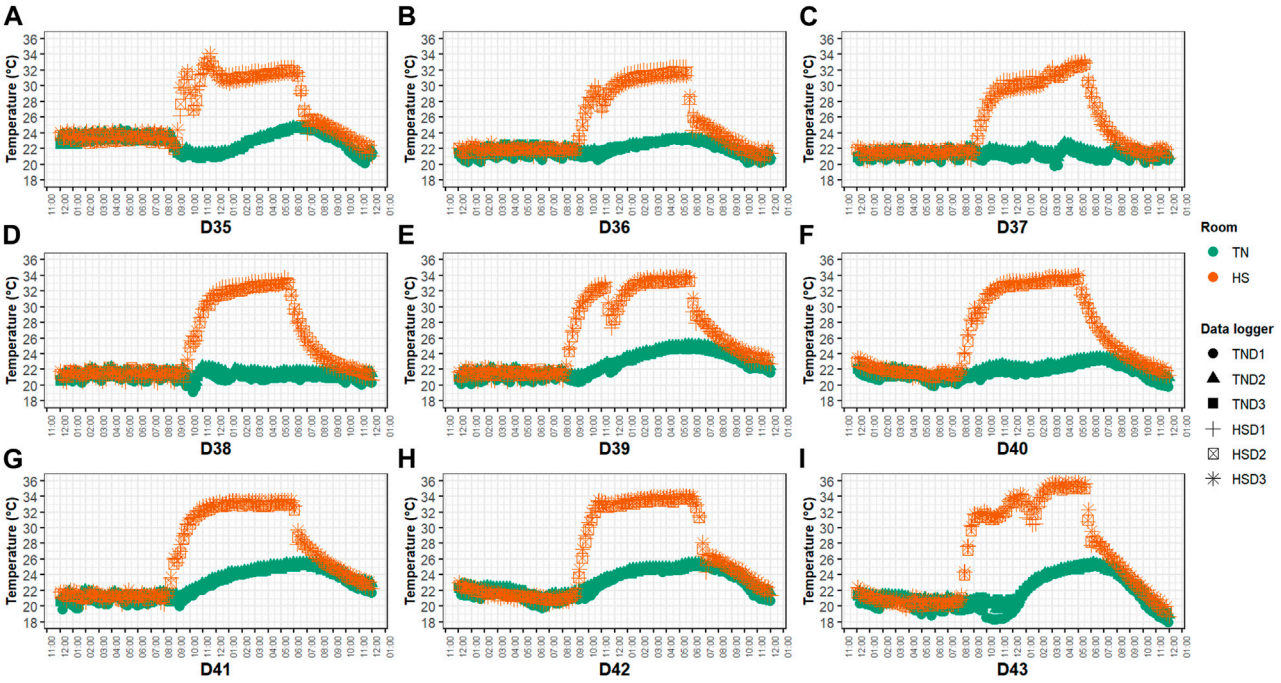

FIGURE 2
Hourly temperature **(A–I)** of the two rooms during the cyclic HS period. Note: D#, day#; TN, thermoneutral conditions; HS, (cyclic) heat stress conditions; TND#, data logger# in room TN; HSD#, data logger# in room HS.

2.3 Lab analysis

For metabolomics analysis, an ^{1}H-NMR solution with D_2O, containing TSP 10 mmol/L and NaN_3 2 mmol/L, was created. Phosphate buffer 1 M was used to achieve a pH of 7.00 ± 0.02, while TSP was used as a reference for NMR chemical-shift and NaN3 avoided bacterial proliferation. Approximately 0.5 g of liver and muscle samples were homogenized (14,000 rpm; 20 s; RT) with 3 mL of a water solution of TCA 7% (w/w). All samples were centrifuged (18,630 g; 900 s; 4°C) and 0.7 mL of supernatant were mixed with 0.1 mL of the ^{1}H-NMR solution. The pH of liver and muscle samples was further adjusted to 7.00 ± 0.02 with drops of NaOH 9 N and 1 N as needed. All samples were centrifuged again at the abovementioned conditions. The ^{1}H-NMR spectra were registered (600.13 MHz; 298 K) with an AVANCE™ III spectrometer (Bruker, Milan, Italy) equipped with TopSpin software v3.5 (Bruker). The signals from broad resonances due to large molecules were suppressed with CPMG-filter (400 echoes with a *t* of 400 μs and a 180°pulse of 24 μs, for a total filter of 330 ms), while the residual signal of water was suppressed by means of presaturation. This was done employing the cpmgpr1d sequence, part of the standard pulse sequence library. Each spectrum was acquired summing up 256 transients constituted by 32,000 data points encompassing a window of 7,184 Hz, separated by a relaxation delay of 5 s. The ^{1}H-NMR spectra were phase-adjusted in TopSpin v3.5 (Bruker) and then exported to ASCII format by means of the built-in script convbin2asc. Spectra were processed with R (R Core Team, 2020) through home-made scripts. Signal assignment was performed comparing their chemical shift and multiplicity with Human Metabolome Database (Wishart et al., 2007) and Chenomx software library v10 (Chenomx Inc. Edmonton, Canada), by means of Chenomx software routines. For all samples, the absolute concentration of molecules was performed with the median water dilution, assessed *via* probabilistic quotient normalization (Dieterle et al., 2006). TSP was used as an internal standard. Differences in water content between samples from the same matrix were considered through probabilistic quotient normalization. The concentration of each molecule was obtained from the area of one of its signals, calculated by the global spectra deconvolution algorithm implemented in MestReNova software v14.2.0–26256 (Mestrelab research S.L. Santiago De Compostela, Spain), by considering a limit of quantification of 5. This was done after applying a baseline adjustment by Whittaker Smoother procedure and a line broadening of 0.3.

Moving to microbiota analysis of the cecal content, DNA extraction was performed adopting a bead-beating procedure and using the QIAmp® DNA Stool Mini Kit (Qiagen, Milan, Italy). Total DNA was fragmented and tagged with sequencing indexes and adapters using the Nextera XT DNA Library Preparation Kit (Illumina, San Diego, CA, United States.). Shotgun metagenomic sequencing was performed with NextSeq 500 (Illumina) 2 × 149 bp in paired-end mode. Filtering of low-quality reads and sequence adapters trimming of raw reads were conducted using the tool AdapterRemoval. The microbial community composition was evaluated with the bioinformatic tool MetaphlAn3 (Truong et al., 2015) at the phylum level. Alpha diversity indices (i.e., observed, Shannon, Simpson, and Inverse Simpson) and Bray-Curtis beta distance were calculated with the R package *phyloseq* (McMurdie and Holmes, 2013).

TABLE 2 Growth performance of groups CON and ARG in the three-phase feeding trial.

Feeding phase	Factor	Dependent variable								
		Chick weight (g/bird)	BW (g/bird)	DWG[a] (g/bird/d)	DFI[a] (g/bird/d)	FI[a] (kg/bird)	Cum. FI[a,b] (kg/bird)	FCR[a]	Cum. FCR[a,b]	Mortality (%)
Starter[c] (0–14 days)	**Group**									
	CON	43.15	437.9	28.20	37.94	0.531	—	1.344	—	0.00
	ARG	43.60	450.4	29.06	36.91	0.517	—	1.270	—	0.00
	SE	1.07	16.69	1.22	2.28	0.03	—	0.06	—	0.00
	***p*-value**	0.327	0.094	0.113	0.292	0.292	—	**0.015**	—	—
Grower[c] (15–27 days)	**Group**									
	CON	—	1,424	75.16	133.1	1.730	2.261	1.772	1.646	0.83
	ARG	—	1,471	77.95	130.0	1.690	2.207	1.670	1.553	0.00
	SE	—	51.59	3.53	9.77	0.13	0.12	0.15	0.10	0.07
	***p*-value**	—	**0.049**	0.079	0.459	0.459	0.295	0.112	**0.042**	0.339
Finisher[d] (28–44 days; cyclic HS from D35 onwards)	**Group**									
	CON	—	3,051	96.51	196.0	3.332	5.592	2.033	1.852	5.00
	ARG	—	3,138	98.21	188.5	3.205	5.412	1.927	1.751	0.83
	Room									
	TN	—	3,222	105.1	208.3	3.541	5.743	1.987	1.811	0.00
	HS	—	2,967	89.64	176.3	2.996	5.261	1.973	1.792	5.83
	Group × Room									
	CON-TN	—	3,188	104.1	217.4	3.696	5.939	2.096	1.894	0.00
	ARG-TN	—	3,257	106.1	199.1	3.385	5.546	1.878	1.728	0.00
	CON-HS	—	2,914	88.93	174.5	2.967	5.246	1.970	1.810	10.00
	ARG-HS	—	3,019	90.35	178.0	3.026	5.277	1.975	1.775	1.67
	SE	—	122.0	4.78	15.47	0.26	0.37	0.20	0.15	0.14
	***p*-value**									
	Group	—	0.111	0.404	0.266	0.266	0.259	0.229	0.122	0.179
	Room	—	**< 0.001**	**< 0.001**	**< 0.001**	**< 0.001**	**0.010**	0.866	0.759	**0.039**
	Group × Room	—	0.732	0.889	0.115	0.115	0.191	0.208	0.297	0.179

[a]Corrected for mortality.

[b]Computed for the period consisting of a feeding phase and its previous one/ones.

[c]n = 12 replicate pens/group.

[d]n = 6 replicate pens/group/room. In "room HS", temperature was raised daily to ~34°C from 9:00 A.M. to 6:00 P.M. from D35 to D43.

Note: p-values less than 0.05 are in bold. BW, body weight; DWG, daily weight gain; DFI, daily feed intake; FI, feed intake; FCR, feed conversion ratio; Cum., cumulative; CON, control group; ARG, arginine-supplemented group; SE, standard error; TN, thermoneutral conditions; HS, (cyclic) heat stress conditions.

2.4 Data analysis

The effect of the factor group on growth performance of the starter and grower phases was assessed with a two-way blocked ANOVA without interaction, considering the room as a fixed factor and using the replicate pen as the experimental unit. The data from the finisher phase, however, were analyzed as a 2 × 2 factorial design using a two-way blocked ANOVA with interaction between the main factors group and room. Tukey's HSD *post hoc* test was used if needed. Mortality rate data were transformed using the arcsine transformation before being analyzed with inferential statistics.

Rectal temperature data were grouped by the day of collection and analyzed through a three-way mixed ANOVA, a type of repeated-

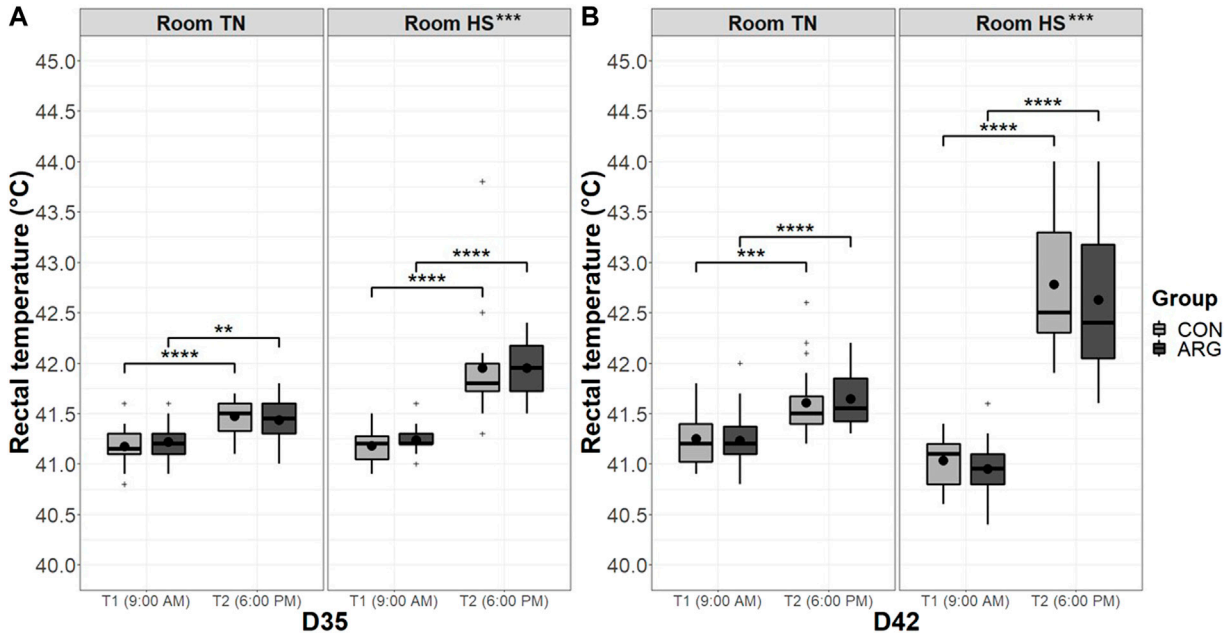

FIGURE 3
Rectal temperature of CON and ARG birds in the two rooms on the first **(A)** and eighth **(B)** day of the cyclic HS period at two time points. Note: The rectal temperature of 18 labeled birds/group/room was measured on the first (D35) and the eighth (D42) day of the cyclic HS period at two time points, namely T1 (9:00 AM) and T2 (6:00 P.M.). Group means are the black dots inside the boxes. **, $p < 0.01$; ***, $p < 0.001$; ****, $p < 0.0001$. TN, thermoneutral conditions; HS, (cyclic) heat stress conditions; T1/2, time point 1/2; D#, day#; CON, control group; ARG, arginine-supplemented group.

measures ANOVA that includes between-subject factors (i.e., group and room) and within-subject factors (i.e., time point). After verifying that there was no statistically significant three-way interaction, rectal temperature data were grouped by the factors group and room to run paired *t*-tests with Bonferroni adjustment between time points. The measured bird represented the experimental unit for rectal temperature data analysis.

Regarding the analysis of metabolomics data, a two-way ANOVA with interaction between group and room and the sampled bird as the experimental unit was carried out. Tukey's HSD *post hoc* test was used where appropriate. Metabolomics data deviating from normality in Shapiro-Wilk test were subjected to Box-Cox transformation (Box and Cox, 1964).

The effects of group, room, and their interaction on alpha diversity measures and beta diversity distance matrix were tested with a two-way ANOVA and a two-way PERMANOVA with interaction between group and room [adonis2 function implemented in the R *vegan* package (Oksanen et al., 2020)], respectively. Similarly, relative abundance data at the phylum and family level were analyzed with a two-way ANOVA followed by a Tukey's HSD *post hoc* test where appropriate. The sampled bird was used as the experimental unit for the statistical analysis of diversity measures and relative abundance data.

These analyses were carried out using R (R Core Team, 2020). *p*-values less than 0.05 were considered significant, while those ranging between 0.05 and 0.1 were considered tendencies.

3 Results

Growth performance results are shown in Table 2. Chicks did not show significantly different weights between groups at placement. Before HS was applied, ARG birds tended to be heavier than CON birds at the end of the starter phase (i.e., BW of 450.4 vs. 437.9; $p = 0.09$). In contrast, FCR of group ARG was significantly lower than that of group CON (1.270 vs. 1.344; $p = 0.02$). Similarly, at the end of the grower phase, ARG birds showed greater BW and lower cumulative FCR than CON birds (1,471 g vs. 1,424 g and 1.553 vs. 1.646; $p < 0.05$). The difference in DWG, however, approached but did not achieve significance (77.95 vs. 75.16 g; $p = 0.08$). In the finisher phase, neither the effect of the factor group nor that of the interaction between group and room was significant. The exposure to HS, however, decreased ($p \leq 0.01$) BW, DWG, DFI, and (cumulative) FI, while increased ($p < 0.05$) mortality regardless of the group.

The rectal temperature of representative birds of groups CON and ARG on the first and eighth day of the cyclic HS period at 9:00 A.M. and 6:00 P.M. is presented in Figure 3. The raw data ranged between 40.4°C and 44.0°C. The mixed ANOVA used to analyze the data grouped by the day of collection did not reveal any significant first-order interaction between the three factors tested (i.e., group, room, and time point). Moreover, the effect of the factor group was not significant, while those of the factors room, time point, and their interaction were highly significant ($p < 0.001$). The pairwise comparisons of time points indicated that the increase in rectal

TABLE 3 Biologically relevant metabolites whose concentration was affected (*p*-values of 0.1 maximum) by either arginine supplementation, HS exposure or both (see Supplementary Tables S1–S3 for more information).

Metabolite	Arginine supplementation[a]			HS exposure[a]		
	Plasma	Liver	*P. major*	Plasma	Liver	*P. major*
Alanine	↓	-	↓	↓	↓	↓
AMP	-	↓	↓	-	-	↑
Anserine	-	-	-	-	-	↑
Arginine	↑	↑	↑	-	-	-
Aspartate	-	-	-	↓	-	-
Beta-alanine	-	-	-	-	-	↑
Betaine	-	-	-	↓	-	-
Carnosine	-	-	-	↓	-	↑
Choline	-	-	-	-	-	↑
Creatine	↑	↑	↑	-	-	-
Fumarate	-	-	-	↓	↓	↓
Glucose	-	-	-	↑	-	-
Glucose-1-phosphate	-	-	↓	-	-	-
Glutamate	-	-	-	-	↑	-
Glutamine	↓	↓	↓	-	-	-
Glutathione	-	↓	↓	-	-	-
Glycerol	-	-	↑	↓	-	-
Glycine	-	-	-	↓	-	↓
Isoleucine	-	-	-	-	↑	↓
Lactate	-	-	↑	-	-	-
Leucine	-	-	-	-	↑	↓
Methionine	-	↑	-	↓	-	-
N, N-Dimethylglycine	-	-	-	↓	↓	↓
Phenylalanine	-	-	-	↑	↑	-
Succinate	-	-	-	↓	↓	-
Threonine	-	-	-	-	↑	↓
Tyrosine	-	-	-	↓	-	↓
UDP	-	-	-	-	↓	↓
Uracil	-	-	-	-	↑	-
Uridine	-	-	-	-	↑	-
Valine	-	-	-	-	-	↓

[a]Compared with concentrations measured in the samples collected from birds fed the control diet or reared under TN conditions, the symbols indicate: ↑, increased; ↓, decreased; -, not affected.

temperature from 9:00 A.M. to 6:00 P.M. was greatly significant ($p < 0.001$) in either room, on both the measurement days, and irrespective of the group.

The concentration of several metabolites was found to be affected by the factors group and room in plasma, liver, and *P. major*, as shown in Supplementary Tables S1–S3. The data of these tables have been compared and summarized in Table 3 focusing on potentially biologically relevant metabolites. Group ARG had a higher concentration of arginine in plasma, liver ($p < 0.001$), and *P. major* ($p = 0.07$), creatine in plasma, liver and *P. major* ($p < 0.05$), methionine in liver ($p = 0.08$), glycerol and lactate in *P. major* ($p < 0.05$), as well as a lower concentration of alanine in plasma ($p < 0.05$)

TABLE 4 Alpha diversity indices of the cecal content of CON and ARG birds in the two rooms at D44.

Factor	Alpha diversity index			
	Observed	Shannon	Simpson	Inverse simpson
Group				
CON	189	3.65	0.94	19.1
ARG	180	3.60	0.95	19.3
Room				
TN	181	3.56	0.94	17.9
HS	189	3.70	0.95	20.5
Group × Room				
CON-TN	184	3.57	0.94	17.5
ARG-TN	177	3.54	0.94	18.3
CON-HS	194	3.72	0.95	20.6
ARG-HS	183	3.66	0.95	20.3
SE	15.64	0.17	0.02	4.51
***p*-value**				
Group	**0.049**	0.403	0.802	0.855
Room	0.085	**0.007**	0.100	0.052
Group × Room	0.589	0.698	0.589	0.667

Note: $n = 12$ birds/group/room. p-values less than 0.05 are in bold. CON, control group; ARG, arginine-supplemented group; TN, thermoneutral conditions; HS, (cyclic) heat stress conditions; SE, standard error.

and *P. major* ($p = 0.08$), AMP in liver and *P. major* ($p < 0.05$), glutamine in plasma ($p = 0.01$), liver ($p = 0.06$), and *P. major* ($p = 0.1$), glutathione in liver and *P. major* ($p \leq 0.01$), and glucose-1-phosphate in *P. major* ($p = 0.07$). The exposure to HS increased the concentration of glucose in plasma ($p < 0.05$), phenylalanine in plasma and liver ($p < 0.05$), glutamate ($p < 0.05$), (iso)leucine ($p = 0.07$), threonine ($p = 0.05$), uracil and uridine ($p < 0.05$) in liver, AMP ($p < 0.05$), anserine, beta-alanine, carnosine ($p \leq 0.001$), and choline ($p = 0.01$) in *P. major*, while decreased that of aspartate, betaine, carnosine, glycerol, and methionine in plasma ($p < 0.05$), succinate in plasma and liver ($p < 0.05$), glycine ($p < 0.01$) and tyrosine ($p \leq 0.09$) in plasma and *P. major*, UDP in liver and *P. major* ($p < 0.01$), alanine, fumarate, and N,N-Dimethylglycine in plasma, liver and *P. major* ($p \leq 0.05$), and (iso)leucine, threonine, and valine in *P. major* ($p \leq 0.002$). On the other hand, the interactive effect of the factors group and room tended to be or was significant on the concentration of a few metabolites, such as carnosine and formate in plasma, sarcosine in plasma and liver, ethanolamine and propylene glycol in liver, and isoleucine and threonine in *P. major*.

As for the results of the cecal microbiota analysis, it can be seen from Table 4 that group ARG showed reduced observed diversity ($p < 0.05$) compared to group CON, while HS increased this alpha index ($p = 0.09$), as well as the Shannon ($p < 0.01$) and Inverse Simpson ($p = 0.05$). No significant interaction between group and room was found for alpha diversity indices. Beta diversity was also affected by group ($p = 0.001$) and room ($p = 0.07$), but not by their interaction ($p = 0.260$). Differences in relative abundance of important cecal bacterial phyla and families for poultry are reported in Table 5 (see Supplementary Table S4 for more information). The data indicate that ARG birds harbored less ($p < 0.01$) Actinobacteria than CON birds, while the other phyla were not significantly affected by the factor group. However, room had a significant but opposite effect on Bacteroidetes and Firmicutes, with the former showing a lower ($p = 0.08$) abundance and the latter a higher ($p < 0.05$) abundance in the ceca of HS birds than those of TN birds. Moving to bacterial families, Bacilli unclassified significantly increased due to arginine supplementation and showed the greatest ($p = 0.001$) abundance in the ceca of ARG birds. Nevertheless, the effect of the factor room seems to prevail, as Enterobacteriaceae, Enterococcaceae, and Lachnospiraceae all increased due to HS exposure (p-values ranging from 0.07 to 0.02). An interactive effect between the factors group and room on the abundance of Bacteroidaceae ($p = 0.07$), Lachnospiraceae ($p = 0.09$), and Lactobacillaceae ($p < 0.01$) was found. The separation of the means showed that CON birds subjected to HS had more ($p < 0.05$) Lactobacillaceae than the other birds.

4 Discussion

While secondary to the main objective of the study, it is worth opening this discussion with an examination of growth performance results. Before the application of HS, arginine supplementation significantly reduced FCR (−5.7%) and increased BW at D27 (+3.3%), which accord with our earlier observations (Zampiga et al., 2018; Brugaletta et al., 2023) and those reported by other authors (Basoo et al., 2012; Xu et al., 2018; Liu et al., 2019; Sirathonpong et al., 2019). This therefore confirms that feeding diets high in arginine improves the performance of broilers under TN conditions. On the other hand, cyclic exposure to high

TABLE 5 Relative abundances (%) of important bacterial phyla and families in the cecal content of CON and ARG birds in the two rooms at D44.

Factor	Phylum				Family								
	Actinobacteria	Bacteroidetes	Firmicutes	Proteobacteria	Bacilli unclassified	Bacteroidaceae	Clostridia unclassified	Clostridiaceae	Enterobacteriaceae	Enterococcaceae	Lachnospiraceae	Lactobacillaceae	Ruminococcaceae
Group													
CON	1.5	6.8	89.3	1.8	0.2	0.8	17.9	2.3	0.6	0.05	8.5	0.9	36.9
ARG	0.8	7.6	88.0	1.5	0.7	0.8	17.5	1.9	0.4	0.10	8.5	0.5	36.1
Room													
TN	1.2	8.3	86.7	1.6	0.4	0.8	17.1	2.2	0.4	0.02	7.9	0.3	37.2
HS	1.1	6.2	90.6	1.7	0.5	0.7	18.3	2.0	0.7	0.12	9.1	1.1	35.8
Group × Room													
CON-TN	1.4	8.1	88.1	1.6	0.2	0.6	17.5	2.6	0.4	0.001	8.4	0.2 b	37.8
ARG-TN	0.9	8.5	85.4	1.6	0.6	1.1	16.7	1.8	0.4	0.05	7.4	0.4 b	36.6
CON-HS	1.7	5.6	90.5	1.9	0.3	1.0	18.2	2.0	0.9	0.10	8.5	1.6 a	36.0
ARG-HS	0.6	6.8	90.7	1.4	0.8	0.5	18.4	2.0	0.5	0.15	9.7	0.6 b	35.7
***p*-value**													
Group	**0.008**	0.495	0.461	0.216	**0.001**	0.979	0.863	0.160	0.234	0.234	0.897	**0.048**	0.654
Room	0.985	0.081	**0.027**	0.730	0.275	0.716	0.486	0.484	0.070	**0.024**	0.064	< **0.001**	0.431
Group × Room	0.254	0.770	0.390	0.323	0.617	0.072	0.767	0.176	0.226	0.956	0.089	**0.004**	0.778

Note: $n = 12$ birds/group/room. p-values less than 0.05 are in bold. Means that fall under the interaction between group and room and show distinct letters are significantly different ($p < 0.05$). CON, control group; ARG, arginine-supplemented group; TN, thermoneutral conditions; HS, (cyclic) heat stress conditions; SE, standard error.

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environmental temperatures considerably impaired growth performance, reducing FI and BW gain, and increased body temperature and mortality, thereby causing some of the adverse effects typically observed in heat-stressed chickens (Brugaletta et al., 2022; Teyssier et al., 2022). Arginine supplementation, however, did not alleviate the deterioration in performance or prevent environmentally induced hyperthermia, as the birds responded similarly to the thermal stress irrespective of the diet they were fed on. While performance and body temperature results are reliable because they are based on a fair number of replicates and rectal temperature measurements, respectively, speculations about mortality data must be made cautiously due to the modest number of birds per pen, which has made mortality rate a variable very susceptible to change in this trial. Data from the literature suggest that arginine supplementation could help broilers counteract the detrimental effects of HS on growth performance (Hassan et al., 2021; Teyssier et al., 2022). Nevertheless, our results seem to contradict that performance of heat-stressed broilers benefits from this nutritional strategy. A likely explanation for this inconsistency is that the favorable effects of arginine supplementation may have been somewhat blunted by the substantial reduction in FI caused by HS, which resulted in the ingestion of insufficient dietary arginine to preserve performance. It should be noted, however, that the reduction in FI was not of the same magnitude for control (−19.7%) and supplemented (−10.7%) birds under HS compared to their TN counterparts. Testing greater levels of dietary arginine to offset the FI loss might therefore be advantageous if further investigations on arginine supplementation for heat-stressed broilers are to be undertaken.

Anyway, the chief goal of the current study was to evaluate the metabolic and microbiota response of broilers to arginine supplementation and HS. Supplemented birds showed a significantly higher concentration of arginine in plasma—supporting again the results formerly reported by our lab (Zampiga et al., 2018; Brugaletta et al., 2023), as well as by Kidd et al. (2001) and investigators working with piglets (Kim et al., 2004) and rats (Holecek and Sispera, 2016)—liver, and *P. major*. These results provide further support for the hypothesis that increasing dietary arginine levels beyond those recommended leads to a considerable increase in its bioavailability, which is of paramount importance for animals unable to synthesize arginine *de novo*, such as chickens (Ball et al., 2007).

Metabolomics analyses also revealed greater concentrations of hepatic and circulating creatine for arginine-supplemented birds, reflecting what we have previously found (Brugaletta et al., 2023), as well as higher levels of creatine in *P. major*. The latter result confirms the observation of Chamruspollert et al. (2002) that breast muscles of broilers fed diets supplemented with arginine showed increased creatine content. Creatine, an AA derivative at the base of which are arginine and glycine, is produced largely in the liver and then transported to target tissues by blood (Walker, 1960; Oviedo-Rondón and Córdova-Noboa, 2020), serving, for example, as a key metabolite for skeletal muscle function and energy homeostasis (Oviedo-Rondón and Córdova-Noboa, 2020). Interestingly, it has been shown that dietary arginine intake affects creatine levels in different parts of the chicken's body (Keshavarz and Fuller, 1971a; 1971b; Chamruspollert et al., 2002) and that creatine supplementation improves growth performance and breast meat yield of broilers (Ringel et al., 2007; Oviedo-Rondón and Córdova-Noboa, 2020; Portocarero and Braun, 2021). In light of this, the increase in creatine levels can be considered a positive outcome of the arginine supplementation tested here.

Histidine-containing dipeptides and betaine were affected by HS exposure. Specifically, heat-stressed birds showed increased concentrations of carnosine and anserine in *P. major*, which is consistent with our earlier findings (Zampiga et al., 2021), while reduced concentrations of carnosine and betaine in plasma. Furthermore, HS raised the levels of beta-alanine and choline in *P. major*. Carnosine consists of histidine and beta-alanine, while anserine is formed by methylation of carnosine. These two dipeptides have been shown to have potent antioxidant activity and several biological functions, such as pH buffering, metal-ion chelation, complexing of dangerous carbonyl compounds, and anticross-linking effect on proteins, thereby protecting cells against stress and ischemia, as recently reviewed by Lackner et al. (2021). Betaine has been found to improve health, performance, carcass composition, and meat quality of poultry (Metzler-Zebeli et al., 2009). Being a powerful osmolyte, betaine can mitigate the effects of HS on cells (Ratriyanto and Mosenthin, 2018). Choline is an important provider of methyl groups and its metabolism is intimately related to that of betaine (a product of choline oxidation) and methionine. Choline is a multifunctional molecule that, among other things, constitutes many phospholipids (e.g., phosphatidylcholine) that maintain integrity and functions of cell membranes (Simon, 1999). Taken together, our present and previous data (Zampiga et al., 2021) support the idea that the accumulation of antioxidant and protective molecules in specific tissues, such as the pectoral muscle, is an essential part of the adaptive response to HS in chickens.

Besides arginine, the liver of arginine-supplemented birds was enriched in methionine, the first limiting AA for chickens (Leeson and Summers, 2001). This finding can be interpreted as a confirmation of what was previously assumed, namely that an improvement in intestinal mucosal health, integrity, function, and morphology mediated by arginine leads to improved digestion and absorption of dietary AAs (Brugaletta et al., 2023). Feeding the diet supplemented with arginine also resulted in reduced levels of the conditionally essential AA glutamine in the blood, similarly to what was observed in our former investigation (Brugaletta et al., 2023), as well as in liver, and *P. major*. Glutamine has been shown to be extremely important in supporting GI tract development and function and promoting gut health (Bortoluzzi et al., 2018; 2020). Its dietary supplementation has been found to attenuate the negative effects of enteric challenges in broilers, such as those of necrotic enteritis, coccidiosis, and *Salmonella* infection (Xue et al., 2018; Oxford and Selvaraj, 2019; Wu et al., 2022), as well as the impacts of HS on intestinal barrier integrity in mice as discussed in the review article by Bortoluzzi et al. (2018). Coster et al. (2004) pointed out that most of dietary glutamine and a quarter of glutamine in the blood are used by enterocytes and intestinal immune cells as a vital nutrient to obtain nitrogen and energy. Consequently, there is a real competition between the gut and extraintestinal tissues for glutamine. These authors also reported that almost all plasma glutamine is derived from the pool of free glutamine in skeletal muscle, the largest reservoir and most important site for the synthesis of this AA. In the liver, however,

glutamine is utilized as a substrate for gluconeogenesis and the synthesis of urea (in mammals), acute phase proteins, and glutathione. The reasons for the reduction in glutamine levels observed in the present study remain to be defined, but it can be hypothesized that intestinal mucosa of arginine-supplemented birds had an increased demand for glutamine, thereby draining arterial glutamine to fuel the accelerated metabolism associated with improved growth rates in the first two feeding phases of this trial. To validate this, it is necessary to analyze the metabolic profile of the intestinal epithelium. If this hypothesis is confirmed in future studies, it might be worth re-evaluating glutamine requirements for broilers fed with arginine above recommended levels.

Except for arginine, methionine, and glutamine, arginine supplementation did not significantly influence hepatic levels of other essential, conditionally essential, or non-essential AAs. The exposure to HS, however, modified the concentrations of many of them. HS decreased the levels of (iso)leucine, threonine, and valine in *P. major*, glycine and tyrosine in plasma and *P. major*, and aspartate and methionine in plasma, whereas increased the levels of glutamate, (iso)leucine, and threonine in liver, as well as those of phenylalanine in plasma and liver. These results broadly confirm that HS deeply alters protein metabolism in chickens, promoting the breakdown of skeletal muscle protein to supply the liver with AAs to be "burned" for energy (Zuo et al., 2015; Lu et al., 2018; Ma et al., 2021). Curiously, both arginine supplementation and HS reduced the concertation of another non-essential AA, that is alanine, in all tissues analyzed, apart from the liver when considering the effect of arginine supplementation. Similarly, HS decreased, in all tissues, the levels of N,N-Dimethylglycine, a betaine derivative involved in choline metabolism and synthesis of the antioxidant tripeptide glutathione by serving as a source of glycine (Simon, 1999; Kalmar et al., 2010). Our research group has found earlier a comparable reduction in N,N-Dimethylglycine in breast muscle of broilers reared under HS conditions (Zampiga et al., 2021). It has been reported that N, N-Dimethylglycine has free-radical scavenging properties (Hariganesh and Prathiba, 2010) and its use as a feed additive has been shown to produce positive effects on the health and performance of broilers (Kalmar et al., 2010; 2011; EFSA, 2011; Prola et al., 2013). These results, particularly the drop in N,N-Dimethylglycine levels caused by HS, and their potential implications for metabolism, growth, and responses to HS and oxidative stress would merit further in-depth investigations in broilers.

Oxidation-related molecules have a prominent role in the present discussion, which is not surprising considering the effects arginine and HS have been shown to have on oxidative stress (Akbarian et al., 2016; Wu et al., 2021). Arginine supplementation reduced the level of glutathione in the liver, as in our previous study (Brugaletta et al., 2023), and in *P. major*. Glutathione, composed of glutamate/glutamine, cysteine, and glycine, is very important for the antioxidant defense system, metabolism of nutrients, and regulation of cellular activities. Being the precursor of glutamate, arginine considerably influences the biosynthesis and levels of glutathione (Castro and Kim, 2020) that, like creatine, is mainly synthesized and provided by the liver (Wu et al., 2004). Considering this, increased hepatic glutathione levels would have been an expected consequence of arginine supplementation. The opposite outcome therefore needs further investigations. Energy-related molecules are also worth discussing. AMP was reduced in liver and *P. major* by arginine supplementation and was increased in *P. major* by HS. This nucleotide plays a pivotal role in many cellular metabolic processes, such as regulation of energy homeostasis by modulating the activity of the enzyme AMP-activated kinase (**AMPK**). Recognized as the master energy sensor for cells (Hardie, 2003; Hardie et al., 2006), AMPK senses energy levels by detecting modifications in the AMP to ATP ratio (Xiao et al., 2011; Chen et al., 2013). Under energy depletion (i.e., increased levels of AMP and decreased levels of ATP), AMP binds to the γ subunits of AMPK leading to the activation of this kinase that results in promotion of catabolic pathways and inhibition of anabolic pathways to generate ATP (Stein et al., 2000). Thus, the data from this study suggest that the liver and *P. major* of arginine-supplemented birds probably were in a good energy balance (low AMP levels), while *P. major* of heat-stressed birds suffered from energy depletion (high AMP levels). According to Baumgard and Rhoads (2013), negative energy balance and catabolic states are two of the most distinctive features of HS conditions. It is therefore intriguing that arginine supplementation may have been able to partially counterbalance the adverse effects of HS on energy homeostasis of broilers by increasing creatine levels and attenuating the increase in AMP levels, particularly in pectoral muscle. Non-etheless, the significant reduction in the levels of fumarate in all tissues and of succinate and UDP in plasma and liver supports the hypothesis that birds exposed to HS had a suboptimal energy balance. Succinate and fumarate are two consecutive intermediates in the citric acid cycle and, as such, their reduced availability can inhibit this central metabolic pathway, interfering with cellular bioenergetic processes. On the other hand, UDP is important in glycogenesis because it is combined with glucose to form UDP-glucose units that can be polymerized to glycogen chains. As UDP levels were reduced by HS, it can be assumed that glycogenesis, a chief anabolic pathway occurring in liver and muscles, was hampered in heat-stressed birds. In contrast, glycogenolysis, and gluconeogenesis may have been promoted to increase hepatic glucose production as reviewed by Rhoads et al. (2013), potentially resulting in the increased level of plasma glucose observed in heat-stressed birds.

Commenting on the results of cecal microbiota analysis, it is interesting to note that arginine supplementation significantly reduced observed diversity and the abundance of Actinobacteria, while increased the abundance of Bacilli unclassified. The change in observed diversity is similar to that obtained in our previous study by supplementing arginine to broilers reared under TN conditions (Brugaletta et al., 2023), but it is opposite to that of Singh et al. (2019) who found an increase in Shannon index in colonic samples taken from mice fed on a diet high in arginine. This inconsistency, however, may be due to the different animal species used and the origin of the intestinal content analyzed. The exposure to HS significantly increased alpha diversity indices (i.e., observed diversity, Shannon, and Inverse Simpson), which is consistent with the changes in ileal alpha diversity reported by Wang et al. (2018). HS was also found to significantly affect beta diversity, reduce the abundance of Bacteroidetes and increase that of Firmicutes, confirming the studies by Shi et al. (2019); Liu et al.

(2020),; Goel et al. (2022). Moreover, the exposure to HS resulted in increased abundances of Enterobacteriaceae, Enterococcaceae, and Lachnospiraceae. The increase in Enterobacteriaceae, one of the most important members of the phylum Proteobacteria, caused by HS partly corroborates the work by Shi et al. (2019) who found that the abundance of cecal Proteobacteria increased in broilers exposed to HS. On the other hand, no results comparable to ours were found in the literature consulted regarding Enterococcaceae and Lachnospiraceae, so the effects of HS on these two cecal bacterial families in broilers are worth investigating further. Overall, the substantial variations in alpha and beta diversities and the results of taxonomic analysis indicate that the HS model applied here considerably affected the microbiota of broilers, broadly supporting previous research on this topic (Suzuki et al., 1983; Burkholder et al., 2008; Song et al., 2014; He et al., 2021; Liu et al., 2022). Interestingly, the factors group and room had a relevant interactive effect only on Lactobacillaceae, which were more abundant in birds fed the control diet and reared under HS conditions compared to the others. However, taking the cue from the study by Singh et al. (2019), further work is needed to elucidate how and to what extent arginine supplementation modulates the GI microbiota and its relationship with the broiler host under HS conditions.

In summary, this study shed light on some intricate metabolic and microbiota changes induced by arginine supplementation and cyclic HS in broilers, while also offering valuable starting points for future investigations that will undoubtedly help researchers better characterize the response of broilers to these two factors and their interaction.

Data availability statement

The datasets presented in this study can be found in online repositories. The names of the repository/repositories and accession number(s) can be found below: Metagenome sequence data—https://www.ncbi.nlm.nih.gov/, Bioproject PRJNA928248.

Ethics statement

The animal study was approved by the Ethical Committee of the University of Bologna (ID: 4387).

Author contributions

GB co-designed the study, collected the samples, analyzed the data, and wrote the manuscript. MZ and RP collected the samples and revised the manuscript. LL performed the ^{1}H-NMR analyses and revised the manuscript. CO performed the lab analysis for the microbiota. VI run the bioinformatic and statistical analyses of microbiota data and revised the manuscript. SP made the experimental diets and collected the samples. MP revised the manuscript. ADC and FS co-designed and supervised the study and revised the manuscript. All authors read and approved the submitted version of the manuscript.

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Conflict of interest

The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

Publisher's note

Supplementary material

The Supplementary Material for this article can be found online at: https://www.frontiersin.org/articles/10.3389/fphys.2023.1155324/full#supplementary-material

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Transcriptome and co-expression network analysis reveals the molecular mechanism of inosine monophosphate-specific deposition in chicken muscle

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The inosine monophosphate (IMP) content in chicken meat is closely related to muscle quality and is an important factor affecting meat flavor. However, the molecular regulatory mechanisms underlying the IMP-specific deposition in muscle remain unclear. This study performed transcriptome analysis of muscle tissues from different parts, feeding methods, sexes, and breeds of 180-day-old Jingyuan chickens, combined with differential expression and weighted gene co-expression network analysis (WGCNA), to identify the functional genes that regulate IMP deposition. Out of the four comparison groups, 1,775, 409, 102, and 60 differentially expressed genes (DEGs) were identified, of which *PDHA2*, *ACSS2*, *PGAM1*, *GAPDH*, *PGM1*, *GPI*, and *TPI1* may be involved in the anabolic process of muscle IMP in the form of energy metabolism or amino acid metabolism. WGCNA identified 11 biofunctional modules associated with IMP deposition. The brown, midnight blue, red, and yellow modules were strongly correlated with IMP and cooking loss ($p < 0.05$). Functional enrichment analysis showed that glycolysis/gluconeogenesis, arginine and proline metabolism, and pyruvate metabolism, regulated by *PYCR1*, *SMOX*, and *ACSS2*, were necessary for muscle IMP-specific deposition. In addition, combined analyses of DEGs and four WGCNA modules identified *TGIF1* and *THBS1* as potential candidate genes affecting IMP deposition in muscle. This study explored the functional genes that regulate muscle development and IMP synthesis from multiple perspectives, providing an important theoretical basis for improving the meat quality and molecular breeding of Jingyuan chickens.

KEYWORDS

Jingyuan chicken, muscle, inosine monophosphate, transcriptome, weighted gene co-expression network analysis

1 Introduction

With further economic and societal developments, the demand for meat products continues to increase, and more focus is being placed on meat quality. The second most consumed meat in China is poultry, and its demand is increasing annually. It has become an essential source of meat in daily life. In recent years, there has been a significant improvement in meat production through the use of modern specialized broiler chickens. This improvement can be attributed to high-intensity selection for growth rate

in poultry breeding efforts (Maharjan et al., 2021). However, the quality of chicken meat has significantly decreased, particularly meat flavor (Bao et al., 2008; Katemala et al., 2021). Therefore, while improving poultry growth rate, cultivating high-quality local chicken breeds with excellent meat quality and unique flavors has become the main research direction for modern poultry molecular breeding.

Animal muscle quality includes various indicators, such as pH, water-holding capacity (WHC), cooking loss, shear force, and flavor. Muscle flavor is an important aspect of meat quality evaluation, and mainly includes umami and aroma (Liu et al., 2017). Inosine monophosphate (IMP) is the most important umami substance in livestock and poultry muscles, and the content of IMP in chicken meat is 4.5 times higher than that of free glutamic acid (Ninomiya, 1998). IMP is an important precursor of meat aromas (Cambero et al., 1992). The influence of IMP on muscles is crucial for meat flavor. Muscle IMP synthesis and metabolic processes involve gene expression, signal transduction, and network regulation; however, many regulatory factors still need to be explored.

Transcriptomics can be used to identify differentially expressed genes associated with target traits from a large amount of genomic information, thus revealing the internal association between gene expression and specific physiological processes (Wang et al., 2009). However, many differentially expressed genes are typically identified in high-throughput sequencing results, and single-gene analysis frequently misses important information. Therefore, the weighted gene co-expression network analysis (WGCNA) method was developed based on global gene-expression patterns. WGCNA has been successfully used to identify gene expression networks and biomarkers of interest in multiple groups of samples (Li et al., 2018; Cheng et al., 2020; Yuan et al., 2020; Yuan and Lu, 2021), thereby alleviating the problem of multiple detections in extensive data analysis. The application of WGCNA to chicken muscle IMP deposition-related gene expression has yet to be reported.

The Jingyuan chicken is an outstanding excellent local breed that has received national recognition for its excellent quality. Known for its slow growth rate and strong resistance, this breed produces meat that is rich in amino and fatty acids, high in muscle IMP content, and incredibly delicious (Yu et al., 2022). This is an ideal animal model for studying the meat quality of slow-growing chickens. Our previous studies have revealed differences in IMP content among Jingyuan chicken parts, feeding methods, sex, and breeds (Zhang et al., 2020; Zhang et al., 2021; Wang et al., 2022). Therefore, in this study, transcriptome sequencing was performed on breast and leg muscles of caged Jingyuan hens, breast muscles of caged Jingyuan roosters, and breast muscles of free-range Jingyuan chickens and Pudong hens (slow-growing local native chickens with excellent meat quality). Multiple differentially expressed genes (DEGs) and metabolic pathways closely related to IMP anabolic processes were identified in the different comparative groups. WGCNA was used to construct co-expressed gene modules related to muscle quality indicators, and *PYCR1*, *SMOX*, *ACSS2*, *TGIF1*, and *THBS1* were identified as potential candidate genes regulating IMP deposition by mRNA-trait association analysis. This study explored the molecular markers regulating muscle IMP synthesis and metabolism in Jingyuan chickens from multiple perspectives, which are economically important for improving the meat quality and molecular breeding of Jingyuan chickens and also provide an important reference for the development and utilization of local chicken breeds.

2 Materials and methods

2.1 Animal and sample collection

Jingyuan chickens were used in this study after 11 generations of purification and rejuvenation and a family-equivalent breeding population was established. Breeding was performed using the closed herd breeding method. To date, two generations of breeding conservation work have been conducted, and all experimental samples were collected at the Jingyuan Chicken National Conservation Farm (Pengyang County, Ningxia). We obtained permission to use Jingyuan chickens and fed them with the same diet as that used on the source farm. After growing to 180 days old, 15 caged Jingyuan roosters and hens, 15 free-range Jingyuan hens, and five free-range Pudong hens were randomly selected. Caged chickens were fed individually in 40 × 40 × 40 cm cages. After fasting for 12 h, the birds were killed under carbon dioxide anesthesia (inhaled 40%). Samples of breast and leg muscles were rapidly collected; one tissue sample was used for the determination of IMP, inosine, and various physical indices (Zhang et al., 2020; Zhang et al., 2021; Wang et al., 2022) (Supplementary Table S1), and another was snap-frozen in liquid nitrogen and stored at −80°C.

2.2 Library construction and transcriptome sequencing

The transcriptome analysis sample grouping information is shown in Table 1. Muscle tissues with different IMP contents between the groups were selected as sequencing samples. Magnetic beads with oligo (dT) were used to enrich the mRNA after assessing the total RNA quality of the muscle tissue. Purified mRNA was used as a template to synthesize single-stranded cDNA using a random hexamer primer, followed by the addition of DNA polymerase I and RNase H to synthesize double-stranded cDNA. The final cDNA library was amplified using PCR. Library quality was evaluated using the Agilent Bioanalyzer 2,100 system (Agilent Technologies, Santa Clara, CA, United States). After passing the library inspection, 15 libraries were submitted to the Illumina HiSeq platform for sequencing and completed by Beijing Nuohe Zhiyuan Technology Co., Ltd. (Beijing, China).

2.3 Differential expression analysis

Raw reads were filtered after sequencing and more than 38,940,706 high-quality clean reads were obtained from each muscle sample. The mapping rate of these reads to the *Gallus gallus* reference genome was >72.14% (Supplementary Table S2). HTSeq (V0.6.1) software was used to analyze gene expression levels in 15 samples, and the FPKM of each gene was calculated (the sum of the FPKM of three replicates was less than 0.1 and can be considered not expressed). The DESeq2 (Love et al., 2014) package was used to perform differential expression analysis between groups, with $p < 0.05$ and $|\log_2(\text{fold change})| > 1$ as thresholds to identify DEGs between groups.

TABLE 1 Transcriptome sequencing sample grouping information.

Group	Sample name	Gender	Muscle	Feeding method	Breed
PJFXL	l27tho, l29tho, l30tho	hen	breast	caged	Jingyuan chicken
PJFTL	l17leg, l18leg, l20leg	hen	leg	caged	
PJMXL	l2tho, l6tho, l10tho	rooster	breast	caged	
PJFXS	PYs2tho, PYs3tho, PYs12tho	hen	breast	free-ranged	
JHFXS	sh3tho, sh4tho, sh5tho	hen	breast	free-ranged	Pudong chicken

2.4 Weighted gene co-expression network analysis

WGCNA is suitable for the analysis of complex trait data and allows for further exploration of key genes affecting the phenotype. In this study, we used FPKM values obtained from mRNA-seq, removed genes with a mean FPKM <1, and applied the WGCNA package tool (Langfelder and Horvath, 2008) to construct co-expression networks. First, the Pearson correlation between genes was calculated to construct a gene co-expression correlation matrix. Subsequently, the optimal soft threshold ($\beta = 12$) was selected according to the criterion of an approximately scale-free topology, and a weighted adjacency matrix was generated. Furthermore, the adjacency matrix was converted into a topological overlap matrix (TOM) using a correlation expression value analysis. Next, the TOM was used to cluster the genes, and the clustered modules were classified using dynamic shearing to identify highly co-expressed gene modules. The key modules of interest were identified based on the first principal component computation module eigengene (ME) of the expression profile and correlated with phenotypic traits. In addition, Pearson correlations between gene expression profiles and phenotypic traits were calculated to estimate gene significance (GS). Finally, the relationship between genes and traits was quantified using the GS module. Then, we analyzed the correlation between GS and module membership (MM), and genes with GS > 0.5 and MM > 0.9 in the trait-specificity module were identified as hub genes.

2.5 DEGs and hub genes functional enrichment analysis

To explore the functions of the DEGs and significant modules, GO function and KEGG pathway enrichment analyses were performed using the Cluster Profiler tool. GO terms and KEGG pathways attaining $p < 0.05$ were significantly enriched for the DEGs and hub genes.

2.6 Key gene identification and correlation analysis

The Upset tool was used to analyze the intersection of DEGs and hub genes, and the Pearson correlation between the intersection of genes with IMP and cooking loss was calculated. The protein interaction network of the differential genes was analyzed using the STRING database (http://string-db.org/), where the minimum required interaction score was set to high confidence (0.700).

2.7 Real-time fluorescence quantitative PCR

The heart, liver, spleen, lungs, kidneys, abdominal fat, breast muscles, and leg muscles of 180-day-old Jingyuan chickens were collected for gene expression profiling. Total RNA was extracted using RNAiso Plus (Takara, Dalian, China) according to the manufacturer's instructions and cDNA was synthesized using the PrimeScript RT Reagent Kit (Perfect Real Time; Takara, Dalian, China). Primer 6.0 was used to design the mRNA primers (Supplementary Table S3). qPCR was performed using a CFX96 real-time PCR detection system (Bio-Rad) according to the instructions for the SYBR@ Green Premix Pro Taq HS qPCR Kit (Accurate Biology, Changsha, China), with β-actin as an internal reference, and three replicates for each sample. The relative expression of mRNA was calculated using the $2^{-\Delta\Delta Ct}$ method, and the results are expressed as the mean ± standard deviation.

3 Results

3.1 Sequencing data evaluation

In this study, 15 mRNA-sequencing libraries were constructed for five groups of muscle samples. A total of 18,931 genes were detected (Supplementary Table S4), of which 15,947 genes were expressed in the PJFXL group, 16,326 in the PJFTL group, 15,786 in the PJMXL group, 15,717 in the PJFXS group, and 15,676 in the JHFXS group. The most abundantly expressed genes were actin alpha 1 (*ACTA1*), troponin I2, fast skeletal type (*TNNI2*), glyceraldehyde-3-phosphate dehydrogenase (*GAPDH*), myosin light chain 1 (*MYL1*), and phosphoglycerate mutase 1 (*PGAM1*).

Principal component analysis (PCA) and intersample correlation analyses showed that the difference between the breast and leg muscles was the most significant (PC1), with significant differences among the PJFXL, PJFXS, and JHFXS groups (Figure 1A). The Pearson correlation coefficient among samples was greater than 0.825 (Figure 1B). This indicated that the transcriptome sequencing results were reliable and could be used for subsequent analyses.

3.2 Screening and identification of DEGs

ptIn each group, 6.33%–7.59% of genes were extremely highly expressed, and the expression levels of most other genes were relatively uniform (Figure 2A). We further analyzed the DEGs between different comparison groups to investigate the key mRNAs regulating IMP-specific deposition in Jingyuan chicken muscle. We

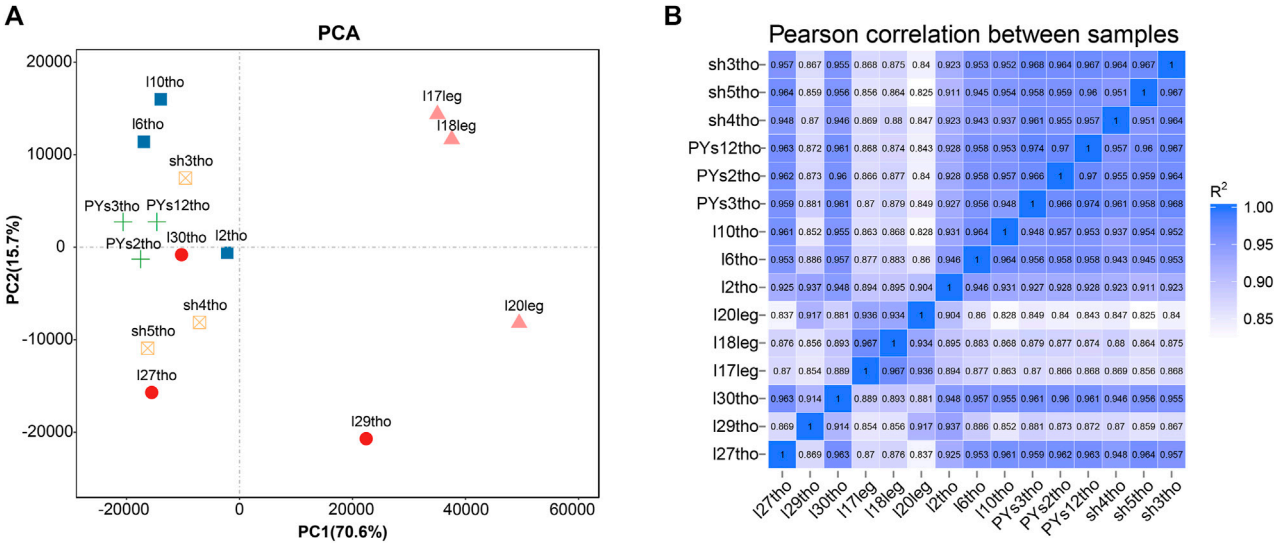

FIGURE 1
Sample principal components and correlation analysis. **(A)** Principal component analysis (PCA) of five groups of muscle samples. **(B)** Heatmap of Pearson correlation between samples based on gene expression.

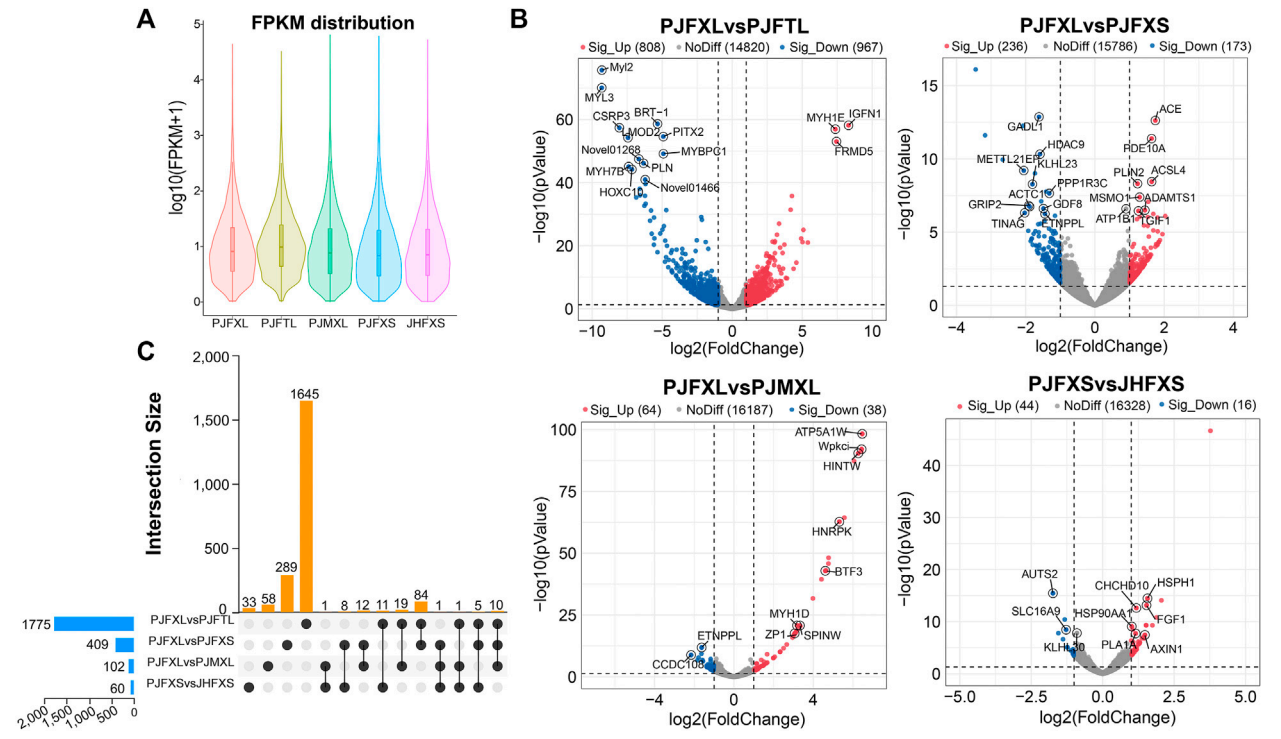

FIGURE 2
Identification of DEGs in five groups of muscle samples. **(A)** Distribution of gene expression levels in different groups. **(B)** Volcano plots of DEGs in the PJFXL vs. PJFTL, PJFXL vs. PJFXS, PJFXL vs. PJMXL, and PJFXS vs. JHFXS groups (Sig_Up: significantly upregulated; Sig_Up: significantly downregulated; NoDiff: no significant difference). **(C)** Upset diagram of four group comparatives DEGs (The horizontal bars on the left indicate the number of DEGs required for each group comparison. The individual points in the middle matrix represent the DEGs specific to each group comparison, and the lines between points represent the DEGs common to different group comparisons. The vertical bars represent the number of DEGs specific to or common to different group comparisons).

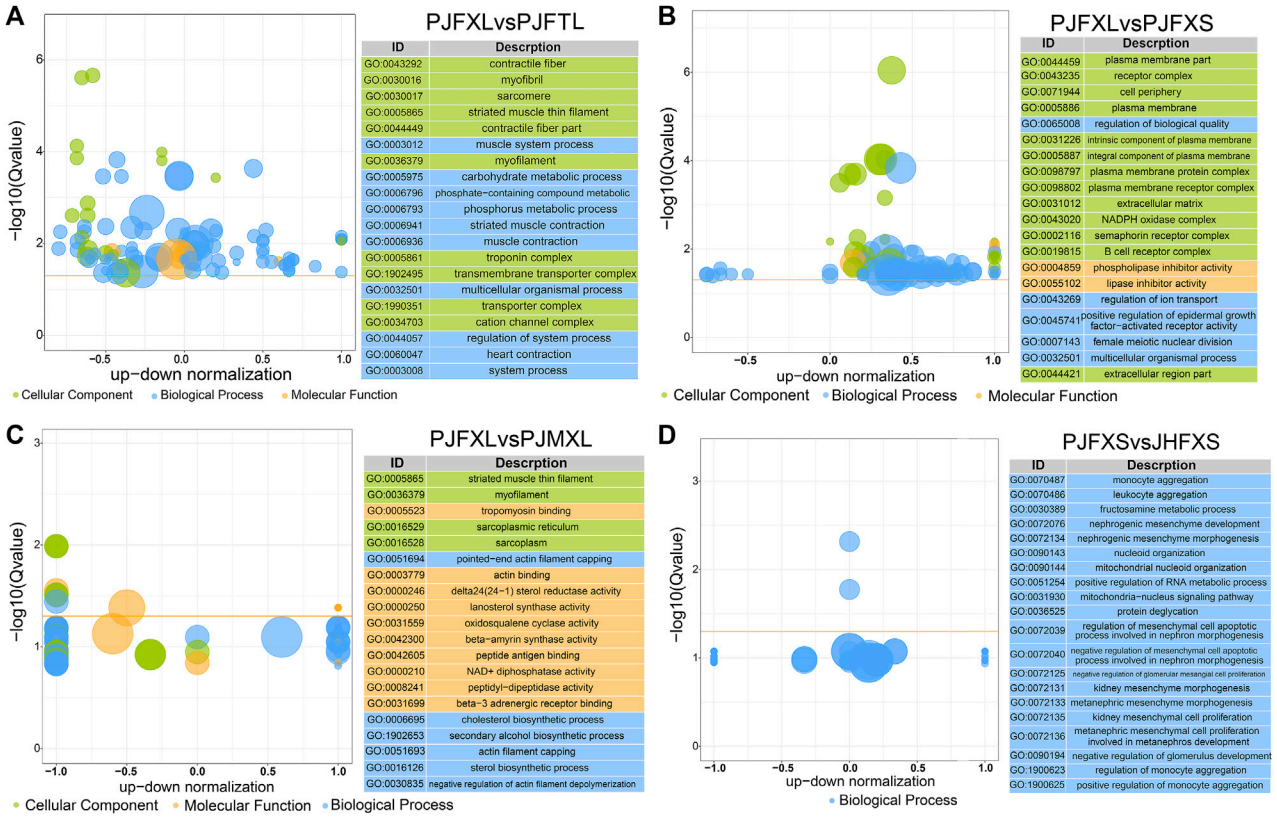

FIGURE 3
GO functional enrichment results of DEGs in **(A)** PJFXL vs. PJFTL, **(B)** PJFXL vs. PJFXS, **(C)** PJFXL vs. PJMXL, and **(D)** PJFXS vs. JHFXS group comparisons (The vertical coordinate is $-\log_{10}$ (Qvalue), the horizontal coordinate is the up-down normalization value: the difference between the number of differentially up-regulated genes and the number of differentially down-regulated genes as a percentage of the total differential genes, and the size of the bubble indicates the number of target genes currently enriched by the GO term).

identified 1775 DEGs in the PJFXL *versus* PJFTL comparison, of which 808 were upregulated and 967 were downregulated. A total of 409 DEGs were identified in the PJFXL *versus* PJFXS comparison, of which 236 were upregulated and 173 were downregulated. A total of 102 DEGs were identified in the PJFXL *versus* PJMXL comparison, of which 64 were upregulated and 38 were downregulated. Sixty DEGs were identified in the PJFXS *versus* JHFXS comparison, of which 44 were upregulated and 16 were downregulated (Figure 2B). Hierarchical cluster analysis revealed significant differences in the expression levels of the DEGs among the comparison groups (Supplementary Figure S1). None of the DEGs were common to all four comparison groups, and there were at most ten DEGs in common among any three groups (Figure 2C).

3.3 GO and KEGG enrichment analysis of DEGs

To gain insight into the functions of the DEGs, we performed GO and KEGG enrichment analyses to reveal the molecular mechanism of IMP anabolism in Jingyuan chicken muscles. GO enrichment analysis showed significantly enriched DEGs in the PJFXL vs. PJFTL comparison in functional terms, such as myofibril, muscle system process, carbohydrate metabolic process, actin binding, and kinase activity (Figure 3A). The DEGs in the PJFXL vs. PJFXS comparison were mainly related to GO terms such as receptor complex, lipase inhibitor activity, and positive regulation of epidermal growth factor-activated receptor activity (Figure 3B). Twelve GO terms were significantly enriched in DEGs in the PJFXL vs. PJMXL comparison, including striated muscle thin filaments, tropomyosin binding, and pointed-end actin filament capping (Figure 3C). Most of the DEGs in the PJFXS vs. JHFXS comparison were enriched in biological processes, and the two significantly enriched GO terms were monocyte and leukocyte aggregation (Figure 3D).

KEGG pathway enrichment analysis showed that DEGs in the PJFXL vs. PJFTL comparison were significantly enriched in 32 signaling pathways, including glycolysis/gluconeogenesis, carbon metabolism, pyruvate metabolism, biosynthesis of amino acids, the PPAR signaling pathway, and the FoxO signaling pathway (Figure 4A), which regulate multiple genes related to muscle development and IMP anabolism. Nine signal pathways were significantly enriched by DEGs in the PJFXL vs. PJFXS comparison, including the p53 signaling pathway, the FoxO signaling pathway, and pentose and glucuronate interconversions are essential to regulate muscle development (Figure 4B). DEGs in the PJFXL vs. PJMXL comparison were significantly enriched in 17 signaling pathways, including steroid biosynthesis and the renin-angiotensin system (Figure 4C). DEGs in the PJFXS vs. JHFXS comparison were enriched in only nine signaling pathways, including circadian rhythm, malaria, and cytokine-cytokine

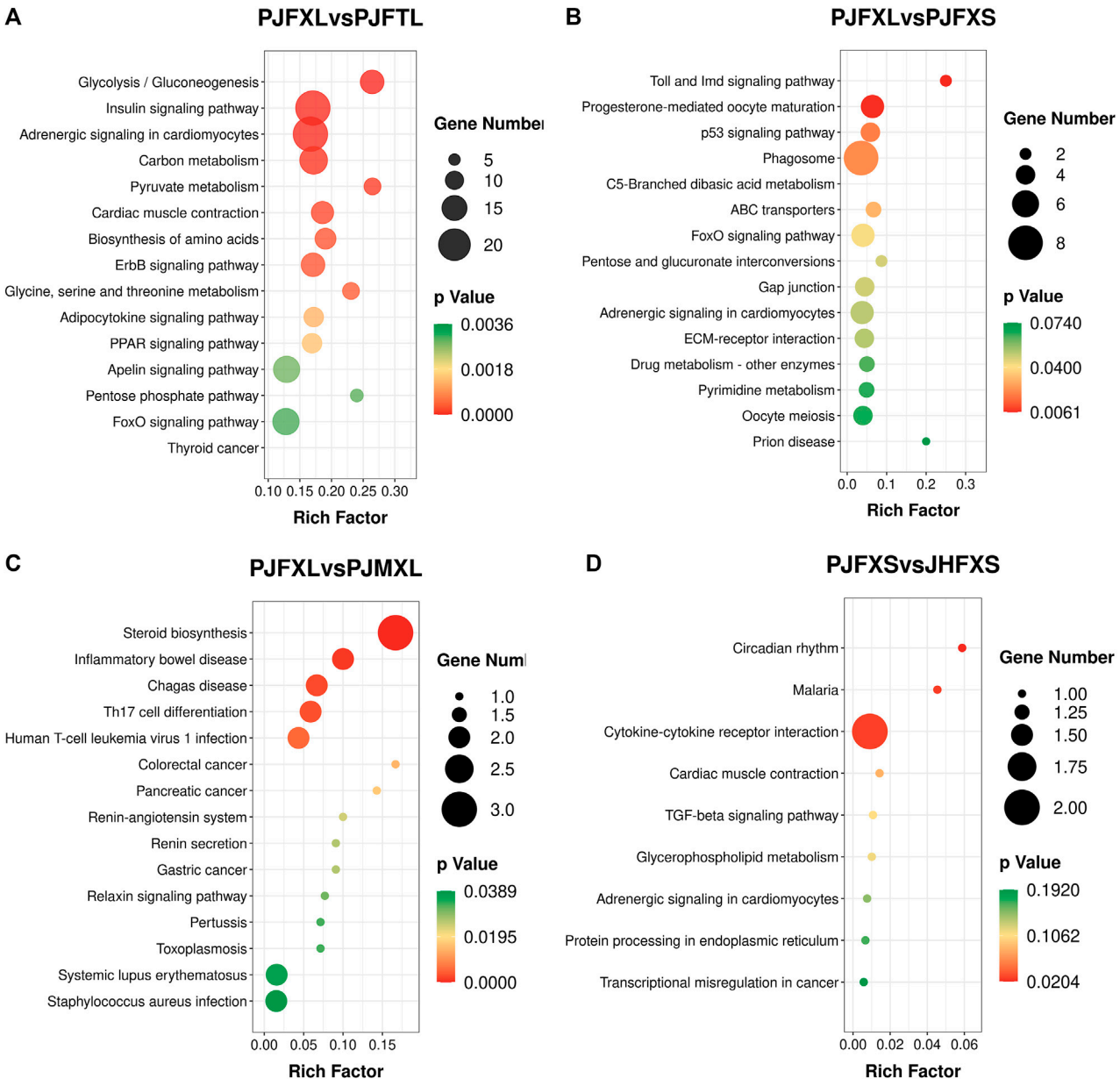

FIGURE 4
KEGG pathway enrichment results of DEGs in **(A)** PJFXL vs. PJFTL, **(B)** PJFXL vs. PJFXS, **(C)** PJFXL vs. PJMXL, and **(D)** PJFXS vs. JHFXS group comparisons (Rich Factor indicates the ratio of the number of differential genes to the number of all genes in the background gene set that are enriched in the pathway).

receptor interaction (Figure 4D). Comprehensive analysis identified *PDHA2*, *ACSS2*, *PGAM1*, *GAPDH*, *PGM1*, *GPI*, and *TPI1* as key genes regulating IMP deposition (Table 2), which are likely to be involved in muscle IMP synthesis and metabolism through a variety of mechanisms, such as energy and amino acid synthesis.

3.4 Weighted gene co-expression network construction

For WGCNA analysis, genes with an average FPKM of less than 1.0 were removed. Based on the gene clustering tree analysis, the 11,640 genes were divided into 12 modules (the gray module in Figure 5A indicates that these genes had a low pattern of variation throughout the experiment and that the pattern of variation could not be used to associate with other genes; therefore, the subsequent analysis was removed). The two largest modules in Figure 5A contained 692 and 536 genes, and the two smallest modules contained 76 and 53 genes, respectively (Supplementary Table S5). A heat map of gene co-expression networks was used to explore the interactions between modules, and multiple modules were found to be interrelated (Figure 5C).

Next, the gene modules significantly associated with meat quality phenotypes were identified. We found that six modules

TABLE 2 Key pathways for significantly enriched of DEGs.

Group	Pathway	Pathway id	*p*-value	Genes
PJFXL vs. PJFTL	Glycolysis/Gluconeogenesis	ko00010	1.72E-06	*PKLR, MINPP1, PDHA2, PGK2, GPI, PGAM1, TPI1, FBP2, PGM1, BPGM, ACSS2, GAPDH, LDHA*
	Pyruvate metabolism	ko00620	0.000125	*ACSS2, PKLR, LDHA, ACYP2, ACYP1, PDHA2, GLO1, ME1*
	Biosynthesis of amino acids	ko01230	0.000299	*CYLY, TPI1, PKLR, PRPS1L1, BCAT1, GAPDH, PSPH, CTH, RPIA, PGK2, CPS1, ARF6, PGAM1*
	Adipocytokine signaling pathway	ko04920	0.001304	*SLC2A1, MAPK10, ACSBG2, PRKAG3, PRKAA2, CD36, ADIPOQ, SOCS3, ACSBG1*
	PPAR signaling pathway	ko03320	0.001487	*FABP6, LPL, CPT2, ACSBG1, ADIPOQ, ACADL, CD36, PLIN1, ACSBG2, GK2, ME1*
	Pentose phosphate pathway	ko00030	0.002986	*FBP2, PGM1, PRPS1L1, RPIA, FBP1, GPI*
	FoxO signaling pathway	ko04068	0.003087	*CDKN1A, MAP2K2, IL10, FOXO3, HOMER2, SETD7, FOXO1, PRKAG3, PIK3R3, CCND1, PRKAA2*
	Focal adhesion	ko04510	0.015515	*TLN2, CHAD, SHC2, PAK1, MYL10, THBS1, VEGFA, MYL2, CCND1, MAPK10*
	Glycerophospholipid metabolism	ko00564	0.034045	*GPD2, MBOAT2, GPAM, PISD, PLA2G2E, GPD1L, PEMT, DGKH, AGPAT2*
	Regulation of actin cytoskeleton	ko04810	0.049843	*MYL2, ENAH, PPP1CC, PIK3R3, FGF4, PAK5, GIT1, FGF9, PDGFA, PAK1, SSH2*
PJFXL vs. PJFXS	p53 signaling pathway	ko04115	0.019724	*RRM2, THBS1, CDK1, CCNB1*
	FoxO signaling pathway	ko04068	0.041711	*PLK3, CCNB3, PLK1, FOXO1, CCNB1*
	Pentose and glucuronate interconversions	ko00040	0.047352	*AKR1B10, KL*
PJFXL vs. PJMXL	Steroid biosynthesis	ko00100	9.24E-06	*LSS, MSMO1, DHCR24*
PJFXS vs. JHFXS	Cytokine-cytokine receptor interaction	ko04060	0.027194	*BMP7, THPO*

(Figure 5B) were significantly and negatively correlated with IMP, including brown ($r = -0.68$, $p = 0.005$) and midnight blue ($r = -0.66$, $p = 0.007$). Inosine was significantly negatively correlated with the cyan module ($r = -0.62$, $p = 0.01$). Six modules (yellow, red, midnight blue, brown, green-yellow, and magenta) were positively correlated with cooking loss. The modules with strong correlations were yellow ($r = 0.75$, $p = 0.001$), red ($r = 0.74$, $p = 0.002$) and midnight blue ($r = 0.7$, $p = 0.004$). There was a significant positive correlation between WHC and the green-yellow module ($r = 0.57$, $p = 0.03$). At the same time, we found that the brown, midnight blue, red, and yellow modules were strongly correlated with both IMP and cooking loss, and the GS of these four modules was the highest among all the co-expression modules (Figure 5D).

3.5 Hub gene screening and functional analysis

After identifying the four important modules, we further explored the hub genes in each module using GS and MM. The brown module obtained 178 hub genes, and the correlation between GS and MM was 0.56 ($p = 2.7e-29$) (Figures 6A–1). Functional enrichment analysis showed that hub genes in this module were significantly enriched in GO terms such as regulation of protein localization to the membrane and regulation of kinase activity (Figures 6A–2). In addition, hub genes were significantly enriched in the adipocytokine signaling, glycerophospholipid metabolism, and arginine and proline metabolism pathways (Figures 6A–3). The midnight blue module obtained 132 hub genes, and the correlation between GS and MM was 0.75 ($p = 2.4e-43$) (Figures 6B–1). Hub genes were significantly enriched in such GO terms as phosphate metabolic process regulation, small molecule metabolic process, and signaling receptor activity (Figures 6B–2). Among the nine KEGG pathways that were significantly enriched, the PPAR signaling pathway, pyruvate metabolism, and glycolysis/gluconeogenesis are necessary for muscle IMP deposition (Figures 6B–3). The yellow module obtained 177 hub genes, and the correlation between GS and MM was 0.76 ($p = 7.7e-59$) (Figures 6C–1). The hub genes in this module were significantly enriched in GO terms such as skeletal muscle cell differentiation and phosphoric ester hydrolase activity (Figures 6C–2). Twelve KEGG pathways were significantly enriched, including the TGF-β signaling pathway, regulation of the actin cytoskeleton, and glycerolipid metabolism (Figures 6C–3). The red module obtained 129 hub genes, and the correlation between GS and MM was 0.87 ($p = 2.9e-68$) (Figures 6D–1). The hub genes in this module were significantly enriched in GO terms such as actin filament binding, growth factor activity, and myosin complex (Figures 6D–2), and significantly enriched in steroid biosynthesis, PPAR signaling pathways, cardiac muscle contraction, calcium signaling pathways, and fatty acid degradation signal pathways (Figures 6D–3).

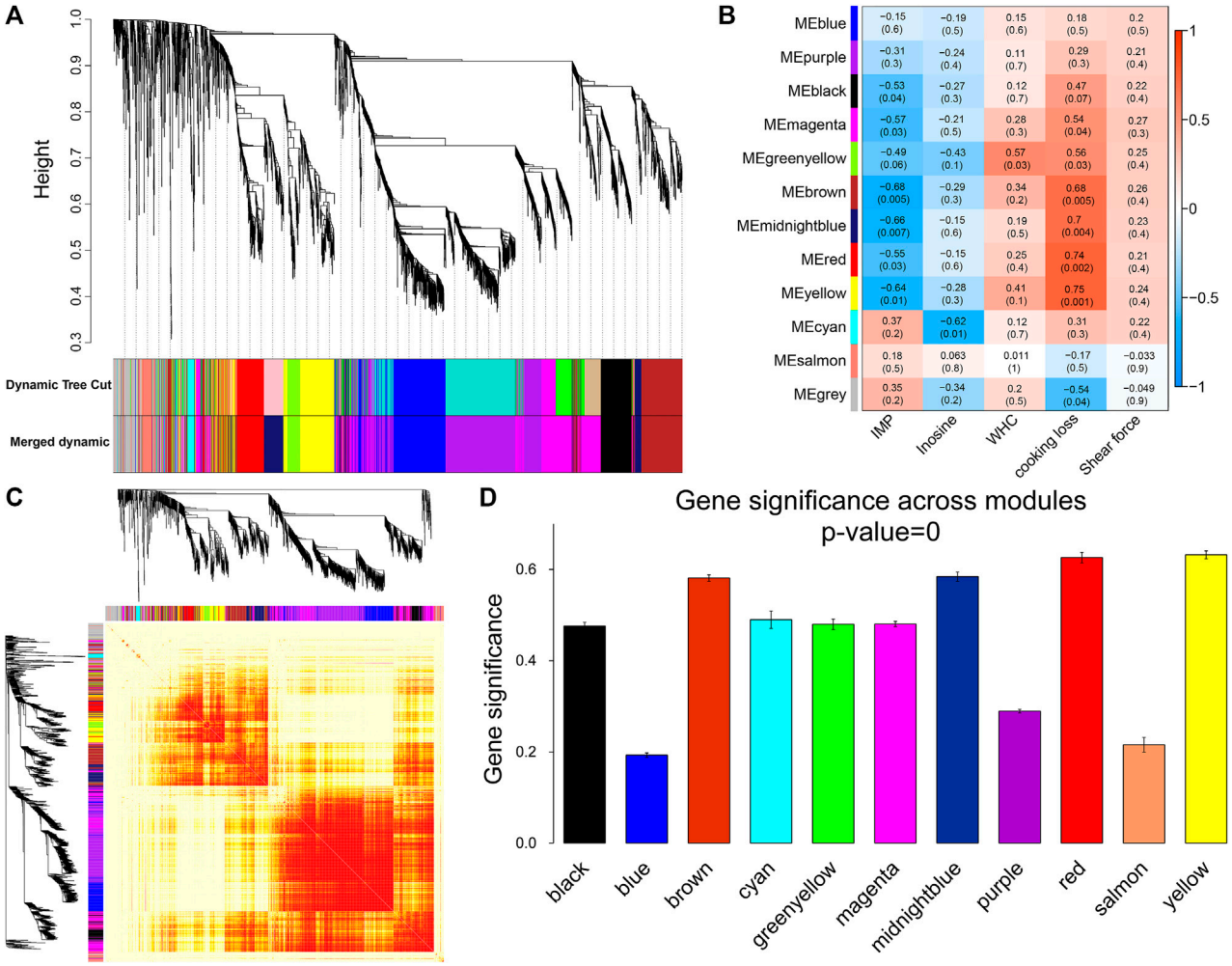

FIGURE 5
Module identification and trait correlation analysis. **(A)** Gene modules based on phylogenetic clustering tree. **(B)** Correlation analysis of 12 modules and muscle phenotypes. **(C)** Heatmap of gene co-expression network (the heatmap area indicates the dissimilarity between genes, the smaller the value, the darker the color). **(D)** Gene significance in the module.

Joint differential expression analysis identified glycolysis/gluconeogenesis, arginine and proline metabolism, PPAR signaling pathway, and pyruvate metabolism as the functional pathways regulating IMP anabolism. The key genes *PYCR1*, *SMOX*, and *ACSS2* may play important regulatory roles in muscle IMP deposition.

3.6 Functional gene identification

Association analysis of hub genes in the four significant modules with intergroup DEGs was performed and 14 intersecting genes *GRIN2C, SOCS3, MSMO1, HSPH1, TMOD1, LMOD2, FNDC5, MAFF, TGIF1, THBS1, PAQR9, FHL1, HBEGF*, and *UBTD1* were common to at least one module and two differential comparison groups (Figure 7A). These 14 genes were positively correlated with cooking loss ($p < 0.05$) and 11 were negatively correlated with IMP ($p < 0.05$) (Figure 7B). The TGF-β signaling pathway genes *TGIF1* and *THBS1* were strongly correlated with both IMP and cooking loss ($p < 0.05$) and were expressed at high levels in the breast and leg muscles of Jingyuan chickens (Figures 7C, D). Protein interaction network analysis showed that *TGIF1* and *THBS1* interacted with nine and ten proteins, respectively (Figures 7E, F), with a transcription regulator activity function. Taken together, *TGIF1* and *THBS1* may regulate the expression of key enzymes involved in IMP anabolism, either directly or indirectly, through muscle biogenesis-related processes.

4 Discussion

The economic value of poultry meat is directly related to its quality. Chicken meat quality may vary greatly depending on the body part, feeding method, sex, and breed. A previous study found that the IMP content in the breast muscle of a caged Jingyuan hen and rooster was significantly higher than that in the leg muscle, and the IMP content in the hen was higher than that in the rooster; however, the difference was not significant (Zhang et al., 2020). The

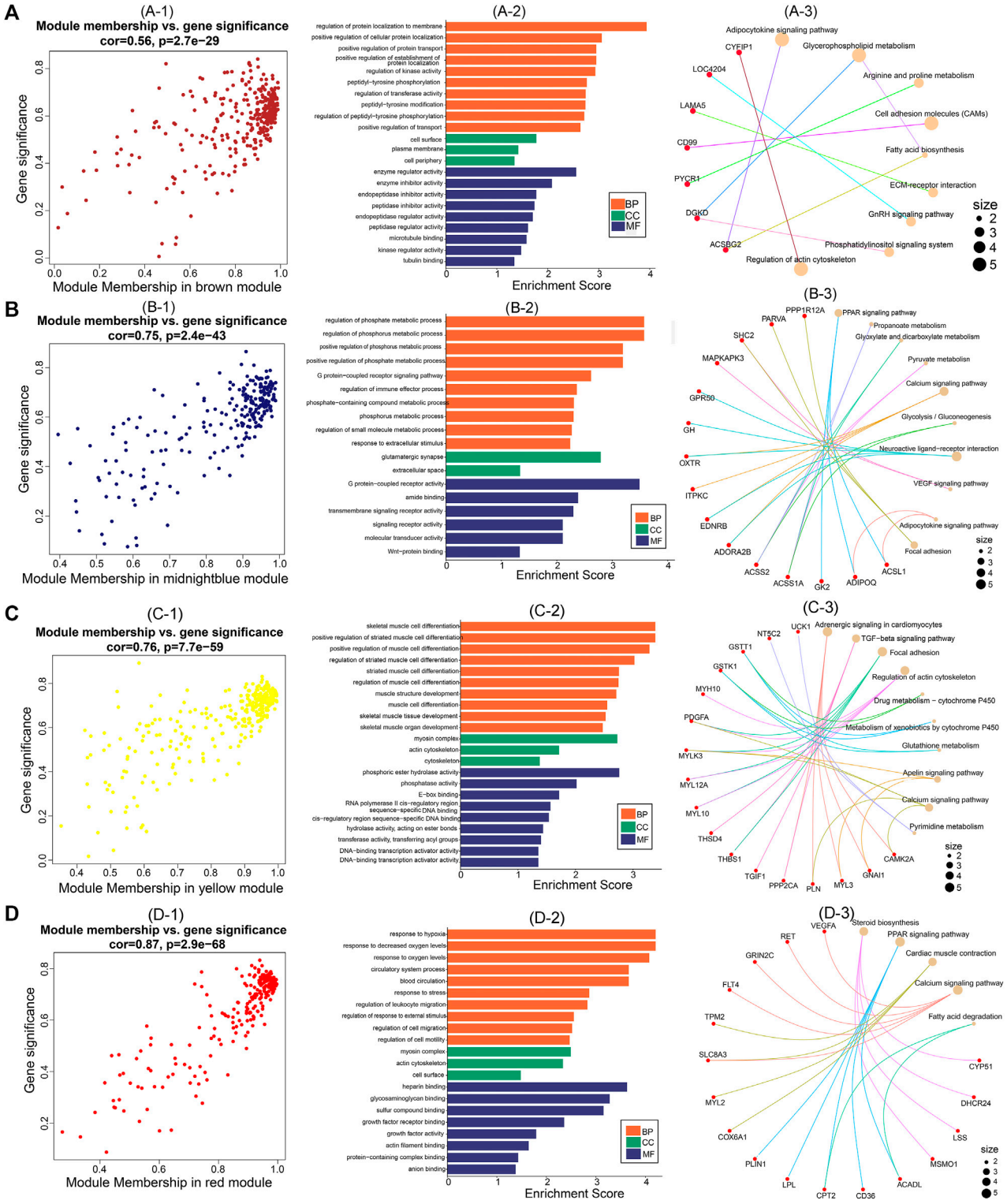

FIGURE 6
Hub genes screening and functional analysis in brown, midnightblue, red, and yellow modules. Scatter plot of gene significance and module membership for **(A)**-1 (brown), **(B)**-1 (midnightblue), **(C)**-1 (yellow), and **(D)**-1 (red) modules. GO functional enrichment analysis (BP: biological processes; CC: cellular components; MF: molecular functions) of hub genes in **(A)**-2 (brown), **(B)**-2 (midnightblue), **(C)**-2 (yellow), and **(D)**-2 (red) modules. KEGG pathway enrichment analysis of hub genes in **(A)**-3 (brown), **(B)**-3 (midnightblue), **(C)**-3 (yellow), and **(D)**-3 (red) modules (red nodes are key genes enriched in the pathway).

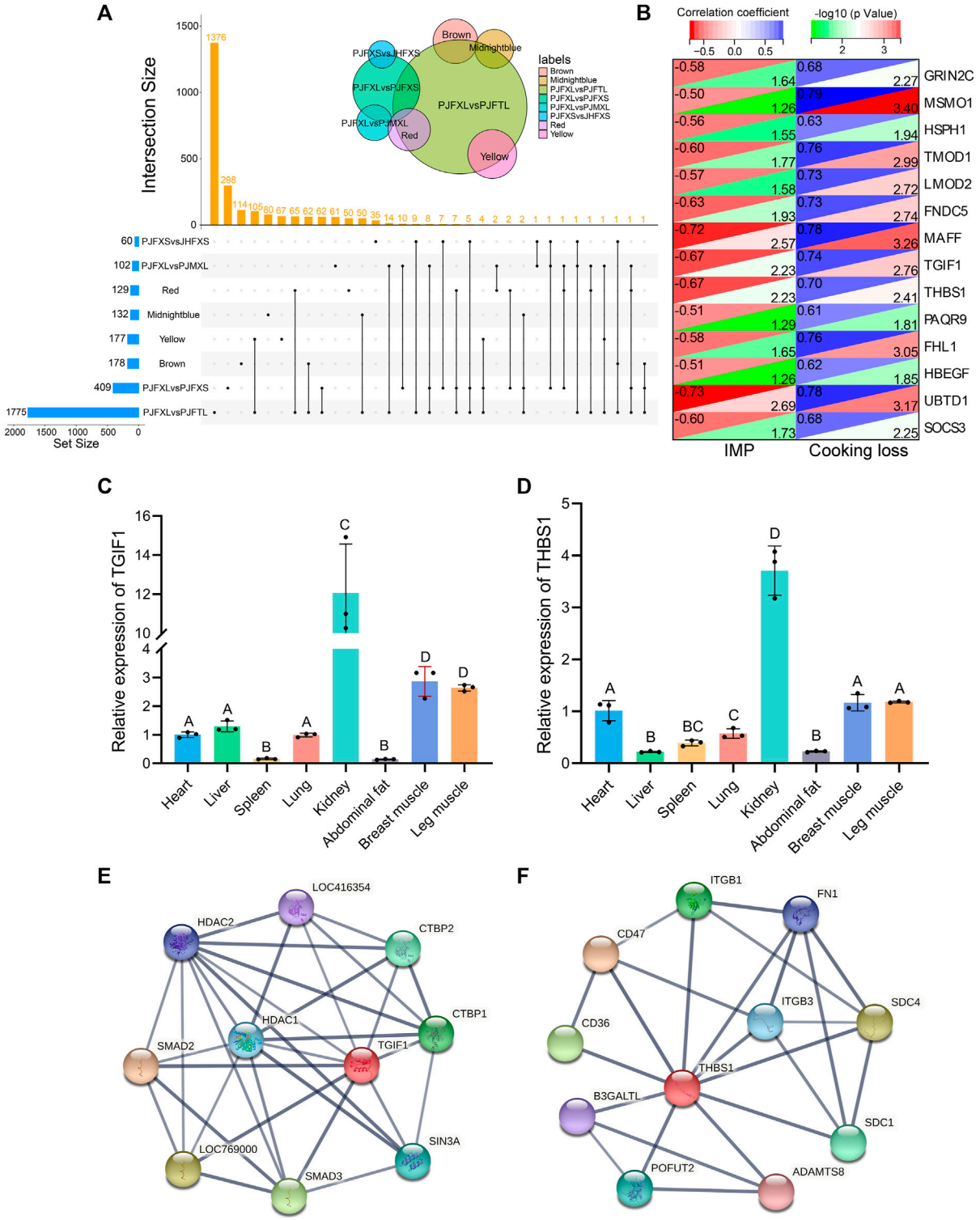

FIGURE 7
Functional gene identification. **(A)** Upset Venn diagram of hub genes and DEGs in four important modules. **(B)** Correlation of 14 DEGs with IMP and cooking loss (The value in the upper left corner of the figure is the correlation coefficient, and the value in the lower right corner is −log10 (*p*-value)). Tissue expression of **(C)** TGIF1 and **(D)** THBS1 in Jingyuan chickens. The protein interaction network of **(E)** TGIF1 and **(F)** THBS1 (Network nodes represent proteins: splice isoforms or post-translational modifications are collapsed, i.e., each node represents all the proteins produced by a single, protein-coding gene locus. Edges represent protein-protein associations: associations are meant to be specific and meaningful, i.e., proteins jointly contribute to a shared function).

IMP content of free-range hens is significantly higher than that of caged hens under different feeding patterns (Wang et al., 2022), probably because of the increase in muscle ATP content resulting from increased activity and enhanced IMP synthesis. Moreover, there were significant differences in IMP content between breeds in the muscles of Jingyuan and Pudong chickens (Zhang et al., 2021). These results are consistent with those of previous studies (Liu et al., 1980; Zhang et al., 2014; Liu et al., 2018; Wang et al., 2018). The reason for these differences may be due to differences in the expression of key enzymes involved in IMP synthesis. Although preliminary studies have explored the gene networks regulating IMP deposition, most have focused only on single-gene expression aspects (Hu et al., 2015; Zhang et al., 2015.; Zhang T. et al., 2018). There is a paucity of relevant studies revealing the molecular mechanisms underlying IMP-specific deposition in chicken muscles from multiple perspectives.

We explored the genetic factors affecting muscle IMP-specific deposition in Jingyuan chickens from several perspectives. We analyzed the transcriptome profiles of Jingyuan chickens for different parts, feeding methods, sexes, and compared them with Pudong chickens to identify key genes associated with muscle IMP. In total, 1,775 DEGs were identified between the breast and leg muscles of caged Jingyuan hens, 409 DEGs in the breast muscles of caged and free-range hens, 102 DEGs in the breast muscles of caged hens and roosters, and 60 DEGs in the breast muscles of free-range Jingyuan and Pudong chickens. Further exploration of the specific biological functions of the DEGs revealed that the DEGs of the four comparison groups could participate in multiple biological processes of muscle development, including several metabolic processes such as energy metabolism and lipid anabolism. It has been shown that the glycolysis/gluconeogenesis pathway is the main form of energy metabolism in the organism, and the energy generated by its metabolic processes plays a critical role in ensuring cell survival and growth. PPARγ can bind and activate the transcriptional activity of pyruvate kinase M in the form of transcription factors, which regulate glycolysis/gluconeogenesis processes (Panasyuk et al., 2012). The PPAR signaling pathway regulates lipid metabolism (Moreno et al., 2010; Cui et al., 2012). Additionally, FoxO is a key pathway involved in gluconeogenesis (Zhang X. et al., 2018). Cytokine-cytokine receptor interactions promote focal adhesions (Wang et al., 2021). Focal adhesions play a vital role in skeletal muscle development and are a signaling center for cell growth and differentiation (Sastry and Burridge, 2000). These results suggest that intergroup DEGs may be involved in the anabolic processes of IMP in the form of energy metabolism, key molecular activities, and direct or indirect participation in muscle quality regulation.

Traditional transcriptome sequencing methods cannot distinguish confounding factors among multiple groups (Barabasi and Oltvai, 2004; Ghaemi et al., 2019). Many DEGs can be identified by transcriptome sequencing; however, reliable evidence to elucidate the gene network associated with meat quality phenotypes is lacking. WGCNA is an effective data-mining method that can cluster massive gene sets into co-expression modules based on gene expression patterns (Zhao et al., 2010). Co-expression modules and key genes that perform biological functions were identified through association analysis with phenotypic traits. In this study, we constructed 11 functional co-expression modules, among which the brown, midnight blue, red, and yellow modules showed a strong correlation with both IMP and cooking loss.

GO and KEGG enrichment analyses explored possible mechanisms by which functional genes regulate muscle development and IMP deposition. GO enrichment analysis revealed significantly enriched functional terms for protein and kinase activity regulation, molecular transduction and metabolism, growth factor activity, transcriptional regulation, and skeletal muscle development. IMP is mainly produced by the degradation of ATP in muscles. Therefore, muscle development is closely related to IMP anabolic processes. IMP is also known as a hypoxanthine nucleotide and the purine metabolic pathway regulates its anabolism. Pyruvate kinase M (PKM), a key gene in the purine metabolic pathway, regulates adenine ribonucleotide biosynthesis (IMP => ADP, ATP) and guanine ribonucleotide biosynthesis (IMP => GDP, GTP) (Yu et al., 2022), as well as a variety of amino acid-assisted regulations in this process. At the same time, PKM is also the key kinase regulating the conversion of glucose to pyruvate in the glycolysis pathway, which has many functions, such as anabolism, cell proliferation, and aerobic glycolysis (Deyle et al., 2012). These results suggest that glycolysis/gluconeogenesis, arginine, proline, and pyruvate metabolism play important roles in IMP-specific deposition processes.

Analysis of the hub genes of the functional modules revealed that *PYCR1*, *SMOX*, and *ACSS2* were the most important genes. Pyrroline-5-carboxylate reductase 1 (*PYCR1*) is a precursor of l-proline synthesis and is involved in the regulation of proline biosynthesis (Alaqbi et al., 2022). A key step in proline biosynthesis is the reduction of Δ^1-pyrroline-5-carboxylate (P5C) to proline, which is catalyzed by P5C reductase (PYCR). Studies have shown that proline biosynthesis is key to sustaining protein synthesis and supporting mitochondrial function, redox balance, signaling, and nucleotide biosynthesis (Burke et al., 2020; D'Aniello et al., 2020; Ding et al., 2020; Tran et al., 2021). Spermine oxidase (*SMOX*) is a multifunctional enzyme that controls polyamine metabolism, plays an important role in muscle differentiation, and maintains muscle fiber size and skeletal muscle mass (Cervelli et al., 2018; Reinoso-Sánchez et al., 2020). Vertebrate *SMOX* is a flavoprotein that specifically oxidizes the natural substrate spermine, with the production of spermidine, hydrogen peroxide (H_2O_2), and the aldehyde 3-aminopropanal (Polticelli et al., 2012; Cervelli et al., 2013). H_2O_2 plays a critical regulatory role in skeletal muscle function. H_2O_2 levels from low to moderate are critical for cell signaling and regulation of gene expression; they act as signals for cell adaptation and are necessary for muscle growth (Powers and Jackson, 2008; Sies, 2017).

Acyl-CoA synthetase short-chain family member 2 (*ACSS2*) is a conserved nucleocytosolic enzyme that affects lipid synthesis and metabolism by selectively regulating genes related to lipid metabolism (Huang et al., 2018). The basic function of the ACS family of enzymes is to convert acetate and coenzyme A (CoA) into acetyl-CoA in an ATP-dependent manner. It was found that *ACSS2* encodes acetyl coenzyme A synthetase, and was expressed at approximately 2-fold higher levels in subcutaneous fat than in intramuscular fat, consistent with a relative preference for acetate in the subcutaneous fat depot (Hudson et al., 2020). STRING database exploration revealed that *ACSS2* has functions similar to those of

adipogenesis-related genes (*ACACA*, *ACOT12*, *SIRT3*, and aldehyde dehydrogenase family members) and can regulate acetyl-CoA metabolism, fatty acid biosynthesis, and adipocyte differentiation. Consistent with the results of Hu et al. (2010), *ACACA* was identified as a key factor in adipogenesis and transport, and played a crucial role in the weight variability of abdominal adipose tissue in growing chickens. IMP deposition is a product of the interaction of multiple biological processes mediated by a complex network of gene regulation in muscles. Although there is no direct evidence that *PYCR1*, *SMOX*, and *ACSS2* are associated with muscle IMP, the association analysis of transcriptome DEGs and co-expression functional modules suggests that *PYCR1*, *SMOX*, and *ACSS2*, which are regulated by the glycolysis/gluconeogenesis, arginine and proline metabolism, and pyruvate metabolism pathways, may be potential candidate genes for regulating IMP deposition.

Based on the combined analysis of the transcriptome and co-expression module, TGF-β signaling pathway-regulated TGIF1 and THBS1 were strongly correlated with both IMP and cooking loss and showed high expression levels in the breast and leg muscle tissues of Jingyuan chickens. TGF-β is a multifunctional secreted protein belonging to the transforming growth factor (TGF) superfamily. TGF-β can inhibit myoblast differentiation during the myoblast state transition by promoting *MYOD* degradation and inhibiting myogenin expression (Schabort et al., 2009). During myogenic differentiation, core proteoglycans in the extracellular matrix can competitively bind TGF-β, resulting in reduced TGF-β binding ability to the receptors TGF-β R1 and TGF-β R2, affecting satellite cell differentiation (Droguett et al., 2006). TGF-β and Wnt pathways also interact to regulate skeletal muscle growth and development (Biressi et al., 2014). Furthermore, the TGF-β signaling pathway typically inhibits adipocyte differentiation (Du et al., 2013). A previous study found that TGF-β3 stimulates adipocyte progenitor proliferation in white adipose tissue, which is related to glucose metabolism (Petrus et al., 2018). This is consistent with our findings that the TGF-β signaling pathway, which regulates multiple molecular activities and energy metabolism, may be a key regulator of muscle development and IMP deposition in Jingyuan chickens.

TGFB-induced factor homeobox 1 (*TGIF1*) is a multifunctional protein that represses TGF-β-activated transcription by interacting with Smad2-Smad4 complexes (Guca et al., 2018). By analyzing open chromatin regions and transcription factor-binding sites in porcine dorsal longissimus muscle, *TGIF1* was identified as a possible transcription factor that affects muscle growth and development (Miao et al., 2021). The *TGIF1* gene was identified in a gene module related to the growth and development of pigeon skeletal muscle, which has a high degree of connectivity and is a hub gene in development-specific modules (Ding et al., 2021). In addition, *TGIF1* is involved in regulating lipid metabolic processes; knockout of *TGIF1* in mice increases the accumulation of intrahepatic lipids and serum levels of cholesterol (Pramfalk et al., 2014), and *TGIF1* represses *ACAT2* and *NPC1L1* (Parini et al., 2018). Thrombospondin-1 (*THBS1*), a multidomain calcium-binding glycoprotein (Adams and Lawler, 2011), is highly expressed during muscle development following injury (Stenina et al., 2003). *THBS1* is also elevated in obesity and is an adipocyte-derived cytokine (adipokine) (Varma et al., 2008). STRING database exploration revealed that *THBS1* functions in cell adhesion, fibronectin binding, and cell surface and is also regulated by KEGG pathways such as extracellular matrix receptor interaction, focal adhesion, and other types of O-glycan biosynthesis. Freedman et al. (2018) found that the extracellular matrix plays an important role in multiple cell proliferation and differentiation processes and can also interact with focal adhesion signaling to participate in the growth and differentiation of skeletal muscle cells (Sastry and Burridge, 2000; Romer et al., 2006). These studies suggest that *TGIF1* and *THBS1* regulate the synthesis and metabolism of IMP in muscles directly or indirectly through biological processes related to myogenesis.

5 Conclusion

Transcriptome analysis of different muscle tissues, feeding methods, and sexes of Jingyuan chickens, and comparison with other breeds, combined with differential expression analysis and WGCNA, helped to identify multiple potential candidate genes regulating muscle IMP deposition, including *PYCR1*, *SMOX*, *ACSS2*, *TGIF1*, and *THBS1*. Glycolysis/gluconeogenesis, TGF-β signaling pathway, and other multiple functional mechanisms are important in IMP-specific deposition. This study explored the genes regulating muscle IMP synthesis and metabolism in Jingyuan chickens from multiple perspectives, providing an important theoretical basis for the improvement of meat quality and molecular breeding of chickens.

Data availability statement

The authors declare that the data supporting the findings of this study are available within the article and its Supplementary Material. All the raw sequences have been deposited in the NCBI database Sequence Read Archive with the accession numbers PRJNA957235 (https://dataview.ncbi.nlm.nih.gov/object/PRJNA957235).

Ethics statement

All animal experiments were approved by the Animal Welfare and Use Committee of Ningxia University and was conducted according to the Guidelines for Animal Use of the Committee on the Ministry of Agriculture of China.

Author contributions

Conceptualization: BY, YG, and JZ; methodology: ZC, JL, and BY; software: ZC and WZ; validation: BY, WZ, and XF; data curation: BY and XF; writing-original draft: BY; writing-review and editing: ZC and JL; visualization: BY; supervision: YG and JZ. All authors have read and agreed to the published version of the manuscript. All authors listed have made a substantial, direct, and intellectual contribution to the work and approved it for publication.

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Conflict of interest

The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

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Supplementary material

The Supplementary Material for this article can be found online at: https://www.frontiersin.org/articles/10.3389/fphys.2023.1199311/full#supplementary-material

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The effects of essential oil from Lippia origanoides and herbal betaine on performance, intestinal integrity, bone mineralization and meat quality in broiler chickens subjected to cyclic heat stress

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Essential oils (EO) affect performance, intestinal integrity, bone mineralization, and meat quality in broiler chickens subjected to cyclic heat stress (HS). Day-of-hatch Cobb 500 male broiler chicks (n = 475) were randomly divided into four groups. Group 1: No heat stress (Thermoneutral) + control diets with no antibiotics; Group 2: heat stress control + control diets; Group 3: heat stress + control diets supplemented with thymol chemotype (45 ppm) and herbal betaine (150 ppm) formulation EO1; Group 4: heat stress + control diets supplemented with phellandrene (45 ppm) and herbal betaine (150 ppm) formulation EO2. From day 10–42, the heat stress groups were exposed to cyclic HS at 35°C for 12 h (8:00–20:00). BW, BWG, FI, and FCRc were measured at d 0, 10, 28, and 42. Chickens were orally gavaged with FITC-d on days 10 (before heat stress) and 42. Morphometric analysis of duodenum and ileum samples and bone mineralization of tibias were done. Meat quality was assessed on day 43 with ten chickens per pen per treatment. Heat stress reduced BW by day 28 (p < 0.05) compared to thermoneutral chickens. At the end of the trial, chickens that received both formulations of EO1 and EO2 had significantly higher BW than HS control chickens. A similar trend was observed for BWG. FCRc was impaired by EO2 supplementation. There was a significant increase in total mortality in EO2 compared with EO1 EO1 chickens had lower FITC-d concentrations at day 42 than the HS control. In addition, EO1 treatment is not statistically different if compared to EO2 and thermoneutral. Control HS broilers had significantly lower tibia breaking strength and total ash at day 42 than heat-stressed chickens supplemented with EO1 and EO2. Heat stress affected intestinal morphology more than thermoneutral chickens. EO1 and EO2 improved intestinal morphology in heat-stressed chickens. Woody breast and white striping were

more common in thermoneutral chickens than heat stress chickens. In conclusion, the EO-containing diet could improve broiler chicken growth during cyclic heat stress, becoming increasingly relevant in antibiotic-free production in harsh climates.

KEYWORDS

betaine, chickens, essential oils, heat stress, performance

1 Introduction

Heat stress (HS) in chickens is a condition that occurs when chickens are exposed to high temperatures and humidity and are unable to dissipate their body heat efficiently (Khan et al., 2021; Khan et al., 2021). Chickens are homeothermic animals, meaning they have a constant body temperature, which is essential for their normal physiological functions (Abioja and Abiona, 2021). However, when the ambient temperature exceeds their comfort range, chickens can become stressed, leading to a range of negative effects (Uyanga et al., 2023; te Pas et al., 2023; Saracila et al., 2023). During heat stress, chickens can experience a range of symptoms including panting, increased water consumption, reduced feed intake, reduced egg production, decreased growth rate, and increased mortality. In severe cases, heat stress can lead to heat stroke, which can be fatal (Nyoni et al., 2019; Balakrishnan et al., 2023).

There are several ways to mitigate the negative effects of heat stress in poultry, including ventilation and cooling schemes, ensuring access to cool water, adjusting feeding schedules to cooler parts of the day, providing shade in outdoor areas and to avoid handling or transporting chickens during the hottest parts of the day (Brugaletta et al., 2023; Rebez et al., 2023).

Essential oils (EO) may reduce chicken heat stress (Pandey et al., 2019; Yilmaz and Gul, 2023a) due to their antibacterial, anti-inflammatory, and antioxidant effects (Basiouni et al., 2023; Mnisi et al., 2023; Rafeeq et al., 2023). Some of the most used essential oils in poultry include oregano, thyme, rosemary, eucalyptus, cumin, mint, lemon, and cinnamon (Yilmaz and Gul, 2023b; AL-Ramamneh, 2023; Shanthi and Diwan, 2023). The active components of these oils include phenols, terpenes, and flavonoids, which are believed to play a role in their beneficial effects on chicken health (Shehata et al., 2022a).

Several investigators have studied the effects of EO on chickens under heat stress conditions (Shehata et al., 2022b; Yilmaz and Gul, 2023a; Mangan and Siwek, 2023). These studies have shown that EO can improve performance, while reducing the negative outcomes of heat stress, such as reduced water intake, oxidative stress, and inflammation (Chowdhury et al., 2023; Das et al., 2023; Rafeeq et al., 2023). Some EO have improved the immune system of chickens, which can help reduce the incidence of disease under heat stress conditions (Perini et al., 2020; Ruff et al., 2021; Yilmaz and Gul, 2023b; Jahja et al., 2023). Furthermore, certain EO can modulate the expression of genes related to stress response and immune function, which may contribute to their beneficial effects on chicken health (Abo Ghanima et al., 2023; Ding et al., 2023; Wu et al., 2023). Hence, the purpose of the present study was to confirm and extend previous research conducted in our laboratory with the EO of *Lippia origanoides* and betaine from an herbal extract on performance, intestinal integrity, bone mineralization, and meat quality in broiler chickens subjected to cyclic heat stress.

2 Materials and methods

2.1 Essential oils and herbal betaine

Promitec Santander S.A. (Bucaramanga, Santander, Colombia) provided two formulations of EO with herbal betaine and feed inclusion was based on the manufacturer's recommendations and analysis. The formulations were:

Formula 1 (EO1): Essential oil of *Lippia origanoides*, thymol chemotype (45 ppm), and herbal betaine (150 ppm). Administration dose: 350 g/Ton food.

Formula 2 (EO2): Essential oil of *Lippia origanoides*, phellandrene chemotype (45 ppm), and herbal betaine (150 ppm). Administration dose: 350 g/Ton food.

The qualitative and quantitative chemical composition of *Lippia origanoides* essential oils for both formulations are summarized in Table 1. Samples of both products were submitted to chromatographic analysis 7890 A (Laboratory of chromatography and mass spectrometry Industrial University of Santander, Bucaramanga, Colombia). The two main components of Formula 1 were thymol (47.5%) and carvacrol (29.9%). On the other hand, the two main components present in Formula 2 were trans-β-Caryophyllene (12.6%) followed by *p*-Cymenene (10.1%). EO's quick absorption and processing by enterocytes suggests encapsulating feed additives to increase efficiency (Gheisar et al., 2015). In the present study, both formulations were spray-dried maltodextrin microencapsulated to improve encapsulation efficiency, bioavailability, and the lifespan of the EO. Table 2 shows the herbal components of the betaine used in both formulations.

The EO1 and EO2 were included in all three diets and administered since day 1. Starter, grower, and finisher mash diets were used in this experiment and were formulated to approximate the nutritional requirements of broiler chickens as recommended by the National Research Council 1994 and adjusted to the breeder's recommendations (Cobb-Vantress Inc, 2018). No antibiotics, coccidiostats or enzymes were added to the feed (Table 3).

2.2 Experimental design

Day-of-hatch Cobb 500 male broiler chicks (n = 475) were purchased from a commercial hatchery. After arriving, all chicks were vaccinated with a coccidia vaccine (Coccivac®-B52, Merck Animal Health, De Soto, KS 66018), neck tagged, and randomly

TABLE 1 Qualitative and quantitative chemical composition of essential oil of *Lippia origanoides* thymol chemotype, formula 1 (EO1) and essential oil of *Lippia origanoides*, phellandrene chemotype, formula 2 (EO2) by chromatographic analysis.

Compounds EO1 (thymol chemotype)	(%)	Compounds EO2 (phellandrene chemotype)	(%)
Thymol	47.5	trans-β-Caryophyllene	12.6
Carvacrol	29.9	*p*-Cymenene	10.1
ɣ-Terpinen	10.5	α-Humulene	8.1
p-Cymenene	10.3	α-Phellandrene	7.6

The LEO (Natbio EsencialPremix®) sample was submitted to chromatographic analysis 7890 A (Laboratory of chromatography and mass spectrometry Industrial University of Santander, Bucaramanga, Colombia). Formula 1 (EO1): Essential oil of *Lippia origanoides*, thymol chemotype (45 ppm), and herbal betaine (150 ppm). Administration dose: 350 g/Ton food. Formula 2 (EO2): Essential oil of Lippia origanoides, phellandrene chemotype (45 ppm), and herbal betaine (150 ppm). Administration dose: 350 g/Ton food.

TABLE 2 Herbal components of the betaine used in Formula 1 and Formula 2.

Each 100 g of powder contains	Quantity (grams)
Trigonella foenum-graecum	25
Foeniculum vulgare	25
Zingiberis officinalis	20
Emblica officinalis	15
Taraxacum officinalis	15

Formula 1 (EO1): Essential oil of *Lippia origanoides*, thymol chemotype (45 ppm), and herbal betaine (150 ppm). Administration dose: 350 g/Ton food. Formula 2 (EO2): Essential oil of Lippia origanoides, phellandrene chemotype (45 ppm), and herbal betaine (150 ppm). Administration dose: 350 g/Ton food.

divided into four groups. Group 1: No heat stress (Thermoneutral-TN) + control diet for starter, grower, and finisher with no antibiotics; Group 2: heat stress control + control diets; Group 3: heat stress + control diets supplemented with EO1 formulation; Group 4: heat stress + control diets supplemented with EO2 formulation. This investigation employed breeder-recommended starter, grower, and finisher diets (Table 3). Diets had no growth boosters. Environmental rooms housed groups. Each room had two 150 × 300 cm pens with feeding and watering systems. Five replicates per treatment were under heat stress circumstances, and four replicates were under thermoneutral (TN) conditions with 25 birds/pen. TN chicks were raised in commercial production conditions (temperature, light). From days 15–42, the temperature was gradually reduced from 32°C to 24°C with relative humidity at 55% ± 5%. From day 10–42, the heat stress groups were exposed to cyclic heat stress at 35°C for 12 h (from 8:00 to 20:00 h). On day 18, eight chickens were randomly selected to orally insert a Thermochron temperature logger (iButton, DS 1922L, Embedded Data Systems, Lawrenceburg, KY). The devices measured body temperature in the gizzard, according to Flees et al. (2017). Chickens' body temperatures were recorded every minute for the first 2 hours and every hour after heat stress. BW, BWG, FI, and FCRc were measured at days 0, 10, 28, and 42. Ten chickens each pen (n = 40 TN; n = 50 heat stress) per group were processed on day 43 to evaluate processing characteristics and meat quality. Protocol 16,084 of the University of Arkansas Institutional Animal Care and Use Committee supervised animal care.

Four random chickens per pen (n = 16 TN; n = 20 heat stress) were orally gavaged with 8.32 mg/kg BW of fluorescein isothiocyanate-dextran (FITC-d, MW 3–5 kDa; Sigma-Aldrich Co.) on day 10 (before heat stress) and day 42 to assess intestinal permeability as stated by Baxter et al. (2017). On the same days, both tibias were collected to evaluate break strength (kg) and total ash (%) as described by Gautier et al. (2017).

On the evaluation day, birds were euthanized, and ileum and duodenum samples were taken (n = 8) for enteric morphometric analysis. Each bird's middle duodenum and lower ileum were excised and fixed in 10% buffered formaldehyde for 48 h. A 5-µm section of each intestine segment was embedded in paraffin, mounted on a glass slide, and stained with hematoxylin and eosin for light microscope analysis. All morphological parameters were measured using ImageJ (http://rsb.info.nih.gov/ij/). The average results from each sample's 10 replicate measurements for each variable were used in statistical analysis as described by Aptekmann et al., 2001 and Sakamoto et al., 2000.

Broilers were reared under chronic heat stress conditions for 42 days. At day of processing (day 43), 10 birds per pen were selected., after a 10-h feed withdrawal period and processed as described by Mehaffey et al. (2006). After a 0.25 h prechill at 12°C, carcasses were immersed in 0°C tanks for 2.5 h with manual agitation. Carcasses were deboned at 3 h postmortem to weigh the breast, tender, wing, and complete leg. Myopathy score (woody breast and white striping), WHC, color, and pH were used to analyze pectoralis major muscles following the procedures described by Kuttappan et al. (2012); Tijare et al. (2016), respectively. After measuring and scoring myopathies, fillets were placed on white plastic storage trays, wrapped in plastic overlay liners, and kept in a walk-in refrigerator at 4°C until 24 h postmortem. Breast fillets were weighed 24 h postmortem to determine drip loss. Drip loss was estimated as a weight-to-deboned weight proportion.

2.3 Data and statistical analysis

Data were analyzed using the JMP Pro 16.0 platform (SAS Institute, United States) according to a Randomized Complete Block Design (RCBD). All data were subjected to analysis of variance (ANOVA). Each pen was considered as the experimental unit for performance parameters. Individual birds were considered as the experimental unit for histological measurements, serum FITC-d analysis, bone quality parameters, and meat quality characteristics. Statistical significance was set at $p \leq 0.05$. If significance was met, means were separated using a Tukey's HSD test.

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TABLE 3 Ingredient composition and nutrient content of the corn-soybean diets used on as-is basis.

Item	Starter control diet	Grower control diet	Finisher control diet
Ingredients (%)			
Corn	51.85	57.85	59.68
Soybean meal	37.66	31.62	27.23
DDGS 8.1% EE	4.00	4.00	6.00
Poultry fat	3.24	3.44	4.38
Limestone	1.08	1.06	1.03
Dicalcium phosphate	1.01	0.88	0.64
Salt	0.35	0.35	0.31
DL-methionine	0.29	0.25	0.22
L-lysine HCl	0.12	0.13	0.12
Mineral premix[a]	0.10	0.10	0.10
Vitamin premix[b]	0.10	0.10	0.10
L-threonine	0.08	0.09	0.09
Choline chloride	0.06	0.06	0.05
Sodium bicarbonate	0.04	0.05	0.03
Antioxidant[c]	0.02	0.02	0.02
Calculated analysis			
ME (kcal/kg)	3,015.00	3,090.00	3,175.00
Ether extract (%)	5.88	6.20	7.28
Crude protein (%)	22.30	20.00	18.70
Lysine (%)	1.18	1.05	0.95
Methionine (%)	0.59	0.53	0.48
Threonine (%)	0.77	0.69	0.65
Tryptophan (%)	0.25	0.22	0.20
Total calcium (%)	0.90	0.84	0.76
Total phosphorous (%)	0.63	0.58	0.53
Available phosphorus (%)	0.45	0.42	0.38
Sodium (%)	0.20	0.20	0.18
Potassium (%)	1.06	0.94	0.87
Chloride (%)	0.27	0.28	0.25
Magnesium (%)	0.19	0.18	0.17
Copper (%)	19.20	18.46	18.85
Selenium (%)	0.28	0.27	0.26
Linoleic acid (%)	1.01	1.13	1.16

[a]Mineral premix supplied the following per kg: manganese, 120 g; zinc, 100g; iron, 120 g; copper, 10–15 g; iodine, 0.7 g; selenium, 0.4 g; and cobalt, 0.2 g (Nutra Blend LLC, Neosho, MO, 64850).

[b]Vitamin premix supplied the following per kg: vitamin A, 20,000,000 IU; vitamin D3, 6,000,000 IU; vitamin E, 75,000 IU; vitamin K3, 9 g; thiamine, 3 g; riboflavin, 8 g; pantothenic acid, 18 g; niacin, 60 g; pyridoxine, 5 g; folic acid, 2 g; biotin, 0.2 g; cyanocobalamin, 16 mg; and ascorbic acid, 200 g (Nutra Blend LLC, Neosho, MO, 64850).

[c]Ethoxyquin.

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TABLE 4 Evaluation of essential oils (EO) and herbal betaine on performance parameters of broiler chickens exposed to cyclic heat stress[a].

Performance	Thermoneutral	Heat stress	Heat stress + EO1	Heat stress + EO2
Body weight (g)				
0	41.1 ± 0.39	41.2 ± 0.23	40.8 ± 0.29	41.3 ± 0.33
10	262.9 ± 8.39^{ab}	268.4 ± 4.97^{a}	242.2 ± 10.81^{b}	253.8 ± 4.92^{ab}
28	1,607.8 ± 23.76^{a}	1,393.2 ± 17.78^{b}	1,325.4 ± 51.49^{b}	1,365.5 ± 28.11^{b}
42	3,187.2 ± 7.89^{a}	2,482.5 ± 84.42^{c}	2,650.3 ± 37.14^{b}	2,639.3 ± 25.70^{b}
Body weight gain (g)				
0–10	221.0 ± 8.72^{ab}	226.4 ± 4.52^{a}	200.4 ± 10.51^{b}	211.8 ± 4.84^{ab}
0–28	1,553.3 ± 23.84^{a}	1,342.0 ± 17.10^{b}	1,274.4 ± 50.91^{b}	1,314.4 ± 28.94^{b}
0–42	3,130.3 ± 8.52^{a}	2,428.4 ± 83.43^{c}	2,592.0 ± 37.81^{b}	2,579.2 ± 23.01 ^{bc}
Feed intake (g)				
0–10	265.8 ± 5.74^{b}	270.0 ± 3.27^{b}	265.7 ± 17.10^{b}	314.6 ± 15.80^{a}
0–28	2,330.1 ± 52.77^{a}	1987.3 ± 35.47 ^{bc}	1936.8 ± 61.41^{c}	2,118.3 ± 62.38^{b}
0–42	5,247.8 ± 43.60^{a}	4,275.0 ± 181.64^{b}	4,490.4 ± 108.15^{b}	5,150.4 ± 166.47^{a}
FCRc				
0–10	1.007 ± 0.039^{b}	1.002 ± 0.01^{b}	1.096 ± 0.06 ^{ab}	1.235 ± 0.07^{a}
0–28	1.435 ± 0.027	1.414 ± 0.03	1.459 ± 0.03	1.548 ± 0.07
0–42	1.626 ± 0.013^{b}	1.692 ± 0.01^{b}	1.691 ± 0.03^{b}	1.844 ± 0.06^{a}
Total Mortality (%)	2.0^{ab}	2.4^{ab}	1.6^{b}	7.2^{a}

[a]Data are expressed as the mean ± SE.

abc Indicates significant differences between the treatments within the rows ($p < 0.05$). Cyclic heat stress started at day 10. EO1: essential oil of *Lippia origanoides*, thymol chemotype (45 ppm) and herbal betaine (150 ppm). Administration dose: 350 g/Ton food. EO2: essential oil of *Lippia origanoides*, phellandrene chemotype (45 ppm) and herbal betaine (150 ppm). Administration dose: 350 g/Ton food.

3 Results

The results of the evaluation of EO and herbal betaine on performance parameters for broiler chickens exposed to cyclic heat stress are summarized in Table 4. By day 28 (18 days after initiating heat stress, groups subjected to heat stress showed a significant ($p < 0.05$) reduction in BW compared with thermoneutral chickens. However, at the end of the trial (day 42), chickens that received both formulations EO1 and EO2, and were exposed to cyclic heat stress, showed a significant improvement ($p < 0.05$) in BW compared to heat stress control chickens. A similar trend was observed for BWG. FCRc was impaired by EO2 supplementation and FI increased only in EO2, not in EO1. There was a significant increase in total mortality in EO2 compared with EO1 (Table 4).

Two hours after introducing heat stress into the experimental groups, chickens' body core temperatures increased significantly ($p < 0.05$) and remained raised during heat stress throughout the trial (data not shown).

Table 5 expresses the evaluation of EO and herbal betaine on broiler chickens exposed to cyclic heat stress on serum FITC-d and bone parameters at days 10 and 42. No significant differences were observed for serum FITC-d or bone parameters at day 10. However, at day 42, a significant difference ($p < 0.05$) in the concentration of FITC-d was observed in chickens that received EO1 compared to HS control. No significant difference between EO1 and EO2 to as concern FITC-d levels. Control HS chickens showed a significant reduction in tibia break strength and total tibia ash (%) at day 42 compared with chickens exposed to heat stress while being supplemented with EO1 and EO2 (Table 5).

The results of the evaluation of EO and herbal betaine on morphometric analysis of the duodenum and ileum mucosa of broiler chickens exposed to cyclic heat stress at day 42 are summarized in Table 6. In the duodenum, heat stress had a severe effect ($p < 0.05$) on villus height, crypt depth, villus width, villus-to-crypt ratio and villus surface area index when compared to thermoneutral chickens. Nevertheless, the severity of the heat stress was reduced ($p < 0.05$) in chickens that were supplemented with EO1 and EO2, and a similar trend was observed in the ileum (Table 6).

Table 7 expresses the results of the evaluation of EO and herbal betaine on final body weight and carcass yields of broiler chickens exposed to cyclic heat stress at day 43. At processing, it was clear that heat stress had a negative impact ($p < 0.05$) on the live weight of the chickens compared to thermoneutral chickens. However, chickens that were supplemented with EO1 or EO2, showed a significant improvement ($p < 0.05$) in BW compared with HS control chickens. No differences were observed ($p > 0.05$) in fat yield (%), hot carcass

TABLE 5 Evaluation of essential oils (EO) and herbal betaine on broiler chickens exposed to cyclic heat stress on serum Fluorescein isothiocyanate–dextran (FITC-d) and bone parameters at days10 and 42[a].

	Thermoneutral	Heat stress	Heat stress + EO1	Heat stress + EO2
Serum FITC-d (ng/mL)				
d 10	39.2 ± 6.42	36.9 ± 10.07	33.6 ± 7.89	37.1 ± 7.71
d 42	50.9 ± 13.46^{c}	114.5 ± 13.45^{a}	67.4 ± 12.46^{bc}	108.8 ± 21.98^{ab}
Tibia break strength (kg)				
d 10	17.3 ± 0.45	16.5 ± 0.32	16.8 ± 0.30	17.1 ± 0.33
d 42	33.2 ± 0.53^{a}	22.3 ± 0.63^{c}	30.2 ± 0.70^{b}	30.9 ± 0.58^{b}
Total ash from tibia (%)				
d 10	53.9 ± 0.65	52.7 ± 0.73	53.2 ± 0.81	53.5 ± 0.71
d 42	56.0 ± 0.90^{a}	52.2 ± 0.83^{d}	54.2 ± 0.93^{b}	53.1 ± 0.76^{c}

[a]Data are expressed as the mean ± SE.
abc Indicates significant differences between the treatments within the rows ($p < 0.05$). Cyclic heat stress started at day 10. EO1: essential oil of *Lippia origanoides*, thymol chemotype (45 ppm) and herbal betaine (150 ppm). Administration dose: 350 g/Ton food. EO2: essential oil of *Lippia origanoides*, phellandrene chemotype (45 ppm) and herbal betaine (150 ppm). Administration dose: 350 g/Ton food.

TABLE 6 Evaluation of essential oils (EO) and herbal betaine on morphometric analysis of the duodenum and ileum mucosa of broiler chickens exposed to cyclic heat stress at day 42[a].

Treatments	Villus height (μm)	Crypt depth (μm)	Villus width (μm)	Villus: crypt ratio	Villus surface area index (mm^2)
Duodenum					
Thermoneutral	976.7 ± 17.0^{a}	187.5 ± 70.4^{a}	179.8 ± 12.5^{a}	5.9 ± 0.8^{a}	538.4 ± 20.1^{a}
Heat Stress	706.8 ± 40.6^{c}	145.0 ± 70.7^{c}	140.1 ± 24.5^{c}	4.1 ± 0.6^{c}	381.8 ± 21.5^{c}
Heat Stress + EO1	853.8 ± 53.7^{b}	160.5 ± 32.7^{b}	158.2 ± 90.3^{b}	5.2 ± 0.5^{b}	405.6 ± 23.0^{b}
Heat Stress + EO2	838.7 ± 83.9^{b}	160.1 ± 37.8^{b}	161.8 ± 19.2^{b}	5.4 ± 0.9^{b}	438.5 ± 24.5^{b}
Ileum					
Thermoneutral	551.3 ± 52.4^{a}	118.6 ± 40.5^{a}	28.5 ± 81.7	5.9 ± 0.5^{a}	18.4 ± 7.2^{a}
Heat Stress	324.1 ± 94.1^{c}	206.7 ± 52.4^{c}	27.0 ± 81.0	1.5 ± 0.5^{c}	15.7 ± 8.1^{b}
Heat Stress + EO1	431.9 ± 93.2^{b}	157.4 ± 33.5^{b}	29.1 ± 61.3	2.7 ± 0.5^{b}	16.8 ± 7.5^{b}
Heat Stress + EO2	425.2 ± 94.2^{b}	149.4 ± 38.6^{b}	33.2 ± 30.4	2.8 ± 0.5^{b}	18.9 ± 9.0^{b}

[a]Values were expressed as means ± SE, representing 8 birds/group and 10 measurements/parameter/bird.
[abc] Indicates significant differences between the treatments within the columns ($p < 0.05$). Cyclic heat stress started at day 10. EO1: essential oil of *Lippia origanoides*, thymol chemotype (45 ppm) and herbal betaine (150 ppm). Administration dose: 350 g/Ton food. EO2: essential oil of *Lippia origanoides*, phellandrene chemotype (45 ppm) and herbal betaine (150 ppm). Administration dose: 350 g/Ton food.

(%), or cold carcass (%) between treatments (Table 7). Thermoneutral chickens and chickens supplemented with EO1, showed a significant reduction ($p < 0.05$) in wing yield. Nevertheless, breast yield in thermoneutral chickens was significantly higher ($p < 0.05$) than in chickens exposed to heat stress. Leg quarter yield was reduced ($p < 0.05$) in thermoneutral chickens compared to heat stress chickens. No differences ($p > 0.05$) were observed in tender yield or rack yield between treatments (Table 7).

The results of the evaluation of EO and herbal betaine on broiler breast myopathy scores and breast myopathy percent incidence of broiler chickens exposed to cyclic heat stress at day 43 are summarized in Table 8. As expected, thermoneutral chickens showed significantly higher incidences ($p < 0.05$) of both myopathies, when compared to heat stress chickens, regardless of the dietary inclusion of EO1 or EO2 (Table 8).

Table 9 expresses the results of the evaluation of EO and herbal betaine on raw meat quality parameters of broiler chickens exposed to cyclic heat stress at day 43. Control HS chickens showed a significant reduction ($p < 0.05$) in drip loss (%) compared with thermoneutral and EO2 supplemented chickens. However, chickens supplemented with EO2 expressed a significant reduction ($p < 0.05$) in the color measurement of relative lightness (L*) compared to thermoneutral chickens. In contrast, color measurements for relative yellowness (b*) were significantly higher ($p < 0.05$) in thermoneutral chickens (Table 9).

TABLE 7 Evaluation of essential oils (EO) and herbal betaine on final body weight (g) and carcass yields (%) of broiler chickens exposed to cyclic heat stress at day 43[a].

	Thermoneutral	Heat stress	Heat stress + EO1	Heat stress + EO2
Live weight	3,310.73 ± 30.02[a]	2,468.60 ± 26.85[c]	2,718.54 ± 26.85[b]	2,673.10 ± 26.85[b]
Fat yield (%)	1.11 ± 0.05	1.05 ± 0.04	1.04 ± 0.04	1.01 ± 0.04
Hot carcass yield (%)	74.73 ± 0.21	74.66 ± 0.18	74.26 ± 0.18	74.22 ± 0.18
Chilled carcass yield (%)	79.37 ± 0.36	79.06 ± 0.32	78.81 ± 0.32	79.22 ± 0.32
Wing yield (%)	8.08 ± 0.08[b]	8.70 ± 0.07[a]	8.30 ± 0.07[b]	8.61 ± 0.07[a]
Breast yield (%)	20.46 ± 0.20[a]	17.50 ± 0.18[b]	18.09 ± 0.18[b]	18.10 ± 0.18[b]
Tender yield (%)	4.03 ± 0.07	4.03 ± 0.06	4.00 ± 0.06	4.14 ± 0.06
Leg yield (%)	23.96 ± 0.19[b]	25.75 ± 0.17[a]	25.32 ± 0.17[a]	25.36 ± 0.17[a]
Rack yield (%)	21.67 ± 0.18	22.03 ± 0.16	21.84 ± 0.16	21.72 ± 0.16

[a]Data are expressed as the mean ± SE.
[abc] Indicates significant differences between the treatments within the rows ($p < 0.05$). Cyclic heat stress started at day 10. EO1: essential oil of *Lippia origanoides*, thymol chemotype (45 ppm) and herbal betaine (150 ppm). Administration dose: 350 g/Ton food. EO2: essential oil of *Lippia origanoides*, phellandrene chemotype (45 ppm) and herbal betaine (150 ppm). Administration dose: 350 g/Ton food.

TABLE 8 Evaluation of essential oils (EO) and herbal betaine on broiler breast myopathy scores and breast myopathy percent incidence of broiler chickens exposed to cyclic heat stress at day 43[a].

	Thermoneutral	Heat stress	Heat stress + EO1	Heat stress + EO2
Woody Breast[1]				
Average	0.74 ± 0.04[a]	0.21 ± 0.04[b]	0.32 ± 0.04[b]	0.27 ± 0.04[b]
0 Occurrence	47.50 ± 5.09[b]	98.00 ± 4.55[a]	94.00 ± 4.55[a]	98.00 ± 4.55[a]
1 Occurrence	52.50 ± 5.09[a]	2.00 ± 4.55[b]	6.00 ± 4.55[b]	2.00 ± 4.55[b]
2 Occurrence	—	—	—	—
White Striping2				
Average	1.18 ± 0.05[a]	0.96 ± 0.04[b]	1.03 ± 0.04 [ab]	0.95 ± 0.04[b]
0 Occurrence	5.00 ± 4.12	16.00 ± 3.69	8.00 ± 3.69	14.00 ± 3.69
1 Occurrence	80.00 ± 5.03	82.00 ± 4.50	86.00 ± 4.50	84.00 ± 4.50
2 Occurrence	15.00 ± 3.11[a]	2.00 ± 2.78[b]	6.00 ± 2.78 [ab]	2.00 ± 2.78[b]

[a]Data are expressed as the mean ± SE., ab Indicates significant differences between the treatments within the rows ($p < 0.05$). Cyclic heat stress started at day 10. EO1: essential oil of Lippia origanoides, thymol chemotype (45 ppm) and herbal betaine (150 ppm). Administration dose: 350 g/Ton food. EO2: essential oil of Lippia origanoides, phellandrene chemotype (45 ppm) and herbal betaine (150 ppm). Administration dose: 350 g/Ton food. For average myopathy scores, thermoneutral treatments only consisted of an n = 40. All remainders had an n = 50. For percent incidence, scores were broken out on a pen basis and consisted of an n = 24. 1Breast fillets were considered a score of 0, 1, or 2 for woody breast if the fillet was flexible throughout, stiff in cranial region, or stiff in the cranial and caudal regions, respectively. 2Breast fillets were considered a score of 0, 1, or 2 for white striping if the fillet displayed no visible stripes, stripes less than 1 mm, or stripes larger than 1 mm, respectively.

4 Discussion

Colombia is a megadiverse nation, as it is home to more than 45,000 unique plant species. A current program for the development of the agro-industrial sector in Colombia investigates sustainable methods for extracting plant metabolites from native plants such as *Lippia. alba*, and *Lipia origanoides*. EO of various *Lippia* species possess antimalarial, sedative, hypotensive, and anti-inflammatory properties (Pascual et al., 2001; Stashenko et al., 2010). Nevertheless, the EO composition changes during plant development, cultivation conditions, or phenotypic plasticity (Antolinez-Delgado and Rodríguez-López, 2008). Thus, chemotype differentiation requires molecular biology and secondary metabolite analysis. In Colombia, infusions of leaves and flowers of *L. origanoides* are used in popular medicine, to treat digestive disorders (Pascual et al., 2001). Chromatographic analyses of EO from *Lippia origanoides* plants growing in the wild throughout various Colombian regions have identified 139 substances (Stashenko et al., 2010). Differential identification of these EO and extracts classifies *L. origanoides* into three chemotypes based on their essential oil primary components (Stashenko et al., 2010). Chemotype A had phellandrene, p-cymene, and limonene, while B and C had carvacrol and thymol (Curado et al., 2006).

The qualitative and quantitative chemical composition of *Lippia origanoides* essential oils for both formulations submitted to

TABLE 9 Evaluation of essential oils (EO) and herbal betaine on raw meat quality parameters of broiler chickens exposed to cyclic heat stress at day 43[a].

	Thermoneutral	Heat stress	Heat stress + EO1	Heat stress + EO2
Drip loss (%)[b]	1.24 ± 0.13[a]	0.75 ± 0.11[b]	0.98 ± 0.12[ab]	1.20 ± 0.11[a]
pH	5.77 ± 0.03	5.76 ± 0.03	5.78 ± 0.03	5.72 ± 0.03
L[ac]	60.96 ± 0.47[a]	59.70 ± 0.42 [ab]	59.56 ± 0.42 [ab]	58.95 ± 0.42[b]
a[ad]	3.31 ± 0.19	3.25 ± 0.17	3.24 ± 0.17	3.21 ± 0.17
b[ae]	8.23 ± 0.27[a]	6.99 ± 0.24[b]	7.23 ± 0.24[b]	7.12 ± 0.24[b]

[a]Data are expressed as the mean ± SE.

ab Indicates significant differences between the treatments within the rows ($p < 0.05$). Cyclic heat stress started at day 10. EO1: essential oil of *Lippia origanoides*, thymol chemotype (45 ppm) and herbal betaine (150 ppm). Administration dose: 350 g/Ton food. EO2: essential oil of *Lippia origanoides*, phellandrene chemotype (45 ppm) and herbal betaine (150 ppm). Administration dose: 350 g/Ton food.

[b]Drip loss—Measured as percent loss in relation to deboned part weight. Presented as percent loss.

[c]L*—CIE, color measurement of relative lightness. Measured 0-100 with 0 being black and 100 being white in color.

[d]a*—CIE, color measurement of relative redness. Measured −60, to +60 with −60, being green and +60 being red in color.

[e]b*—CIE, color measurement of relative yellowness. Measured −60, to +60 with −60, being blue and +60 being yellow in color.

chromatographic analysis 7890 A revealed that the two main components of *L. origanonides* in Formula 1 were thymol (47.5%) and carvacrol (29.9%). On the other hand, the two main components of *L. origanonides* present in Formula 2 were trans-β-Caryophyllene (12.6%) followed by *p*-Cymenene (10.1%), confirming clear differences in the primary components of essential oils in both chemotypes of *L. origanonides*.

The EOs have gained popularity as a potential strategy for mitigating the effects of heat stress in poultry (Raza et al., 2022). In this study, we evaluated the potential effects of two formulations of EO from *L. origanoides* chemotype thymol (EO1) and *L. origanoides* chemotype phellandrene (EO2) combined with herbal betaine on chickens under cyclic heat stress conditions. The EO from *L. origanoides* have shown a wide range of biological activities, including antimicrobial, anti-inflammatory, and antioxidant properties on chickens under heat stress conditions, suggesting that the EO contained in *L. origanoides* can reduce the severity of the negative effects of heat stress in broiler chickens (Turcu et al., 2018).

The mechanisms behind the effects of EOs on the performance of chickens under heat stress are not yet fully understood. However, several potential mechanisms have been proposed. Essential oils can improve gut morphology, increase the activity of digestive enzymes, and enhance the production of beneficial gut microbes. They have also been shown to have antioxidant and anti-inflammatory properties in chickens leading to improved performance (Gholami-Ahangaran et al., 2022).

Betaine (TMG) detoxifies homocysteine and methylates. Many naturally occurring betaines protect cells from osmotic stress as organic osmolytes (Van Puyvelde et al., 2023). Betaine inside cells prevents dehydration (Denaxa et al., 2023). Enzymes, proteins, and membranes are unaffected by this buildup. Betaine is a growing biological methyl donor (Bekdash, 2023). Betaine increases energy metabolism enzyme activity and reduces liver fat in hens (Sun L et al., 2023). Betaine-supplemented hens have improved growth rate, feed efficiency, and carcass quality, especially during heat stress or disease outbreaks (Suliman et al., 2023; Sun S et al., 2023).

EO1 and EO2 from Lippia origanoides were combined with herbal betaine in this investigation. Maltodextrin microencapsulated both formulations to protect and improve EO bioavailability. Heat stress decreased performance compared to thermoneutral chicks in this study. EO1 and EO2 had higher BW than HS chickens at the end of the research. BWG improved significantly in EO1-supplemented chickens. EO2-supplemented birds devoured more feed, lowering FCRc, but BWG was not different.

Essential oils are replacing antibiotics in poultry health and growth (Booth and van Vuuren, 2023). Chicken health and performance depend on intestinal permeability, the gut lining's ability to absorb nutrients and block harmful substances from entering the bloodstream (Gilani et al., 2021; Rocchi et al., 2022).

Thymol increases chicken intestine tight junction protein expression and localization, improving intestinal barrier function (Roussel et al., 2015; Wei et al., 2017). Thymol may also modulate tight junctions through its anti-inflammatory effects (Yao et al., 2018; Pham et al., 2023) contributing to maintain the integrity of tight junctions in the chicken intestine.

Moreover, several investigators have demonstrated that betaine also improves gut integrity by regulation of tight junctions in the intestinal epithelium of chickens (Shin et al., 2018; Shin et al., 2018; Wu et al., 2020; El-Chami et al., 2021). In this investigation, chickens under heat stress that received EO1 had a substantial reduction in serum FITC-d at day 42, a well-known intestinal permeability biomarker.

Strong bones require complicated bone mineralization. Bone health affects eggshell quality, mobility, and overall health in hens (Talaty et al., 2009). EO from Lippia origanoides improved bone mineralization in chicken tibias exposed to cyclic heat stress (Ruff et al., 2021). By increasing calcium absorption and decreasing bone resorption, thymol may improve bone mineralization. Alagawany et al. (2018) examined how thymol affects laying hen bone quality. For 12 weeks, chickens drank 0.05%, 0.10%, or 0.15% thymol. Thymol enhanced bone microstructure and mineral density. Thymol may improve bone health by increasing osteoblast and decreasing osteoclast activity (Ghanima et al., 2020).

Betaine supplementation increases bone mineral density and bone microstructure, which can prevent fractures and improve skeletal health by making minerals like calcium and phosphorus more available (Kuo et al., 2023). Compared to HS control hens, cyclic heat stress-exposed chicks fed with EO1 and EO2 had significantly higher tibia breaking strength and total ash.

The duodenum is essential for digestion and absorption. Duodenum cells are columnar. The lamina propria contains blood arteries, lymphatic vessels, and immunological cells. Lamina propria smooth muscle cells move villi to receive nutrition (Pelicano et al., 2005). Villi cover enterocytes. These villi increase duodenal nutrition absorption surface area. Villus height depends on villus cell proliferation, differentiation, and tip cell shedding. Infections, inflammation, nutrient deficiencies, and environmental contaminants affect villi height. Villi height decreases with malabsorption and gastrointestinal illnesses (Babbin et al., 2006).

Thymol improves duodenal villi shape and function (Mo et al., 2023). Thymol protects duodenal villi from oxidative stress (He et al., 2017). When free radicals outnumber antioxidants, oxidative stress ensues (Basiouni, et al., 2023). Free radicals destroy cells and tissues, causing inflammation and disease. Antioxidants help to neutralize free radicals and protect cells from damage (Shehata et al., 2022b). Similarly, betaine supplementation has been shown to increase villi height in the duodenum of chickens by increasing the secretion of mucus and improving the integrity of the intestinal mucosal barrier in chickens under long-term heat stress (Liu et al., 2019). The results of the present study confirm the adverse effects of heat stress on the villus height. Nevertheless, chickens that received both formulations of EO showed significantly higher villus in both the duodenum and ileum compared to HS control chickens.

Intestinal crypts, which produce intestinal stem cells, are invaginations between villi. Broiler chickens' intestinal health is determined by their villus height to crypt depth ratio, which indicates the small intestine's ability to absorb nutrients (Peng et al., 2022). Thymol supplementation improves this histomorphological ratio in broiler chickens (Galli et al., 2020). According to earlier studies, the increased villus-to-crypt depth ratio in the duodenum and ileum of hens given EO1 or EO2 may improve nutritional absorption, development, and production (Peng et al., 2016; Magouz et al., 2022).

Carcass yield is the amount of chicken flesh received after slaughtering and processing. It affects chicken farming profitability and the amount of meat sold. Chickens fed EO or betaine exhibit higher carcass yields (Gumus and Gelen, 2023; Pardo et al., 2023; Zheng et al., 2023). Heat stress reduces carcass yield, increasing leg meat yield at the expense of breast meat yield and meat quality (Zaboli et al., 2019; Greene et al., 2021). In this investigation, EO and betaine did not improve chickens' cyclic heat stress severity. Heat stress lowered feed intake and growth compared to thermoneutral chicks. Thus, thermoneutral chickens had more myopathies. Myopathies also contain a yellow viscous fluid that increases instrumental yellowness (b*; Sihvo et al., 2014; Maynard et al., 2023). In this experiment, thermoneutral broilers had a greater b* value than heat stress broilers regardless of EO treatment.

In summary, the dietary formulation with the tested EOs may be a viable nutritional strategy to support the growth performance of broiler chickens exposed to cyclic heat stress, which is becoming increasingly important in antibiotic-free production carried out in adverse climate. However, the potential benefits of using essential oils in combination with other management strategies for heat-stressed chickens should not be ignored. Further studies to evaluate the effects of these formulations on mitochondria function, gene expression of inflammatory and tight junction proteins, and intestinal and respiratory microbiomes are currently being investigated.

Data availability statement

The original contributions presented in the study are included in the article/supplementary material, further inquiries can be directed to the corresponding author.

Ethics statement

All animal handling procedures were followed according to the Institutional Animal Care and Use Committee at the University of Arkansas under protocol number 16084.

Author contributions

GT-I, BH, CO, and DG conceptualized the study. RS-C, AS, CM, AU, BM, and JA-I handled the methodology. LG, LL, KM, and IL were in charge of the software. EG and MC validated the study. RS-C, AS, and JL performed the formal analysis. CO, DG, and GT-I conducted the investigation. RS-C, AS, and JL prepared and wrote the original draft. XH-V, VP-G, and GT-I contributed to the writing, review, and editing of the manuscript. GT-I, BH, and CO were in charge of the project administration and funding acquisition. All authors contributed to the article and approved the submitted version.

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Conflict of interest

Authors AU, BM, and JA-I, were employed by the company Promitec.

The remaining authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

Publisher's note

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Impact of chronic heat stress on behavior, oxidative status and meat quality traits of fast-growing broiler chickens

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This research aimed to investigate, through a multifactorial approach, the relationship among some *in-vivo* parameters (i.e., behavior and blood traits) in broilers exposed to chronic HS, and their implications on proximate composition, technological properties, and oxidative stability of breast meat. A total of 300 Ross 308 male chickens were exposed, from 35 to 41 days of age, to either thermoneutral conditions (TNT group: 20°C; six replicates of 25 birds/each) or elevated ambient temperature (HS group: 24 h/d at 30°C; six replicates of 25 birds/each). In order to deal with thermal stress, HS chickens firstly varied the frequency of some behaviors that are normally expressed also in physiological conditions (i.e., increasing "drinking" and decreasing "feeding") and then exhibited a behavioral pattern finalized at dissipating heat, primarily represented by "roosting," "panting" and "elevating wings." Such modifications become evident when the temperature reached 25°C, while the behavioral frequencies tended to stabilize at 27°C with no further substantial changes over the 6 days of thermal challenge. The multifactorial approach highlighted that these behavioral changes were associated with oxidative and inflammatory status as indicated by lower blood γ-tocopherol and higher carbonyls level (0.38 vs. 0.18 nmol/mL, and 2.39 vs. 7.19 nmol/mg proteins, respectively for TNT and HS; $p < 0.001$). HS affected breast meat quality by reducing the moisture:protein ratio (3.17 vs. 3.01, respectively for TNT and HS; $p < 0.05$) as well as the muscular acidification (ultimate pH = 5.81 vs. 6.00, respectively; $p < 0.01$), resulting in meat with higher holding capacity and tenderness. HS conditions reduced thiobarbituric acid reactive substances (TBARS) concentration in the breast meat while increased protein oxidation. Overall results evidenced a dynamic response of broiler chickens to HS exposure that induced behavioral and physiological modifications strictly linked to alterations of blood parameters and meat quality characteristics.

KEYWORDS

broiler chicken, heat stress, behavior, blood parameter, oxidation, meat quality

Abbreviations: BHT, butylated hydroxytoluene; DFD, dark, firm, and dry; DNPH, dinitrophenylhydrazine; HS, heat stress; MUFA, monounsaturated fatty acids; PCA, principal component analysis; PC, principal component; PUFA, polyunsaturated fatty acids; SFA, saturated fatty acids; TBARS, thiobarbituric acid reactive substances; TNT, thermoneutral.

1 Introduction

The report of the International Panel on Climate Change (IPCC, 2022) depicted a concerning scenario in which, without a substantial reduction of greenhouse gas emissions, global warming could exceed the threshold established in the Paris Climate Agreement (i.e., maintain global warming under 2°C, preferably 1.5°C, respect to pre-industrial levels) within 2040. Albeit the negative effects of global warming on agriculture and livestock production were already evident in the past decades (FAO, 2016; Corwin, 2021), such forecasted increase of global temperature represents an additional challenge to satisfy, in an efficient and sustainable manner, the protein demand from a growing world population. Indeed, in tropical areas as well as in temperate climates during summer, the rise of environmental temperature can increase the risk for poultry and other livestock species to experience heat stress (**HS**), which takes place when animals are not capable of dissipating excess heat into the surrounding environment (Akbarian et al., 2016). Regardless of the duration (chronic vs. acute; Akbarian et al., 2016), HS is recognized as one of the most frequent and difficult to manage stressors that can occur during the rearing cycle (Aggarwal and Upadhyay, 2012), with negative implications on animal welfare, physiology and health as well as production sustainability, yields and product quality (Zaboli et al., 2019; Liu et al., 2020; Kumar et al., 2021; Brugaletta et al., 2022).

Broiler chickens are particularly susceptible to HS due to the presence of feathers, absence of sweat glands, high body mass-to-body surface area ratio, rapid metabolism and elevated core temperature (Syafwan et al., 2011). Decreased feed consumption as well as growth depression and physiological alterations (e.g., oxidative damage, fat deposition, protein catabolism, and histological modifications of the gastro-intestinal tract) were observed in broilers experiencing HS (Kikusato and Toyomizu, 2019; Lu et al., 2019; Mazzoni et al., 2022). Moreover, the artificial selection for high productive performance has modified energy partitioning in modern broilers favoring anabolic processes (e.g., efficient conversion of dietary energy into body growth; Zampiga et al., 2018), while negatively affecting their ability to deal with environmental stressors such as HS (Pawar et al., 2016).

In general, birds respond to high ambient temperatures by adjusting their behavior and physiological homeostasis in order to reduce body temperature (Lara and Rostagno, 2013). The identification of these changes is crucial to recognize and minimize the consequences of HS. It is important to underline that behavioral changes are the first bird response to thermal stress because of the lower energetic cost compared to other physiological adjustments (Branco et al., 2020). For instance, in HS conditions chickens spend more time resting, drinking, and panting and less time feeding, walking, and standing (Mack et al., 2013). The evaluation of the dynamic modifications of behavioral traits in response to the temperature increase can represent a valid information to rapidly identify thermal discomfort in broiler chickens, preserving animal welfare and health as well as productivity, with potential applications in the "precision livestock" context. On the other hand, the behavioral observations after a prolonged period of chronic HS can provide indications on the potential adaptive mechanisms adopted by the birds to cope with such conditions. In addition, only few studies have considered the overall relationships existing among animal behavior and oxidative balance, as well as their consequences on breast meat quality traits, in modern broilers exposed to chronic HS. Based on these considerations, the aim of this research was to investigate, through a multifactorial approach, the relationship among some *in-vivo* parameters (i.e., behavior and blood traits) in broilers exposed to chronic HS, and their implications on proximate composition, technological properties, and oxidative stability of breast meat.

2 Material and methods

2.1 Animal housing and environmental conditions

The present research is part of the experimental trial reported in Zampiga et al. (2021), where animal housing is described in detail. Briefly, the trial was conducted in the research facility of the University of Bologna (Italy) according to EU Regulation for the protection of meat-type chickens (European Commission, 2007), the protection of animals at the time of killing (European Commission, 2009), and the protection of animals used for scientific purposes (European Commission, 2010). The Ethical Committee of the University of Bologna authorized the experimental protocol (ID: 1031/2019).

For this research, two different rooms were used in order to rear the chickens under thermoneutral (**TNT**) or HS conditions. The rooms presented the same characteristics regarding artificial lighting and ventilation systems, and were equipped with six pens/room showing identical features. Each pen was 3.3 m^2 and furnished with one circular pan feeder and five nipples. A total of 300 one-day-old Ross 308 male chickens were purchased and vaccinated at the hatchery (Marek, Newcastle, Gumboro, and coccidiosis), and then randomly allocated in either the TNT or HS room (25 chickens/pen). For the whole rearing cycle (0—41 days), the ambient temperature of the TNT room was settled consistently with the recommendations of the breeding company guidelines (i.e., placement: 30°C; 3 days: 28°C; 6 days: 27°C; 9 days: 26°C; 12 days: 25°C; 15 days: 24°C; 18 days: 23°C; 21 days: 22°C; 24 days: 21°C; 27–41 days: 20 °C). In the HS room, chickens were reared in thermoneutral conditions until 35 days of age, and then exposed to a temperature of 30°C for 24 h/d up to slaughter (41 days). Overall, the temperature increase in HS room was progressive, approximately 1°C–1.5°C per h on average. Such conditions (i.e., temperature, time of exposure, *etc.*) were chosen in order to simulate a chronic heat wave occurring the last week before slaughtering, so when modern broilers are particularly susceptible to high temperatures. Overall, the conditions adopted in this study were effective to induce a thermal stress response in the birds without significantly impacting livability (Zampiga et al., 2021). From 35 to 41 days, the temperature of both rooms was recorded through the use of three data loggers positioned at the beginning, middle, and end of each room. During the trial, the range of relative humidity was 40%–55% in both rooms. All birds received the same commercial basal diet (based on corn, wheat and soybean meal; mash form) according to a 3-phase feeding program: starter (0—14 days), grower (15—28 days) and finisher (29—41 days). For the entire period of trial, birds had free access to fresh water and feed, which were distributed *ad-libitum*.

TABLE 1 Ethogram used to analyze the behavior of broiler chickens raised in either thermoneutral (TNT) or chronic heat stress (HS) conditions (n = 36/group) from 35 to 41 days of age.

Category	Behavior	Description
STATIC	Roosting	Lying position, the ventral body region is in contact with the floor
	Resting	The body is in line with the ground, the head is erected and eyes opened. Only the feet are in contact with the floor
	Sleeping	The head is in a low posture (under the wing or on the bedding) and eyes closed
ACTIVE	Walking	Moving more than three steps
EAT	Feeding	Pecking inside the feeder
	Drinking	Pecking the drinker
HEAT	Panting	Showing fast, laboured breathing with an opened beak
	Elevating wings	Wings are spaced from the body

2.2 Behavioral observations

Behavioral observations were conducted in three representative pens/room (i.e., beginning, middle, and end of each room) at 35, 36 and 41 days of bird age. Twelve chickens per pen (in total 36 birds/treatment) were randomly selected and individually labelled with stick spray on their back. To avoid any interference between the observer and the birds, the behavior was assessed with the Noldus Technology (Wageningen, the Netherlands), which consists of two software: Media Recorder and Observer XT. The Media Recorder allows to record a video with a camera (Basler, Wageningen, the Netherlands), which was then analyzed with the Observer XT using the instantaneous scanning sampling method (Altmann, 1974) following a pre-defined ethogram (Table 1). At 35 days, a video of 8 h length was recorded in the three pens of the HS room to evaluate the changes in bird behavioral pattern in response to the gradual increase of environmental temperature from 18°C to 30°C. In particular, as the temperature increase was progressive, videos were analyzed at every change in temperature (1°C) for 1 min. At 1 and 6 days of HS exposure (corresponding to 36 and 41 days of bird age), two videos a day, taken from 9 a.m. to 10 a.m. and from 3 p.m. to 4 p.m., were recorded in the same three pens of the TNT and HS room. According to Cartoni Mancinelli et al. (2020) and Cartoni Mancinelli et al. (2022), each video was analyzed by a single researcher with experience in poultry behavior and trained in the use of Noldus software, using the scan sampling method for a total of 60 observations of 5 s each *per* hour (n = 36 birds/group). All data are presented as the frequency of each behavior in the analyzed video (n./time).

2.3 Blood parameters

As reported in Zampiga et al. (2021), 12 birds/group (2 birds/replication) were chosen at 41 days based on the average body weight of each experimental group (2,900 and 2,450 g, respectively for TNT and HS; Δ = ± 50 g for both groups). At slaughtering in a commercial plant (using electrical stunning as described below; European Commission, 2009), blood samples were obtained from the 12 selected broilers/group. Blood was collected in heparinized vacutainers and centrifuged (1,500 × g for 10 min at 4°C) to collect plasma, while serum was obtained by spontaneous separation in tubes kept 2 h at ambient temperature. Both plasma and serum samples were stored at −80°C until analyses. As reported in Mattioli et al. (2019), the plasma lipid peroxidation was determined through a spectrophotometer (Shimadzu Corporation UV-2550, Kyoto, Japan) set at 532 nm according to the absorbance of thiobarbituric acid reactive substances (**TBARS**) and a tetraethoxypropane calibration curve in sodium acetate buffer (pH = 3.5). Accordingly, the results were expressed as nmol of malondialdehyde (**MDA**)/mL of plasma. The method proposed by Dalle-Donne et al. (2003), based on the use 2,4-dinitrophenylhydrazine (**DNPH**) as reactive, was applied to determine the protein carbonyl groups. Furthermore, the serum carbonyl content (reported as nmol/mg of protein) was evaluated at 366 nm of absorbance using 22,000 M 1/cm as a molar absorption coefficient. Before that analysis, the serum was diluted to 1:40 with phosphate-buffered saline.

The concentrations of tocols (α-tocopherol and its isoforms γ and δ, and α-tocotrienol) and retinol were determined following the Schüep and Rettenmaier (1994) protocol. In detail, the plasma (0.2 mL) was mixed with 4 mL of an ethanol solution containing 0.06% butylated hydroxytoluene (**BHT**) and 1 mL of water. Water/potassium hydroxide (60%) was used to saponify the mixture at 70°C for 30 min, which was then extracted with hexane/ethyl acetate (9/1, v/v). After centrifugation, a volume of 2 mL of the supernatant was transferred into a glass tube, dried under N_2, and re-suspended into 200 µL of acetonitrile. The pellet was re-extracted twice and the filtrate (50 µL) was injected into an HPLC (pump model Perkin Elmer series 200, equipped with an autosampler system, model AS 950-10, Jasco, Tokyo, Japan) on a Sinergy Hydro-RP column (4 µm, 4.6 × 100 mm; Phenomenex, Bologna, Italy) setting 2 mL/min as flow rate. The identification of the tocopherols and tocotrienols was done by means of a fluorescence detector (model Jasco, FP-1525) with excitation and emission wavelengths of 295 and 328 nm, respectively. External calibration curves, constructed with increasing quantities of pure standard solutions (Sigma-Aldrich, Bornem, Belgium) in ethanol, were used to quantify the related tocopherols. The same HPLC device, equipped with a UV-VIS spectrophotometer detector (Jasco UV2075 Plus) set at λ 325 nm, was used to assess the retinol. For the identification and quantification of retinol, the sample was compared with a pure commercial standard in chloroform (Extrasynthese, Genay, France; Sigma-Aldrich, Steinheim, Germany).

The Folch et al. (1957) method was adopted for serum lipid extraction, while the esterification was carried out following the methodology proposed by Christie (1982). The heneicosanoic acid methyl esters (Sigma Chemical Co.) were used as the internal standard for the trans-methylation procedure with recovery rates of 89% ± 4%. A Varian gas chromatograph (CP-3800), equipped with a flame ionization detector and a capillary column (100 m length × 0.25 mm × 0.2 µm film; Supelco, Bellefonte, PA, United States), was utilized for the analysis of the fatty acid composition (described in detail in Mattioli et al., 2021). The flow rate was 2 mL/min with helium as carrier gas and the split ratio was equal to 1:80. The oven temperatures were as follows: 40°C for 1 min, then 163°C for 10 min (rate of 2°C/min), 180°C for 7 min (rate of 1.5°C/min), 187°C for 2 min (rate of 2°C/min), and finally 230°C for 25 min with a rate of 3°C/min. The temperatures of the injector and detector were 270°C and 300°C, respectively. For each sample, the identification of the individual fatty acid methyl esters was carried out by comparing the peak retention times with those of the standard mixture (FAME Mix Supelco). The results were expressed as percentage of each individual fatty acid methyl ester on the total fatty acids methyl esters detected. Finally, the sum of the total saturated fatty acids (**SFA**), total monounsaturated fatty acids (**MUFA**), and total polyunsaturated fatty acids (**PUFA**) of the n-3 and n-6 series were calculated using the average amount of each fatty acid.

2.4 Meat quality traits

At 41 days, all birds were sent to a commercial processing plant (using the same, conventional truck; transport time ~2 h) and then slaughtered according to routine practices. Both the groups were exposed to the same processing conditions. Birds were stunned by means of an electrified water-bath (200–220 mA, 1,500 Hz; European Commission, 2009) and killed through neck vessels severing, which was performed through an automatic device. After bleeding, carcasses were scalded (approximately 50°C for 210 s), mechanically plucked, eviscerated and processed (i.e., removal of head, neck, abdominal fat, and feet), and finally air-chilled to reach a core temperature of about 4°C–5°C. After 24 h, 15 carcasses per experimental group were individually weighed and dissected to obtain the *P. major* muscles. Breast yield (%) was calculated accordingly and then meat used for the analytical determinations. Breast meat proximate composition was evaluated following the procedures reported by the Association of Official Analytical Chemists (AOAC, 1990). Moisture and ash evaluation was performed in duplicate, whereas the total fat and crude protein content was determined by means of the Folch et al. (1957) and the Kjeldahl copper catalyst (AOAC, 1990) methods, respectively. As concerns the technological properties of breast meat, color (L* = lightness, a* = redness, and b* = yellowness; CIE, 1978) was measured in triplicate on the muscle ventral (bone side) surface at 24 h post-mortem by means of a reflectance colorimeter (Chroma Meter CR-400; Minolta Corp., Milan, Italy). The iodoacetate method (Jeacocke, 1977) was applied to evaluate ultimate pH (pHu) of breast muscles. Briefly, 2.5 g of meat were minced and then homogenized for 30 s at 13,500 rpm by means of an Ultra-Turrax T25 basic (IKA-Werke, Germany) in solution (25 mL, pH 7.0) of 5 mM sodium iodoacetate and 150 mM potassium chloride. Finally, a Jenway 3510 pH-meter (Jenway, Cole-Parmer, Staffordshire, United Kingdom), calibrated at pH 4.0 and 7.0, was used to assess homogenate pH. For drip loss analysis, meat samples weighing approximately 80 g (8 cm × 4 cm × 2 cm) were obtained from the cranial portion of each *Pectoralis major* muscle, weighed, and then stored in plastic boxes over sieved plastic racks for 48 h at 4°C ± 1°C. Then, the samples were weighed back after blotting the excess surface fluids and drip loss was expressed as percentage of weight lost during refrigerated storage. Each sample utilized for drip loss analysis was then packaged under vacuum and cooked in a water bath upon reaching 80°C in the inner core. Samples were then cooled down at room temperature and weighed to calculate cook loss. Finally, cooked subsamples (4 cm × 2 cm × 1 cm) were used for shear force analysis, which was assessed through a TA. HDi Heavy Duty texture analyzer (Stable Micro Systems Ltd., Godalming, Surrey, United Kingdom) equipped with a 25 kg loading cell and an Allo-Kramer shear probe. Shear force results were expressed as kilogram per gram of meat.

2.5 Oxidation markers, antioxidants content and fatty acid proportions in the breast muscle

All oxidative parameters and the fatty acid profile of the breast muscle were assessed in triplicate. The content of α, γ and δ-tocopherol, α and γ-tocotrienol, and retinol were determined using an HPLC system following the method proposed by Hewavitharana et al. (2004). Briefly, 2 g of sample were included into a solution containing 5 mL distilled water and 4 mL ethanol, which was then vortexed for 10 s. Four mL of hexane containing BHT (200 mg/L) were included into the solution that was mixed and centrifuged (8,000 × g for 10 min). Then, 3 mL of supernatant were dried by N_2 stream and dissolved into 200 µL of acetonitrile. A total of 50 µL was injected into the HPLC equipment and analyzed as indicated for the plasma. The peroxidability index was computed according to the formula defined in the work of Arakawa and Sagai (1986): (% monoenoic × 0.025) + (% dienoic × 1) + (% trienoic × 2) + (% tetraenoic × 4) + (% pentaenoic × 6) + (% hexaenoic × 8). As described before, the Folch et al. (1957) method was applied to extract the meat lipid fraction for fatty acid analysis. The gas chromatograph conditions were the same adopted for the serum fatty acids evaluation. As for the meat oxidative profile, TBARS were analyzed following the procedure proposed by Bao and Ertbjerg (2015), whereas protein carbonylation level was determined through the DNPH-based method (Soglia et al., 2016).

2.6 Statistical analyses

The STATA software (StataCorp LP., United States) was used for the statistical analysis of data concerning the animal behavior, in which the bird was considered as the experimental unit. For these traits, two different aspects were evaluated: i) the bird behavioral changes in response to the increase of environmental temperature at 35 days (from 18°C to 30°C), and ii) the effect of the prolonged

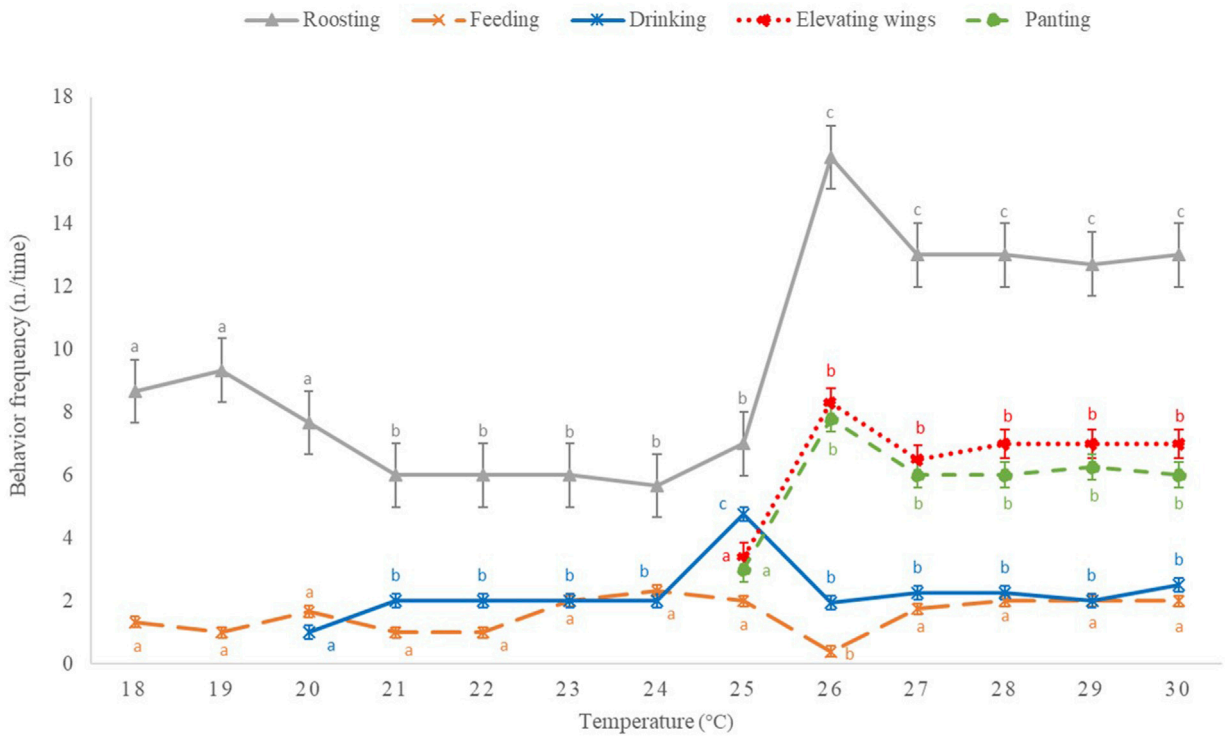

FIGURE 1
Frequency of the main behaviors expressed (n./time, time = minute) by broiler chickens during the 8 h of temperature increase from 18°C to 30°C (n = 36/group). Results are expressed as mean and standard error of the mean. $^{a-c}$: For each behavior, different letters indicate significant changes ($p < 0.05$) in its occurrence according to temperature variation.

exposure to HS (1 and 6 days) on behavior. The first dataset was analyzed by means of a mixed model considering the effect of HS accounting for the repeated measures performed on the same broiler chicken at different times. In the second dataset, the effect of HS on behavior was tested by one-way ANOVA with the fixed effect of the ambient condition (TNT vs. HS) and the repeated effect of the bird. As no significant differences ascribable to the time of recording (AM or PM) and HS exposure (1 and 6 days) were found, these effects were not included in the statistical model and thus the environmental condition was the only experimental factor. Significance was designated at $p < 0.05$ and the Bonferroni *post hoc* test was used. Moreover, a multivariate analysis was performed (Principal Component Analysis; **PCA**) to simultaneously assess the global trend of behavior (roosting, resting, sleeping, feeding, drinking, panting and elevating wings) and blood parameters (carbonyls and γ-tocopherol concentration). Panting and elevating wings were considered as a unique variable in the PCA being typical behaviors associated to HS in chickens. To reduce the number of Principal Components (**PC**), only those with eigenvalues > 1 were retained. The one-way ANOVA option of the GLM procedure of SAS software (SAS Institute Inc., United States) was applied for the analysis of data concerning meat quality traits and oxidative profile. The main effect of temperature was tested (TN vs. HS) and the single breast and bird were considered as the experimental unit, respectively. The Tukey's HSD test with a significant level of p <0.05 was selected for means separation.

3 Results

3.1 Behavioral observations

Behavioral changes, both in terms of activity and frequency, were observed when the environmental temperature increased (Figure 1). In particular, when the temperature reached 25°C chickens exhibited the highest frequency of "drinking" (4.8 n./time). At this temperature, "panting" and "elevating wings" behaviors were observed for the first time. At the temperature of 26°C, the prevalent behaviors were "roosting" (16.1 n./time) followed by "elevating wings" and "panting" (8.3 and 7.8 n./time, respectively), while "feeding" was the only behavior not expressed by the chickens. Over 26°C, birds seemed to stabilize their behavior showing high frequency of "roosting", "elevating wings" and "panting" followed by "drinking" and "feeding."

The behavioral pattern of TNT and HS broilers exposed to chronic HS (1 and 6 days of exposure) are reported in Figure 2. Specifically, when compared to HS birds, TNT ones exhibited greater variability in the static behaviors with a higher frequency of "resting" and "sleeping" (6.64 vs. 1.32 and 2.48 vs. 0.16 n./time, respectively; $p < 0.001$). On the contrary, birds belonging to the HS group spent more time in "roosting" when compared to TNT (49.4 vs. 18.9 n./time, respectively; p <0.001). Concerning the eating behaviors, the HS group showed a higher frequency of "drinking" compared to the TNT one. On the contrary, TNT birds exhibited the highest frequency of "feeding." The HS chickens also showed behaviors like "panting" and "elevating wings" that were not observed in the TNT ones.

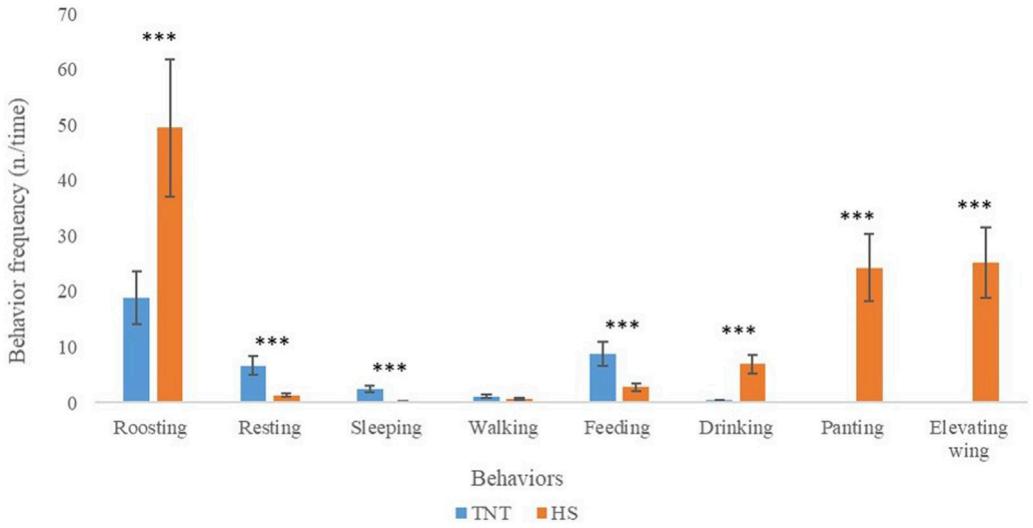

FIGURE 2
Behavioral frequencies (n./time, time = 2 h of length video/d) of broiler chickens raised in either thermoneutral or chronic heat stress conditions from 35 to 41 days of age (n = 36/group). Results are expressed as mean and standard error of the mean and represent the average of independent measurements carried out after 1 and 6 days of HS exposure. *** = $p < 0.001$.

TABLE 2 Blood oxidative parameters of 41 days-old broiler chickens raised in either thermoneutral (TNT) or chronic heat stress (HS) conditions (n = 12/group) from 35 to 41 days of age.

	TNT	HS	SEM	p-value
Oxidative markers				
TBARS (nmol MDA/mL)	36.21	35.47	4.50	ns
Carbonyls (nmol/mg proteins)	2.39	7.19	2.50	***
Antioxidant content				
Retinol (nmol/mL)	6.32	5.89	1.00	ns
α-tocotrienol (nmol/mL)	0.005	0.110	0.012	ns
δ-tocopherol (nmol/mL)	0.15	0.31	0.11	***
γ-tocopherol (nmol/mL)	0.38	0.18	0.16	***
α-tocopherol (nmol/mL)	18.78	19.19	5.76	ns

***= $p < 0.001$; ns = not significant.
TBARS, thiobarbituric acid reactive substances.
MDA, malondialdehyde.
SEM, standard error of the mean.

3.2 Blood parameters

The blood oxidative parameters and the fatty acid profile of TNT and HS broilers were reported in Tables 2 and 3, respectively. The HS significantly affected the carbonyls content, which was 3-fold higher in HS birds than in TNT ones (7.19 vs. 2.39 nmol/mg proteins, respectively; $p < 0.01$). A different trend was observed for the tocopherol isoforms, with a higher δ-tocopherol concentration found in HS chickens ($p < 0.01$). On the contrary, the γ-tocopherol was higher in TNT than in HS group ($p < 0.01$), whereas no substantial difference was detected for α-tocopherol. As for the blood fatty acid profile, significant differences were only found on the Σ MUFA content, which was lower in HS birds (12.75% vs. 15.57%, respectively for TNT and HS, $p < 0.001$). This is mainly due to the lower concentration of oleic acid (C18: 1 n-9) in HS birds (13.92% vs. 10.90%, respectively for TNT and HS; $p < 0.001$).

3.3 Association among behavioral measures and blood parameters

The plot of multivariate analysis (Table 4; Figure 3) showed the interaction of behavioral and blood parameters in TNT and HS chickens. The first two extracted PC with eigenvalues greater than 1.00 explained 77.8% of the total variance (Table 4). A positive value in the PC1 was observed for "roosting," "drinking," blood carbonyls, "panting and elevating wings." Conversely, negative loadings were found for "feeding," "resting," "sleeping" and blood γ-tocopherol. In PC2, "roosting," "feeding," "resting", "drinking", "panting and elevating wings" showed positive value whereas "sleeping, blood carbonyls and blood γ-tocopherol exhibited a negative one. In Figure 3, the scores revealed a clear separation of the two experimental groups (TNT and HS). In particular, the HS birds were mostly discriminated by blood carbonyls, "drinking", "roosting", "panting and elevating wings", whereas the TNT chickens were mainly characterized by "feeding", "sleeping", "resting" and blood γ-tocopherol.

3.4 Meat proximate composition and quality traits

Broilers exposed to chronic HS conditions exhibited lower carcass weight (p <0.01) and breast yield (p <0.05) than those raised in the TNT environment (Table 5). Regarding the

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TABLE 3 Blood fatty acids proportion (% of total fatty acids) in 41 days-old broiler chickens raised in either thermoneutral (TNT) or chronic heat stress (HS) conditions (*n* = 12/group) from 35 to 41 days of age.

	TNT	HS	SEM	*p*-value
C14:0	0.40	0.50	0.34	ns
C16:0	12.2	13.1	0.10	ns
C17:0	0.16	0.14	0.27	ns
C18:0	12.5	13.5	0.03	ns
C24:0	1.90	2.54	0.67	ns
ΣSFA	27.2	29.7	0.25	ns
C14:1	0.08	0.06	0.90	ns
C16:1	0.99	1.02	0.02	ns
C17:1	0.05	0.10	0.04	ns
C18:1 n-9	13.9	10.9	0.02	***
C24:1	0.47	0.64	0.92	ns
ΣMUFA	15.6	12.8	0.19	***
C18:2 n-6 [LA]	41.2	39.7	0.84	ns
C18:3 n-6 [GLA]	0.05	0.01	0.91	ns
C20:4 n-6 [AA]	6.77	7.27	0.01	ns
C22:2 n-6	0.24	0.12	0.34	ns
Σn-6	48.0	47.0	0.04	ns
C18:3 n-3 [ALA]	1.34	1.55	0.90	ns
C18:4 n-3	0.08	0.03	0.10	ns
C20:3 n-3	0.07	0.02	0.03	ns
C20:5 n-3 [EPA]	1.05	0.83	0.02	ns
C22:6 n-3 [DHA]	1.10	0.82	0.95	ns
Σn-3	3.65	3.26	0.08	ns
ΣPUFA	52.0	50.4	0.19	ns
n-6/n-3	13.3	15.3	0.89	ns
Others	5.30	6.90	1.02	ns

***= $p < 0.001$; ns = not significant.
SEM, standard error of the mean.
LA, linoleic acid; GLA, gamma-linolenic acid; AA, arachidonic acid; ALA, alpha-linolenic acid; EPA, eicosapentaenoic acid; DHA, docosahexaenoic acid.

chemical composition of breast meat (Table 5), no remarkable difference was detected for the percentage of total lipid and ash between the TNT and HS groups. However, HS samples exhibited lower moisture and higher protein content when compared to the TNT ones (75.0% vs. 76.0%; $p < 0.05$, and 23.7% vs. 22.4%; $p < 0.01$, respectively). Consequently, a lower moisture:protein ratio was detected in breast meat from HS birds (3.01 vs. 3.17, respectively from HS and TNT; $p < 0.05$). HS substantially affected the main quality traits and technological properties of chicken breast meat (Table 5). Meat color was not affected by the environmental conditions, albeit lightness tended ($p = 0.06$) to be lower in HS samples which, on the other hand, exhibited higher pHu compared to the TNT ones (6.00 vs. 5.81, $p < 0.01$). Water holding capacity was found to be enhanced in HS fillets, as suggested by their lower drip and cooking losses ($p < 0.05$ and $p < 0.001$, respectively) when compared to TNT group that, in turn, exhibited higher shear force ($p < 0.05$).

3.5 Oxidation markers, antioxidants content and fatty acid proportions in the breast muscle

The results regarding the effects of HS on meat oxidative profile and antioxidant content are reported in Table 6. If compared to TNT, meat samples belonging to HS group exhibited significantly lower TBARS level and higher carbonyls content (5.23 vs. 4.70 mg MDA/kg of meat; $p < 0.01$, and 1.52 vs. 1.77 nmol/mg protein; $p <$

TABLE 4 Eigenvalue, explained variance and loadings of the first two Principal Components (PC) of the multivariate analysis regarding the interaction between behavioral and blood parameters in TNT (thermoneutral) and HS (heat stress) chickens at 41 days of age.

Variable	PC1	PC2
Eigenvalue	5.24	4.98
Proportion	65.6	12.3
Cumulative	77.8	
Roosting	0.391	0.057
Feeding	−0.358	0.480
Resting	−0.381	0.435
Drinking	0.333	0.130
Sleeping	−0.338	−0.256
Panting and elevating wings	0.405	0.146
Blood carbonyls	0.347	−0.119
Blood γ-tocopherol	−0.247	−0.676

0.05, respectively). However, no significant difference in breast antioxidants content (Table 6) and fatty acid proportion (Table 7) was found between the TNT and HS birds.

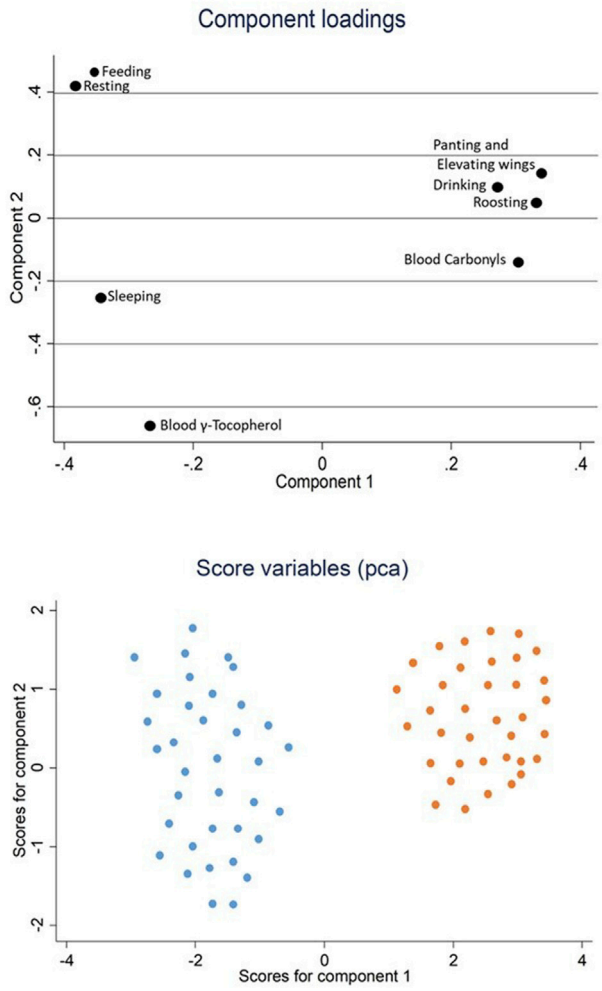

FIGURE 3
Plot of multivariate analysis related to the interaction between behavioral and blood parameters of broiler chickens raised in either thermoneutral (TNT; blu dots) or heat stress (HS; orange dots) conditions at 41 days of age (n = 36/group).

4 Discussion

Raising broiler chickens in chronic HS conditions led to several changes in behavioral, oxidative and meat quality traits. Behavioral parameters could be considered useful tools for assessing the welfare state of an animal. It is well known that, when exposed to a stressful stimulus, animals tend to modify their behavior to adapt themselves to the new condition (Mack et al., 2013). In this study, we found that these behavioral changes could be divided into two different types: 1) frequency modification of normally expressed behaviors, and 2) activation of behaviors not previously expressed. As expected, during the 8 h in which the environmental temperature was increased from 18°C to 30°C (35 days of bird age), a higher frequency of "drinking" behavior was exhibited by the chickens once a temperature of 25°C was reached. However, such activity was not enough to counteract the progressive increase of temperature, so that new distinctive behaviors ("panting" and "elevating wings") were expressed by the birds. Indeed, an increased frequency of "panting and elevating wings" was revealed starting from a temperature of 25°C. It should be noted that this behavioral pattern was observed only in HS broilers, indicating that chickens raised under heat stress tend to increase their body surface while exposing featherless areas in order to dissipate heat with the "elevating wings" and by increasing the evaporation through "panting" (Elshafaei et al., 2021). Moreover, a drastic decrease of "feeding" behavior was observed when the environmental temperature approached 26°C. The behavior pattern seems to stabilize at 27°C and it is characterized by a high frequency of "roosting." The 8-h monitoring confirmed that behavioral changes are closely related to the rising of temperatures. In particular, two temperature thresholds were identified: the first at 25°C, where the animals activate new behaviors to dissipate heat, and the second one at 27°C when animals tend to stabilize their behavior pattern, exhibiting an increased frequency of static behaviors that are typical of heat-stressed chickens. This aspect was also confirmed by the evaluation of behavior pattern of birds undergoing a prolonged exposure to HS. Indeed, in our study no significant differences were observed in the behavior of chickens after 1 or 6 days of thermal stress, indicating that the animals tend to adapt their behavior to the new condition without substantial changes over time. In fact, although the "roosting" behavior is peculiar of broiler chickens (Bizeray et al., 2002), the HS condition drastically increased its frequency. It is reported that "roosting" facilitates heat exchange with the litter that generally presents a lower temperature than the bird (Branco et al., 2020). The higher frequency of "roosting" in HS chickens was associated with a reduced expression of "feeding" behavior. This outcome corroborates the results shown in our companion paper (Zampiga et al., 2021), in which a 33% reduction of feed intake was reported for HS birds compared to TNT ones. Similarly, Talebi et al. (2022) showed that both broilers

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TABLE 5 Chemical composition and meat quality traits of breast meat from broiler chickens (41 days-old) raised in either thermoneutral (TNT) or chronic heat stress (HS) conditions (n = 15/group) from 35 to 41 days of age.

	TNT	HS	SEM	*p*-value
Carcass weight (g)	2,031	1,753	50.8	**
Breast yield (%)	39.0	35.3	0.82	*
Moisture (%)	76.0	75.0	0.25	*
Crude protein (%)	22.4	23.7	0.29	**
Total lipid (%)	1.38	1.55	0.11	ns
Ash (%)	1.40	1.49	0.06	ns
Moisture:protein ratio	3.17	3.01	0.04	*
Lightness (L*)	56.9	54.8	0.56	0.06
Redness (a*)	1.12	1.14	0.11	ns
Yellowness (b*)	6.05	5.94	0.21	ns
pH_u	5.81	6.00	0.03	**
Drip loss (%)	1.61	1.19	0.08	*
Cooking loss (%)	22.8	15.3	0.90	***
Shear force (kg/g)	3.11	2.71	0.10	*

*= $p < 0.05$; ** = $p < 0.01$; *** = $p < 0.001$. ns = not significant.
SEM, standard error of the mean.

TABLE 6 Meat oxidative profile and antioxidants content in 41-d-old broiler chickens raised in either thermoneutral (TNT) or chronic heat stress (HS) conditions (*n* = 12/group) from 35 to 41 days of age.

	TNT	HS	SEM	*p*-value
Oxidative markers				
TBARS (mg MDA/kg of meat)	5.23	4.70	0.10	**
Carbonyls (nmol/mg of protein)	1.52	1.77	0.05	*
Antioxidant content				
Retinol (μg/g)	4.79	3.11	0.57	ns
γ-tocotrienol (μg/g)	0.12	0.09	0.02	ns
α-tocotrienol (μg/g)	0.01	0.01	0.01	ns
δ-tocopherol (μg/g)	0.02	0.02	0.01	ns
γ-tocopherol (μg/g)	0.10	0.07	0.01	ns
α-tocopherol (μg/g)	5.10	3.76	0.61	ns
Peroxidability index	15.0	18.1	2.04	ns

*= $p < 0.05$; ** = $p < 0.01$; ns = not significant.
SEM, standard error of the mean.

and laying hens spend less time eating when exposed to high environmental temperatures. Moreover, in the study of Attia et al. (2018), HS negatively affected feed intake and, in turn, weight gain and feed efficiency. In line with our results, Branco et al. (2020) showed that, through the use of a Generalized Sequential Patterns (GSP) algorithm, chickens exposed to HS remained inactive thus evidencing that the environmental conditions were not favorable. Moreover, the ambient temperature was reported to be positively correlated with the "drinking" and negatively with the "running" (Bell and Weaver, 2002). Active behaviors such as "walking" or "eating" are known to cause an increase in body temperature (Marìa et al., 2004). Hence, the decreased activity of HS broilers can be considered as an adaptive response to reduce heat generation. It is important to underline that the longer is the time of thermal discomfort, the greater are the changes in the physiological status, productive performance and meat quality characteristics (Xing et al., 2019).

The multifactorial approach adopted in the present study allowed to point out the complex relationship existing among the *in-vivo* aspects considered (i.e., behavior and blood oxidative status), yet demonstrating a clear distinction between the TNT and HS group. Specifically, HS chickens were characterized by "drinking," "roosting," "panting" and "elevating wings" behaviors associated with carbonyls blood concentration. On the contrary, TNT birds were identified by "feeding," "resting" and "sleeping" behaviors associated with γ-tocopherol blood level. The physiological and behavioral response of broilers chickens is a complex and dynamic outcome aimed at achieving homeostasis and adapting them to the new environmental conditions. The results of this study highlighted such dynamic trend. In fact, the multifactorial approach could be defined as an "image capture" of the *in-vivo* mechanisms adopted by the animals to overcome the HS and thus to return to their physiological homeostasis status. In this study, HS broilers showed higher blood carbonyls and lower γ-tocopherol concentration compared to thermoneutral birds. Many authors (Sykes and Fataftah, 1986; Ali et al., 2010) indicated that the blood carbonyls level can be considered as a biomarker of oxidative stress and protein peroxidation, whereas the γ-tocopherol exerts many beneficial functions especially as anti-inflammatory agent. So that, through such approach it is possible to speculate that the behaviors mostly characterizing the HS chickens (namely, "panting and elevating wings") are strictly related to an inflammatory and oxidative status induced by the thermal challenge. The presence of inflammatory processes in HS birds is also confirmed by the higher heterophil-to-lymphocyte ratio detected in birds belonging to this experimental group (Zampiga et al., 2021). During the inflammatory process, the two isoforms of the cyclooxygenase enzymes (COX-1 and COX-2) convert the arachidonic acid into various prostanoids such as prostaglandins (PGs) (Verma et al., 2021), whose biosynthesis plays a key role in both the development and the propagation of the inflammatory signals (Ricciotti and FitzGerald, 2011). The γ-tocopherol is able to limit the synthesis of the inflammation mediator Prostaglandin E2 (PGE2) by inhibiting the enzyme COX-2 (Jiang et al., 2000). The prolonged exposure to chronic HS condition induces several metabolic alterations in broiler chickens. In fact, in normal physiological conditions, an energy unbalanced status can be restored through the mobilization of body fat (Lu et al., 2017). On the contrary, fat mobilization is suppressed under HS and the energy deficit, induced by the reduction of feed intake, enhances glucose metabolism (Akşit et al., 2006; Lu et al., 2017). Accordingly, a significantly lower MUFA content was detected in the blood of HS birds compared to TNT, which could be ascribed to a decreased synthesis of these compounds as a consequence of reduced feed intake. Moreover, TNT and HS birds exhibited the same fatty acid profile in both blood and meat, likely suggesting that no substantial

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TABLE 7 Fatty acid proportion (% of total fatty acids) in the breast muscle of 41 days-old broiler chickens raised in either thermoneutral (TNT) or chronic heat stress (HS) conditions (N = 12/group) from 35 to 41 days of age.

	TN	HS	SEM	*p*-value
C14:0	0.33	0.31	0.16	ns
C16:0	18.6	17.8	0.51	ns
C17:0	0.25	0.27	0.02	ns
C18:0	8.49	8.62	0.45	ns
C24:0	0.13	0.16	0.14	ns
ΣSFA	27.8	27.2	0.62	ns
C14:1	0.06	0.06	0.01	ns
C16:1	2.40	2.46	0.20	ns
C17:1	0.14	0.17	0.01	ns
C18:1 n-9	26.6	26.0	0.84	ns
C18:1 *cis11*	2.23	2.13	0.06	ns
C24:1	0.01	0.01	0.01	ns
ΣMUFA	31.5	30.9	0.97	ns
C18:2 n-6 [LA]	28.6	28.3	0.43	ns
C18:3 n-6 [GLA]	0.26	0.29	0.02	ns
C20:4 n-6 [AA]	4.22	4.81	0.51	ns
C22:4 n-6	0.05	0.03	0.32	ns
C22:5 n-6	0.06	0.08	0.05	ns
Σn-6	33.2	33.5	0.48	ns
C18:3 n-3 [ALA]	2.41	2.57	0.09	ns
C18:4 n-3	0.15	0.05	0.05	ns
C20:3 n-3	0.13	0.10	0.02	ns
C20:5 n-3 [EPA]	1.07	1.24	0.14	ns
C22:5 n-3 [DPA]	0.86	1.01	0.11	ns
C22:6 n-3 [DHA]	0.39	0.54	0.06	ns
Σn-3	5.03	5.53	0.28	ns
ΣPUFA	39.1	39.9	0.80	ns
n-6/n-3	6.86	6.19	0.27	ns
Others	1.69	1.89	0.08	ns

ns = not significant.
SEM, standard error of the mean.
LA, linoleic acid; GLA, gamma-linolenic acid; AA, arachidonic acid; ALA, alpha-linolenic acid; EPA, eicosapentaenoic acid; DHA, docosahexaenoic acid.

variations occurred in terms of lipid mobilization between control and stressed birds.

Regarding the meat oxidative status, HS broilers showed higher carbonyls coupled with lower TBARS content. The latter outcome was rather unexpected considering that fatty acids profile and antioxidants concentration did not exhibit significant variations in response to the environmental conditions. HS has been associated with increased muscle protein degradation and catabolism (Lu et al., 2018) with consequent changes in amino acids metabolism (Tabiri et al., 2002; Ma et al., 2018). Thus, it has been hypothesized that HS may promote protein degradation in the muscle, thereby increasing the concentration of some amino acids in the blood (Rhoads et al., 2011; Zampiga et al., 2021). Some amino acids containing nucleophilic groups (e.g., histidine, cysteine, and lysine) can be indirectly carbonylated by binding with non-protein reactive carbonyl species, such as MDA, thus resulting in an MDA concentration-dependent increase of the protein carbonyl content (Wu et al., 2009). Considering that such modifications occur on the N-terminal of the peptides (Butterfield and Stadtman, 1997; Zhao et al., 2012), it could be supposed that the higher mobilization of

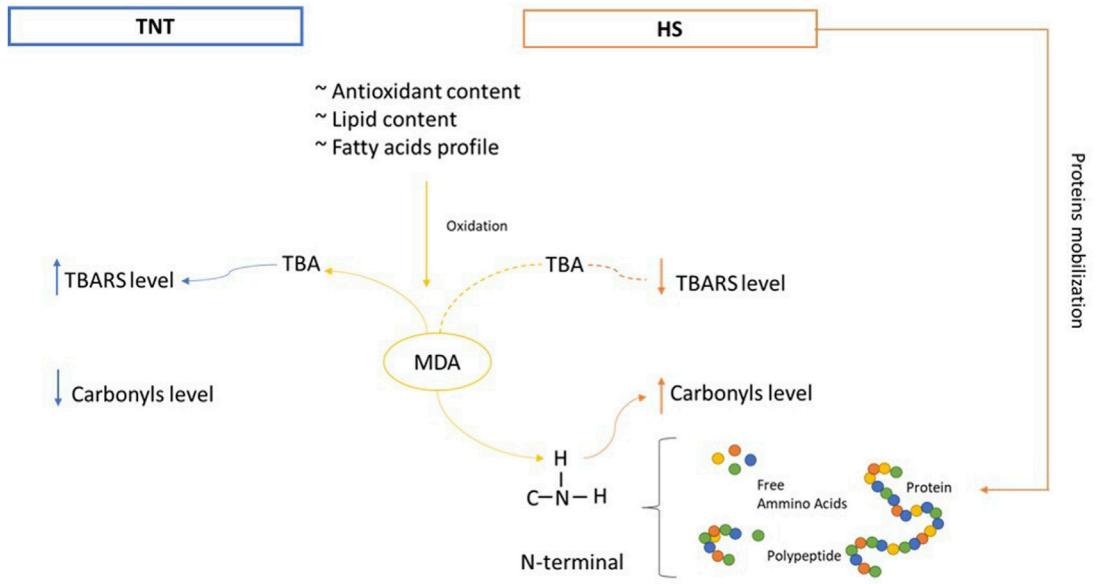

FIGURE 4
Hypothetical oxidative mechanism occurring in meat of broiler chickens raised in thermoneutral (TNT) or chronic heat stress (HS) conditions from 35 to 41 days of age. Antioxidants and lipids content as well as fatty acids profile are similar in the two groups. In both TNT and HS conditions, lipid oxidation occurs to the same extent, with the production of MDA (malondialdehyde). In TNT, MDA reacts with the TBA (thiobarbituric acid) to detect the TBARS level (thiobarbituric acid reactive substances). In HS, MDA reacts with the N-terminal group of free amino acids and polypeptides derived from protein mobilization. Carbonyls level increase while the reaction between MDA and TBA occurs to a lesser extent, thus resulting in a reduced TBARS detection.

proteins in response to the thermal challenge, which is supported by our previous investigation (Zampiga et al., 2021), might have increased the number of N-terminal groups available for the reaction with MDA. This phenomenon may ultimately result in a greater carbonylation level and reduced amount of MDA detectable through TBA-spectrophotometric assay (Figure 4). However, further insights are necessary to confirm such hypothesis.

It is widely recognized that HS is one of the most critical environmental factors affecting broiler growth performance due to the reduction in both feed intake and efficiency (Shakeri et al., 2020; Shakeri and Le, 2022). Within this context, broilers exposed to chronic HS exhibited a remarkable depression of both carcass weight and breast yield (−14% and −10%, respectively), which is consistent with the reduced "eating" behavior observed in the HS group and also with the lower final body weight and feed intake previously reported in Zampiga et al. (2021). Concerning meat proximate composition, breast muscles belonging to HS group were characterized by lower moisture and higher protein content. The lower amount of water found in HS group might be associated with an increased evapotranspiration that could result in dehydration and altered body water homeostasis, which can occur in heat-stressed birds especially when relative humidity is low (Yahav et al., 1995). On the other hand, the higher crude protein level detected in the breast muscles of HS group is rather counterintuitive considering the reduced feed intake and the increased catabolic processes associated with the thermal challenge. Possibly, such increase could indirectly result from a "concentration effect" determined by the lower water content rather than a greater *in-vivo* protein synthesis. Moreover, the reduced moisture:protein ratio found in the HS group suggests that a significantly lower quantity of water per amount of protein is present in the meat of HS birds, further supporting the hypothesis of a potential dehydration effect. In the EU legislation, the acceptable moisture: protein ratio for broiler breast meat is 3.19 ± 0.12 (range: 3.07–3.31) (Dias et al., 2020). Therefore, the average value detected in HS birds is out of range with possible relevant commercial issues for both internal EU market and exporters Countries.

HS can also alter muscular metabolism *in-vivo*, inducing several well-known final meat quality alterations whose extent eventually depends on both the duration and the magnitude of the stress (Wang et al., 2017; Gonzalez-Rivas et al., 2020). As for meat technological properties, it is widely accepted that exposing animals to acute HS immediately before slaughter accelerates muscle glycogenolysis and produces a rapid drop of muscular pH resulting in pale, soft and exudative meat, a condition responsible for impaired meat technological quality that can be frequently observed in poultry (Van Laack et al., 2000; Zampiga et al., 2020), pigs (Bowker et al., 2000), and cattle (Kim et al., 2014). By contrast, studies carried out on ruminants (Kadim et al., 2008) and pigs (D'Souza et al., 1998) disclosed that the exposure to chronic HS causes a reduction of muscular glycogen reserves and a feeble lactic acid production, thus resulting in higher pHu, darker color and greater water holding capacity of meat, i.e., common traits of dark, firm, and dry (**DFD**) meat. In poultry, the development of a DFD-like meat condition is more frequent in chickens kept in cold rather than heat stress conditions during pre-slaughter operations (Leishman et al., 2021), even though a prolonged exposure to high environmental temperatures can generate breast meat with high ultimate pH likely because of the stress-induced reduction of muscle glycogen reserves (Dai et al., 2012; Imik et al., 2012; Zeferino et al., 2016). Gregory (2010) indicated that extreme heat can provoke an adrenergic stress response with consequent increase of peripheral vasodilatation and muscle glycogenolysis, potentially

resulting in meat with high pHu and darker color in case of protracted stress. In our study, muscles belonging to HS group exhibited a reduced extent of acidification as suggested by the higher pHu. Lower glucose levels *in-vivo* were found in the breast muscle of HS broilers by Zampiga et al. (2021), who hypothesized a boost of carbohydrate utilization in the glycolytic pathway of heat-stressed animals. On the other hand, the significant reduction of feed intake induced by the thermal challenge in HS broilers (−33% compared to TNT; Zampiga et al., 2021) could have also played a role on muscle glycolytic potential and thus on its acidification capacity. The higher pHu (6.00) found in HS samples, being far from the isoelectric point of myofibrillar proteins, likely enhanced their ability to retain constitutional water by increasing their net negative charge (Schreurs, 2000; Warriss, 2000). Accordingly, HS fillets exhibited an improved water holding capacity, as suggested by the remarkably lower drip and cooking losses (−26.1% and −33.0%, respectively) if compared to TNT ones. Moreover, shear force assessed on cooked meat was found to be lower in HS group, thus corroborating the inter-relationship between water holding capacity and meat tenderness, which establishes that a higher moisture content retained within the muscle results in a greater tenderness of meat (Hughes et al., 2014). Overall, it might be hypothesized that exposing birds to chronic HS during the last week before slaughtering could have affected muscle glycolytic potential and its acidification pattern, with consequences on color, water holding capacity and tenderness of breast meat. Although further insights are necessary to elucidate these dynamics, the depletion of muscle glycogen reserves *in-vivo* could be the result of the prolonged stress and its effects on metabolic traits and feed consumption. As mentioned above, the reported data were obtained in chronic HS performed to simulate the environmental conditions that could be experienced by the birds during an extreme heat wave in tropical regions or in temperate climates during summer. It is important to underline that the discordant results available in the literature could be related to the different heat stress conditions, including type (e.g., constant vs. cyclic), duration (e.g., days to weeks), and intensity (e.g., 28°C–34°C), which could remarkably affect the variables studied herein (Azad et al., 2010; Lu et al., 2019; Zaboli et al., 2019).

In conclusion, the present study highlights the changes in behavior, blood parameters, oxidative status and meat quality in broiler chickens subjected to chronic HS conditions. It was possible to identify a dynamic behavioral response of the animals to the rise of the environmental temperature, initially consisting in modifications of the frequency of some behaviors also expressed in thermoneutral conditions (i.e., increase of "drinking" and decrease of "feeding"), and then in the manifestation of behaviors aimed at dissipating heat, such as "panting" and "elevating wings." Such modifications become evident when the temperature reached 25°C, while the behavioral frequencies tended to stabilize at 27°C with no further substantial changes over the 6 days of HS. The new behavioral patterns exhibited by HS chickens were linked to alteration of the blood parameters suggesting the presence of an oxidative (protein-induced) and inflammatory state. Chronic HS also affected the final meat quality by reducing muscular acidification, which led to abnormal meat water holding capacity and tenderness. Surprisingly, muscle TBARS concentration was lower in HS birds although protein oxidation occurred to a greater extent possibly due to an increased protein mobilization in response to the thermal challenge. However, further research is needed to better investigate this aspect.

Data availability statement

The raw data supporting the conclusions of this article will be made available by the authors, without undue reservation.

Ethics statement

The animal study was approved by Ethical Committee of the University of Bologna. The study was conducted in accordance with the local legislation and institutional requirements.

Author contributions

ACM, SM, FeS, MP, and MZ co-designed and conceptualized the study. ACM, SM, and MZ collected the samples. GB and FrS performed meat quality evaluations, including TBARS and protein carbonylation, and analyzed the data. ACM and SM performed the behavioral evaluations and analyses of blood parameters and oxidative status. CC analyzed these data. FeS, CC, and MP supervised the study. FeS and MZ managed the project. ACM and GB wrote the manuscript and all authors contributed to manuscript reviewing and editing. All authors contributed to the article and approved the submitted version.

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Conflict of interest

The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

Publisher's note

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Greater numbers and sizes of muscle bundles in the breast and leg muscles of broilers compared to layer chickens

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Meat-type (broiler) and egg-type (layer) chickens were bred by intensive selection over the years, resulting in more numbers and larger sizes of myofibers. Although the characteristics are important parameters in muscle growth and meat quality, muscle bundle characteristics have not been studied in poultry. Therefore, this study aimed to compare the histological characteristics of myofibers and muscle bundles in muscles between male broiler (Ross broiler breed) chickens and layer (Hy-Line) chickens. Chicken muscles, *pectoralis major* (PM) and *gastrocnemius* (GM), were sampled at the age of 49 days and stained to analyze histological characteristics. Expectedly, body weights (BWs) and weights of PM and GM muscles in 49-day-old broilers were significantly heavier than those in layers. Within PM, broilers exhibited greater number and cross-sectional area (CSA) of myofibers than layers (3.3- and 3.3-fold, respectively). The total number and CSA of PM muscle bundles were approximately 1.5 and 6.6 times greater, respectively, in broilers than layers. Moreover, broilers exhibited 2 times greater number of myofibers per bundle of PM muscle than layers. Within GM, myofiber number and CSA were 2.3- and 2.4-fold greater, respectively, in broilers than layers. In addition, the total number of muscle bundles and bundle CSA were 2.5- and 2.1-fold greater, respectively, in broilers than in the layers. The novel findings of the current study provide evidence that greater muscle mass of broilers occurs by both hyperplasia and hypertrophy of muscle bundles and myofibers.

KEYWORDS

broiler, layer, muscle hyperplasia, muscle hypertrophy, muscle bundle

1 Introduction

Poultry meat and egg consumption are steadily increasing as consumers face increasing health concerns related to red meat consumption. As a meat-type poultry breed, broilers have been genetically selected to have a fast-growing performance and high meat yield, whereas layers selected for egg production and backyard chickens, such as Hubbard JA57, have a slow-growing performance. As a result, they show significant differences in body and muscle weight, especially in the breast muscle, and these differences most likely involve the quantity or size of myofibers (Scheuermann et al., 2004).

TABLE 1 Ingredient and calculated nutrient composition of the dry matter of diets fed to both layer and broiler chickens.

Corn (kg 100 kg^{-1})	41.9
Soybean meal, 48% (kg 100 kg^{-1})	44.4
Meat and bone meal, 55% (pork) (kg 100 kg^{-1})	5
Blended fat (kg 100 kg^{-1})	2.9
D,L-Methionine (kg 100 kg^{-1})	0.25
L-Lysine (kg 100 kg^{-1})	0.15
Salt (kg 100 kg^{-1})	0.4
Limestone (kg 100 kg^{-1})	0.7
Dicalcium phosphate, 18.5% (kg 100 kg^{-1})	2.85
Copper sulfate, fine 25.2% (kg 100 kg^{-1})	0.05
Amprolium, 2.5% (kg 100 kg^{-1})	1
Selenium, 90.8 mg/lb (kg 100 kg^{-1})	1
Choline chloride (kg 100 kg^{-1})	0.15
L-Lysine (kg 100 kg^{-1})	0.15
Vitamin A (IU/kg^{-1})	13,200

Muscle bundle characteristics have been compared and related with growth characteristics of muscle among different breeds of livestock species (Albrecht et al., 2006). Greater muscle mass in fast-growing animals is generally associated with increased number (hyperplasia), increased size (hypertrophy), or both of myofibers and bundles, which can be attributed to various factors, such as animals, species, body weight, breed, age, sex, growth rate, and physical activity (Kiessling, 1977; Scheuermann et al., 2004; Albrecht et al., 2006; Choi and Kim, 2009; Choi et al., 2013a; Choi et al., 2013b; Choi et al., 2014; Kokoszyński et al., 2018; Kokoszyński et al., 2022; Kim et al., 2022; Weng et al., 2022). The low-weight quail line exhibited a lower number, but similar size of myofibers, compared to the random bred control (RBC) quail line, providing a unique muscle hypoplasia model in avian species (Choi et al., 2014). In contrast, the heavy-weight quail line has a greater size of myofibers in the breast muscle, but no difference in total fiber number compared to the RBC quail line (Choi et al., 2013b). Additionally, myofiber hypertrophy appeared in the fast-growing duck line compared to ducks in the slow-growing line (Huo et al., 2021). In chickens, commercial broiler lines with higher breast yield compared to Leghorn egg-type chickens of the same age and sex showed myofiber hyperplasia and hypertrophy (Scheuermann et al., 2004). Although muscle bundle characteristics are important parameters contributing to muscle growth and meat quality in livestock animals (Scheuermann et al., 2004; Albrecht et al., 2006; Chandraratne et al., 2006; Lee et al., 2018; Choi et al., 2019), these factors have not been extensively studied in chickens. Therefore, the objective of this study was to compare histological traits of myofiber and muscle bundle in the pectoralis major (PM) and gastrocnemius (GM) muscles between male broiler chickens and layer chickens.

2 Materials and methods

2.1 Animal care

Commercially available chickens (broiler and layer; Ross broiler breed and Hy-Line, respectively) and experiments were approved by The Ohio State University Institutional Animal Care and Use Committee (IACUC; protocol no. 2020A00000094). All animals were raised under the same environmental conditions such as room temperature and the size of brooder cages. In addition, we fed the same diet to both layer and broiler chickens to eliminate diet effects (Table 1). Chickens were euthanized by cervical dislocation after CO_2 inhalation according to the IACUC protocol.

2.2 Collection of muscle samples

A total of 12 male chickens (age, 49 days; broiler, n = 6; layer, n = 6) were used in this study. Body weight (BW), PM muscle weight (PMW), and GM muscle weight (GMW) were measured, and percentages of PMW and GMW were calculated in relation to BW. After measurement of PMW, CSA of the left PM muscle was measured in an area cut from the lower left to the upper right at the half-point of the muscle (Scheuermann et al., 2004). Whole right GM muscles were fixed and then cut in the middle of the muscle to prepare paraffin blocks.

2.3 Histological processing and measurement of myofibers and muscle bundles

PM and GM muscles fixed with 10% neutral-buffered formalin were embedded in paraffin and then cross-sectioned into 10-μm slices. The sections were stained using a hematoxylin and eosin stain method following our previous study (Kim et al., 2022). All stained sections were assessed in terms of myofiber and muscle bundle characteristics, including total number, average CSA, and myofiber number per bundle, using image analysis (Image-Pro Plus software, Media Cybernetics, Silver Spring, MD). For each sample, at least 500 different fibers and 30 bundles were randomly selected and measured to determine these parameters at ×10 and ×40 magnification. Average CSA and total number of myofibers were calculated according to previous studies (Scheuermann et al., 2004; Kim et al., 2022). Total bundle number was calculated by dividing the PM muscle CSA by the mean bundle area of each sample. The average of the bundle CSA was determined by dividing the total bundle area by the total bundle number measured.

2.4 Statistical analysis

To compare carcass traits and histological characteristics between broiler and layer chickens, the data were analyzed by *t*-tests using GraphPad Prism software, version 6.02. All data

TABLE 2 Comparison of body weight, carcass traits, and histological traits of the *pectoralis major* and *gastrocnemius* muscles between broiler and layer chickens at 49 days post-hatch.

	Broiler (n = 6)	Layer (n = 6)	Level of significance
Weight and carcass traits			
Body weight (g)	4,097 (396.3)[1]	512.3 (67.7)	***
PM muscle weight (g)	946.7 (112.9)	36.5 (8.63)	***
PM muscle percentage (%)	23.1 (1.19)	7.06 (0.64)	***
GM muscle weight (g)	41.3 (5.45)	4.98 (0.62)	***
GM muscle percentage (%)	1.01 (0.09)	0.97 (0.06)	NS
Histological traits of PM muscle			
Total myofiber number (× 1,000)	1,380 (442.1)	415.2 (141.2)	**
Myofiber CSA (μm^2)	3,377 (1 330)	1,026 (170.5)	**
Total bundle number	4,941 (1,175)	3,215 (1,174)	*
Bundle CSA (μm^2, × 1,000)	906.0 (291.1)	138.1 (38.9)	***
Myofiber number per bundle	276.7 (47.6)	138.5 (46.4)	***
Histological traits of GM muscle			
Total myofiber number (× 1,000)	308.2 (82.8)	134.5 (24.1)	***
Myofiber CSA (μm^2)	1,400 (368.2)	589.2 (95.0)	***
Total bundle number	2,937 (670.1)	1,152 (52.4)	***
Bundle CSA (μm^2, × 1,000)	143.7 (29.0)	67.6 (7.00)	***
Myofiber number per bundle	106.1 (22.1)	116.5 (17.3)	NS

Level of significance: NS, no significance; * $p < 0.05$; ** $p < 0.01$; *** $p < 0.001$.
[a]Standard error of least-square means.
PM, *pectoralis major*; GM, *gastrocnemius*; CSA, cross-sectional area.

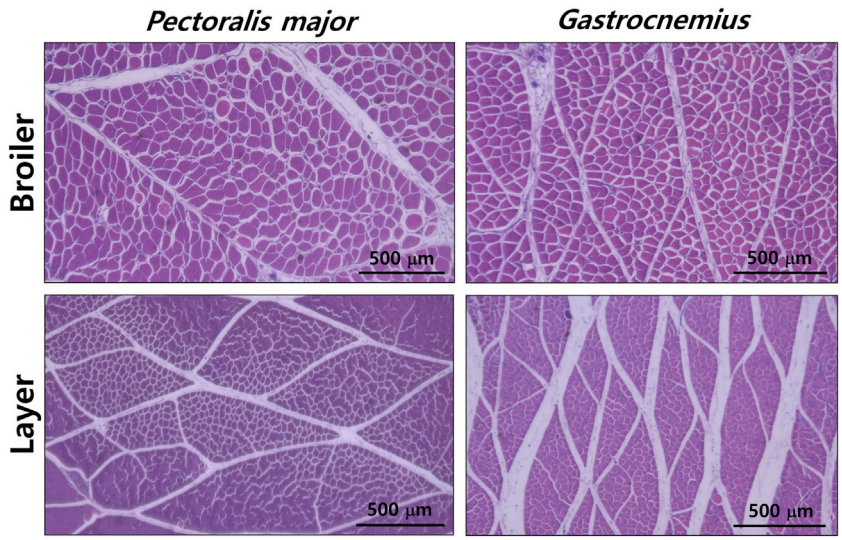

FIGURE 1
Histological differences in *pectoralis major* and *gastrocnemius* muscles between male broiler and layer chickens at 49 days post-hatch. Scale bar: 500 μm.

were expressed as means ± SEM. The results with $p < 0.05$ were considered significant.

3 Results

As expected, at 49 days post-hatch, broilers exhibited heavier body weight than layers (4,097 vs 512.3 g, $p < 0.001$) (Table 2). PMW (946.7 vs 36.5 g, $p < 0.001$) and percentage of PM (23.1% vs 7.06%, $p < 0.001$) were 25.9- and 3.3-fold greater in broilers than in layers, respectively. GMW of the broiler was approximately 8.3 times greater than that of the layer (41.3 g vs 4.98 g, $p < 0.001$), although there was no difference in the percentage of the GM muscle between the breeds (1.01% vs 0.97%, $p > 0.05$).

PM muscles of broiler chickens had a 3.3-fold greater total number (1,380,000 vs 415,200, $p < 0.01$) and CSA (3,377 vs 1,026 μm^2, $p < 0.01$) of myofibers compared to those of layer chickens. In the GM muscle, broilers showed more number (308,200 vs 134,500, $p < 0.001$) and greater size (1,400 vs 589.2 μm^2, $p < 0.001$) of myofibers compared to layers. PM muscles of broilers were 1.5-, 6.5-, and 2.0-fold greater in total bundle number (4,941 vs 3,215, $p < 0.05$), bundle CSA (906,000 vs 138,100 μm^2, $p < 0.001$), and myofiber number per bundle (276.7 vs 138.5, $p < 0.001$), respectively, compared to those of layers. Similar to PM muscles, greater number (2,937 vs 1,152, $p < 0.001$) and CSA (143,700 vs 67,600 μm^2, $p < 0.001$) of GM muscle bundles were found in broilers compared to layers; whereas two breeds did not differ in myofiber number per bundle (106.1 vs 116.5, $p > 0.05$). Their representative images are presented in Figure 1.

4 Discussion

It is generally reported that fast-growing broiler chickens are 2- to 3-fold greater in growth rate, and in particular, their breast muscles grow 8-fold faster than those in layer-type chickens (Buzala and Janicki, 2016). The findings that greater percentage of PM muscle in broilers, but similar percentage of GM muscle between two breeds, clearly provide evidence for the general selection for greater yield of breast muscle but not for leg muscle. Our previous study reported that 33-day-old broiler chickens had 2.7-fold greater myofiber CSA of PM muscle than layer chickens at the same age (Kim et al., 2022). In addition, myostatin knock-out chickens showing a rapid growth rate exhibited heavier body weight and greater myofiber CSA of the *semitendinosus* muscle than wild-type chickens with a slower growth rate (Kim et al., 2020). Similar to these results, in this study, broilers showing a heavier body weight had a greater total number and CSA of myofibers of the PM and GM muscles compared to layers showing a lighter body weight. This suggests that greater PMW and GMW of broilers could have resulted from both myofiber hyperplasia and hypertrophy.

A muscle fascicle is a bundle of different numbers of myofibers surrounded by connective tissue (Albrecht et al., 2006). The bundle characteristics, especially bundle size and fiber number per bundle, are related to muscle growth and meat quality of cattle (Albrecht et al., 2006; Choi et al., 2019). Clear differences in the number and size of muscle bundles and myofiber number per bundle were observed among the cattle breeds that have different muscle characteristics (Albrecht et al., 2006). In our previous study, we reported that a specific line of broiler chickens had a larger bundle CSA of PM muscle with myofiber hypertrophy rather than myofiber number per bundle than a specific line of layers (Kim et al., 2022). As total bundle numbers have not been investigated for both PM and GM muscles of avian species, this is the first study reporting bundle characteristics, including number, size, and myofiber number per bundle, of both PM and GM muscles of broiler and layer chickens. Broilers used in this study had a greater number and CSA of both the muscles, and PM muscle bundles of broilers showed a greater number of myofibers than those of layers ($p < 0.05$). Therefore, PM muscle bundles of broilers are characterized by a greater number and size of muscle bundles with a higher number of myofibers per bundle due to myofiber hyperplasia and hypertrophy. Greater GM muscle mass of broilers is caused by the greater number and size of muscle bundles rather than myofiber number per bundle.

Taken together, broiler and layer chickens show clear differences in histological characteristics of myofibers and muscle bundles of PM and GM muscles. These findings support that fast-growing broilers have greater muscle mass due to both hyperplasia and hypertrophy of myofibers and muscle bundles. Further investigations are needed to identify factors regulating the size and number of muscle bundles and to expand the possible influence of muscle bundles on the understanding of poultry meat quality.

Data availability statement

The original contributions presented in the study are included in the article/Supplementary Material; further inquiries can be directed to the corresponding author.

Ethics statement

The animal study was approved by The Ohio State University Institutional Animal Care and Use Committee (protocol no. 2020A00000094). The study was conducted in accordance with the local legislation and institutional requirements.

Author contributions

BL: investigation, methodology, visualization, and writing–original draft. D-HK: conceptualization, formal analysis, methodology, validation, visualization, and writing–original draft. JL: conceptualization, investigation, methodology, validation, and writing–original draft. MC: methodology, resources, and writing–review and editing. YC: investigation, methodology, resources, and writing–review and editing. KL: conceptualization,

funding acquisition, project administration, supervision, validation, and writing–review and editing.

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Conflict of interest

The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

Publisher's note

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In vivo oxidative stress associated with growth-related myopathies in chicken and potential health impact: an opinion paper

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1 Introduction

Chicken meat, particularly the breast portion, offers high-quality protein, providing adequate amounts of all of the essential amino acids with a Protein Digestibility Corrected Amino Acid Score value ranging between 0.91 and 0.95 (Boye et al., 2012). Among land animal meats, chicken breast is fairly low in lipid and collagen which is more favorable for protein digestibility (Barrón-Hoyos et al., 2013; Marangoni et al., 2015). Global demand for poultry meat has been steadily rising and it has been projected at 145 tons by 2029, with chicken expected to account for 50% of the meat consumed (USDA, 2021). A steady increase in chicken breast meat consumption within the next decade has been predicted although alternative proteins, e.g., insect and plant-based proteins, has gained increasing attention. The main reason behind its high demand is its affordable price for all classes of consumers. In addition, for the past decades, meat consumption has shifted from predominantly red meat to white meat as a healthier choice.

For the past decades, commercial broilers have been intensively selected through a breeding selection for production efficiency to meet high consumer demand (Barbut and Leishman, 2022). The success in breeding selection, however, has coincided with increased abnormalities among broilers, including growth-related myopathies, namely, White striping (WS), Wooden breast (WB) and Spaghetti meat (Barbut, 2019; Petracci et al., 2019; Soglia et al., 2019; Barbut and Leishman, 2022). These myopathies can be found together or individually in all broiler chicken breeds with a large variation in occurrence and severity across global regions (Lorenzi et al., 2014; Malila et al., 2018; Barbut, 2019; Soglia et al., 2019; Che et al., 2022).

The issue of growth-related myopathies in broilers are globally recognized among poultry community. The industry has found an increasing prevalence of those abnormalities in the past decade with mild WS as the "new norm" of chicken breast meat. Originally found only in the breast (*Pectoralis major*), occurrence of WS and WB has now been observed in other cuts, including chicken filet (*Pectoralis minor*) and thigh (Petracci et al., 2019). A number of studies previous investigated the approaches, including selecting the hybrids with slower growth rate (Gratta et al., 2019), slowing the growth with manipulating the amino acids in the feed (Meloche et al., 2018; Zampiga et al., 2019; Lackner et al., 2022), and terminating the birds at the younger ages (Abreu et al., 2022) with an attempt to reduce the prevalence of the myopathies. Although the previous studies addressed the experimental reduction of the myopathies, the issue at the industrial scale does still exist.

2 An association between growth-related myopathies in broilers and occurrence of *in vivo* oxidative stress

The actual etiology of the myopathy is still under investigation. Yet, previous histological studies consistently show chronic muscle fiber damage as shown by accumulated macrophages, large-rimmed vacuoles, nuclei internalization, deposition of adipocytes, thickened endomysium and perimysium, inconsistent size of rounded myofibers, infiltration of lymphocytes and macrophages, and necrosis in the affected muscles (Kuttappan et al., 2012; Sihvo et al., 2014; Papah et al., 2017; Malila et al., 2018; Salles et al., 2019; Hosotani et al., 2020; Praud et al., 2020). The occurrence of fiber necrosis, fibrosis and adipose tissue filtration increased as the severity degree of the myopathies increased (Praud et al., 2020). The muscle fiber damage has been hypothesized as an adverse consequence when the muscle fibers were outgrown their supportive systems, particularly vascularization (Mutryn et al., 2015; Alnahhas et al., 2016; Kindlein et al., 2017; Papah et al., 2017; Sihvo et al., 2018; Lake and Abasht, 2020). In male Cobb 500 broilers, an increased intercapillary distances together with reduced ratio of capillary to muscle fibers were correlated with WB severity level (Kindlein et al., 2017). The early pathogenesis of WB was likely associated with endothelial cell dysfunction, particularly in the capillaries and venous ends of the vasculature (Abasht et al., 2021). In the breast muscle of commercial broilers, multifocal perivascular and perivenous aggregates of lipid-laden macrophages were observed at 1 week of age prior to the development of myopathic lesions at 2 weeks of age (Papah et al., 2017). Limited oxygenation (Kindlein et al., 2017) in combination with the sequellae of phlebitis and impaired venous drainage (Papah et al., 2017; Abasht et al., 2021) may lead to local accumulation of metabolic waste and reactive oxygen species (ROS), triggering muscle fiber degeneration. In addition, lipid accumulation in the affected *Pectoralis major*, resemblance to type 2 diabetes, may exert cellular stress to the cells and further suppress glycolysis and gluconeogenesis in the affected birds (Lake and Abasht, 2020). Accumulated lipids can enhance oxidative stress through the lipid peroxidation of fatty acids (Li et al., 2022). In addition, ROS can readily react with other biomolecules, particularly lipids, proteins and DNA (Min and Ahn, 2005). Malondialdehyde, a product of lipid oxidation, has been shown to be responsible for secondary carbonylation of myoglobin and myofibrillar proteins along with cross-linking of myofibrillar proteins from rabbit skeletal muscle (Wang et al., 2020; Yin et al., 2022).

Differential gene expression patterns associated with development of growth-related myopathies suggested alteration of several biological processes, including metabolisms of nutrients, programmed cell death, to muscle regeneration (Mutryn et al., 2015; Zambonelli et al., 2016; Malila et al., 2020). Among the key stress-related transcription factors, transcript abundance of hypoxia-inducible factor 1 (HIF-1), particularly alpha subunit (HIF1A), along with antioxidant enzymes, particularly superoxide dismutase isoform 2 and 3, were increased in the myopathic muscles (Malila et al., 2019; Marchesri et al., 2019; Malila et al., 2022). The findings implied molecular activities against cellular oxidative stress. However, chronic hypoxia within the affected breast muscle appeared to weaken HIF signaling and disrupt the processes of autophagy and mitophagy (Hosotani et al., 2020). In turn, such pathological condition attenuated adaptability of the muscle to hypoxia. The stress environment might trigger aberrant activity of fibro-adipogenic progenitors, resulting in fibrosis (Malila et al., 2022). Metabolic intermediates, i.e., fumarate, and malate, from tricarboxylic acid cycle, were accumulated in the affected breast muscles, suggesting the back flux of oxaloacetate converted into malate and fumarate under limited oxygenation condition (Boerboom et al., 2018). An increased conversion of L-arginine into citrulline was observed in WS breast muscle, presumably to produce nitric oxide (Boerboom et al., 2018). When the level of nitric oxide was elevated, tyrosine residue on polypeptide chains could undergo oxidation, resulting in nitrotyrosine which was associated with inflammation diseases (Cai and Yan, 2013).

3 Potential effects of consuming oxidized lipids and proteins

Although whether consumption of oxidized lipids and proteins would cause any chronic diseases in human is still inconclusive, a growing evidence demonstrated that diets containing excessive oxidative products showed potential to disturb *in vivo* cell redox status (Estévez and Luna, 2017; Estévez and Xiong, 2019; Hu et al., 2021). The chronic oxidative stress is not only responsible for the virulence and severity of the disease but also the oxidative DNA damage of the epithelial cells (Hardbower et al., 2013). Damages of DNA may interrupt transcription leading to the aberrant cellular response to the oxidative stress or the obtain of erroneous protein structure and functions (Huang and Anh, 2019). Unless the damage to DNA is repaired, it can induce long-term physiological conditions, including inflammation, atherosclerosis, aging and cancer (Huang and Anh, 2019). The intake of high-fat diets and oxidized lipids has been known to be associated with pathological conditions (Estévez and Xiong, 2019). Previous studies in animal models suggested that consumption of diet containing oxidized oils elevated the risk of cellular oxidative stress (Dalle-Donne et al., 2006). An association between 4-hydroxyhexenal, a lipid peroxidation product, and the progression of Alzheimer's disease was addressed (Bradley et al., 2012).

In contrast to oxidized lipids, the investigation regarding impacts of consuming dietary oxidized proteins have recently gained attention (Estévez and Xiong, 2019). As reviewed by Soladoye et al. (2015), Estévez and Xiong, (2019), and Domínguez et al. (2022), an accumulation of oxidized proteins and their products (e.g., heterocyclic aromatic amines or advanced-glycation end products) was linked with pathological conditions of certain diseases (e.g., Parkinson's, Alzheimer's, type II diabetes, and renal failure). Protein carbonylation, an irreversible modification associated with oxidative damage, has been widely used as a biomarker for protein oxidation (Cai and Yan, 2013). The modification occurs on multiple amino acid residues on selected protein targets, including arginine, histidine, lysine, proline, threonine and cysteine (Soladoye et al., 2015). Hence, quantity and quality of essential amino acids were reduced (Soladoye et al., 2015). Gut proteases may not recognize the target sites on the oxidized proteins, leading to reduced protein digestibility and bioavailability (Soladoye et al., 2015). Protein carbonyls may induce polymerization among the oxidized proteins or between their derivatives and other polypeptide chains. On the contrary, in severe condition, the carbonyls can also attack the peptide backbone, resulting in breakdown of the polypeptides into several carbonyl-containing peptides (Stadtman and Levine, 2003). In addition, lipid-derived protein carbonyls can promote a pro-oxidative environment in the muscle tissue. An example was the role of 4-hydroxynonenal in the formation of formation of protein adducts in heart, liver and skeletal muscle of rats (Keller et al., 2020).

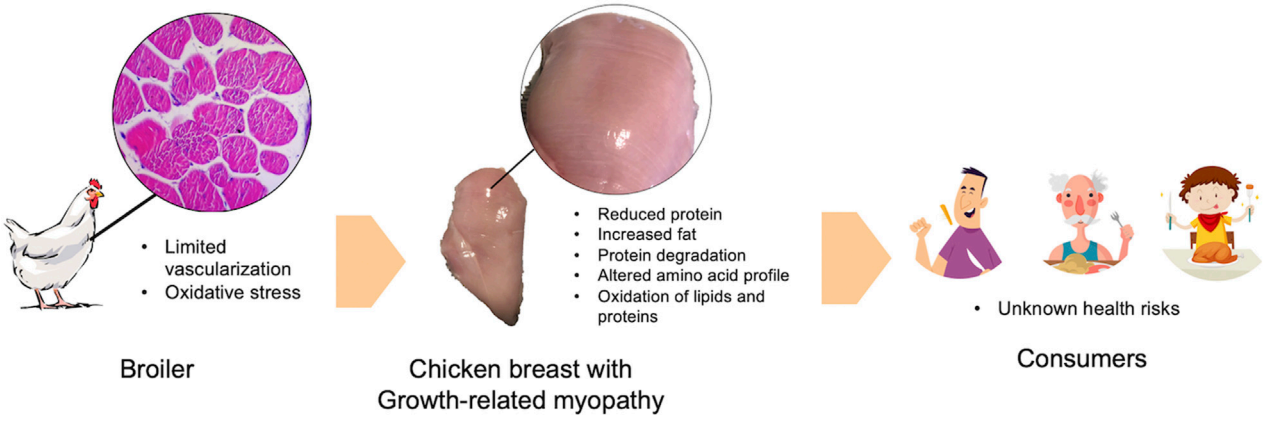

FIGURE 1
A schematic diagram depicts an association between growth-related myopathies and oxidative stress. Impacts of growth-related myopathies and the assumption of health consequence are included.

4 Health consequences of consuming growth-related myopathies in chicken breast meat: should it be concerned?

Previous studies consistently demonstrated a decreased proportion of protein and increased fat in raw chicken breast meat severely affected with WS and WB condition (Petracci et al., 2014; Soglia et al., 2016; Baldi et al., 2018; Malila et al., 2018; Soglia et al., 2018; Adabi and Suncu, 2019; Mudalal, 2019; Carvalho et al., 2021). The deviation of chemical composition can shift energy distribution of the affected chicken breast meat towards energy contribution from fat (Petracci et al., 2014). The change was more pronounced as the severity elevated. Profile of essential amino acids were also altered in the myopathic chicken meat (Adabi and Suncu, 2019; Soglia et al., 2019; Dalle Zotte et al., 2020; Thanatsang et al., 2020). Proteins might undergo degradation into free amino acids in the affected breast (Soglia et al., 2019) which might be potentially lost with dripping and purging fluids during storage and cooking, respectively. In addition, antemortem oxidative stress condition would result in increased ROS and free radicals promoting oxidation of lipids and proteins in food (Estévez and Xiong, 2019). Given that growth-related myopathies were associated with oxidative stress (Thanatsang et al., 2020; Li et al., 2022), one may assume that when chicken meat with high severity of WS and WB abnormalities was consumed, consumers might increase risk of an expose to oxidized lipids and proteins (Figure 1). However, no investigation has been conducted on such aspect.

So far, because the issue of growth-related myopathies has been emerged for about a decade, the published research has been emphasized on the impacts on technological properties and underlying their etiology with the best attempt to establish effective solutions. Apart from those aforementioned altered macronutrients, other health consequences due to consumption of those growth-related myopathies have not been investigated. Whether such altered macronutrients would significantly exert health impacts remains unclear. It is worth noting that in most countries, the severe WB are rejected and only focal mild cases are utilized as human food. Additionally, muscle damages were widely detected on the superficial part of the affected breast and the lesions were less pronounced at the deeper regions (Sihvo et al., 2014; Soglia et al., 2017; Baldi et al., 2018); hence, it is reasonable to hypothesize that consumption of the whole breast meat may less likely exert any adverse health consequences. However, media and internet began to criticize chicken breasts with growth-related myopathies. Such information may someday gain wide attention and eventually exert any negative perception and fear towards chicken meat and poultry industry. Therefore, it is essential that scientific community begins to gather the reliable scientific evidence regarding the influence of growth-related myopathies on protein quality, protein bioavailability and health impact particularly among susceptible consumers (e.g., elderlies and patients) at a long-term exposure.

Author contributions

The author confirms being the sole contributor of this work and has approved it for publication.

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Conflict of interest

The author declares that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

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Relationship of knob morphometric analysis with production performance and meat quality in Yangzhou goose (*Anser cygnoides*)

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The development of the knob in Chinese geese (*Anser cygnodies*) is an outcome of extensive and prolonged selection and breeding. The knob serves not only as a visual indicator of sexual maturity in geese but also holds significance as a crucial packaging trait that attracts attention of consumers attentions, who tend to distinctly prefer geese with larger knobs. Consequently, investigating the formation of the knob holds practical value, as it will help achieving external traits aligned with consumers' preferences. To understand the relationship between knob size, production efficiency, and meat quality in Yangzhou geese, we examined histological and anatomical characteristics in 500- and 120-day-old geese with large and small knobs. Notably, knob size had a pronounced impact on key anatomical and structural parameters, such as chest depth, leg muscle water-binding capacity, and insoluble collagen composition in Yangzhou geese ($p < 0.05$). In addition, we measured testosterone and estrogen levels in male and female geese, respectively, as well as growth hormone, and found that birds of both sexes with a large knob had higher sex and growth hormone levels in the body. This study established a fundamental theoretical basis for advancing the enhancement of goose knob traits.

KEYWORDS

knob, meat quality, packaging traits, production efficiency, Yangzhou geese

Introduction

China is the largest goose-producer in the world, with annual production reaching 4.29 million tons in 2021 (FAO-STAT., 2023). Although goose meat is mentioned as the top of the World Health Organization's "Healthy Food List," increasing goose consumption remains a persistent challenge (Akhtar et al., 2021).

In geese, the knob located posterior to the beak serves as an indicator of sexual maturity (Hudson et al., 2017) but it is also a vital packaging trait that captures consumers' attention (Gumułka and Rozenboim, 2013; Ji et al., 2021). The presence and appearance of the knob, therefore, is a distinctive factor in goose sales that enables consumers to readily discern the gender, age, and other attributes of the birds.

The various resources are mainly distributed in Europe and Asia, and can be roughly classified into European geese and Chinese geese (Moniem et al., 2020). Compared with European geese, Chinese geese have a slender neck and grow into adults with a fleshy bulge at the top of the bill, known as a knob, which is a distinguishing feature (Zhang et al., 2023). On the whole, knobs tend to be larger in males than in females, and they are consistently larger in adults than in juveniles (Lee, 2021). Furthermore, at the same age, a larger knob indicates greater closeness to maturity and better meat quality (Weng et al., 2021; Razmaitė et al., 2022). Hence, investigating the relationship between knob formation and production performance is of practical significance for harnessing and maximizing the potential of this trait, ultimately contributing to the growth and promotion of goose meat consumption (Zhang et al., 2014; Liu et al., 2019; Ni et al., 2022). The dimensions of the knob are predominantly determined by the breed, age, and sex of geese, as well as by various other factors (Mawdesley-Thomas et al., 1967; Wright et al., 2012). Nonetheless, the knob size significantly influences production performance and meat quality metrics, an aspect that remains unexplored in the existing literature.

In this study, we characterized 120-day-old Yangzhou geese and performed an in-depth analysis of their histological and anatomical properties depending on the knob size. The primary objective was to elucidate the relationship between the knob size and the production performance and meat quality of Yangzhou geese. The ultimate aim was to foster the refinement, application, and preservation of the desired goose knob traits. These efforts have successfully established a robust theoretical foundation for driving future advancements in goose breeding (Yang et al., 2005).

Materials and methods

Ethics statement

All animal experiments were approved by the Animal Care and Use Committee of the Yangzhou University (approval number: 132-2020). The study was conducted in accordance with the local legislation and institutional requirements.

Animals and sample collection

The geese were sourced from the Yangzhou Tiange Goose Industry Development Co., Ltd. (Yangzhou, Jiangsu, China). This experiment was performed using standard group breeding, and we then selected our target group from the standard group. The space of each goose house was maintained at 40 × 20 m, and the number of geese in each pen was maintained at approximately 500. The goose houses were semi-enclosed and received natural light during the day and artificial light at night from 19:00 to 21:00. In addition, each goose house was equipped with a pool designed to meet the water-playing habits of the geese and ensure animal welfare to a certain extent. A staff member regularly changed the water in the pool to ensure the hygiene and cleanliness of the water and reduce the occurrence of diseases. Usually, a combination of concentrated roughage and green fodder is used to supply the goose house, allowing the geese to eat freely. Concentrated roughage included standardized concentrate feed, corn, rice husks, and various mineral and vitamin preparations, while green feed was grown by the goose factory itself, harvested on time, and then fed in the goose houses for the geese to eat freely.

Once they met the market standard at 120 days, 15 geese with large knobs and 15 with small knobs, comprising a total of 30 individuals (n = 15), were selected for Experiment One. This subset of geese was utilized for the investigation of the correlation between knob size variations and performance, bone structure, histological characteristics, and meat quality. In order to determine knob and beak size, we established a set of methods for the determination of knob and beak sizes of mute swans in China (Horrocks et al., 2009), which has been published (Ji et al., 2021). The length, height, and width of the knob were measured in millimeters. The knob length (Kl) was measured between the most anterior part of the knob and the phenotypic boundary of the knob. The knob width (Kw) was measured at the widest part of the knob. The knob height (Kh) was measured between the fronto-nasal junction and the most dorsal part of the knob. Knob size was assessed using the product of knob length, width, and height. The criteria for classifying knob size for the 120-day-old geese were defined as large knobs having a volume (V) > 250,000 mm^3 and small knobs having V < 15,000 mm^3. For the 500-day-old geese, large knobs were defined as V > 40,000 mm^3 and small knobs as V < 25,000 mm^3. Before slaughter, the geese underwent a 12 h fasting period, after which they were euthanized via neck bleeding. After slaughtering, the head of each goose was removed. Subsequently, follicle-free skin derivatives from the knob (or forehead) of each goose's head was extracted and fixed in 4% paraformaldehyde. The remaining portion was sealed in plastic, the appropriate label was applied, and it was stored in a −20°C refrigerator. Afterwards, the breast and leg muscles were meticulously dissected using a scalpel. The entire leg and chest muscles were isolated separately. A standard sample was chosen for hematoxylin and eosin (HE) tissue sectioning, and the remaining meat samples were sealed in bags and stored undisturbed at 4°C for 24 h to assess meat quality. The 24-h resting period is used to neutralize the meat's acidity. Following the completion of correlation testing, individual samples undergo an immediate, secure handling process (Baéza et al., 2022). Experiment Two: selected 50 large-knob female geese and 50 small-knob female geese, and paired each group with 10 male geese with typical knob size. They were raised under identical feeding conditions until they reached 300 days of age, at which point we initiated measurements. These relevant performance evaluations continued until they reached 500 days of age. The primary objective of this study was to investigate the relationship between knob size and egg-production performance, as well as to assess the impact of knob size on hormone levels within the body.

Blood collection

Before commencing the sampling work, we first conducted blood collection and weighing. The geese were restrained in the supine position, their wings were spread, and the inferior wing vein was exposed, wiped, and disinfected with alcohol, whereupon 1–2 mL of blood was collected into an anticoagulant vacuum blood collection tube and allowed to stand on ice for 30 min. Then, the tubes were centrifuged at 3,000 rpm for 15 min, and the upper layer of yellow transparent liquid was collected and stored at −20°C for use (Mewis et al., 2014).

Measurements of body weight and size in geese with large and small knobs

The geese were weighed before slaughter. Body size measurements, specifically body oblique length, chest width, chest depth, sternal length, back width, tibial length, tibial circumference, semi-diving length, and neck length, were performed according to NY/T823-2004.

Determination of knob histological index

Knob morphological traits were determined using HE staining (Ji et al., 2021). The skin samples were fixed in 4% paraformaldehyde for 24 h. The samples were placed in an embedding cassette and rinsed with running water for 30 min to remove the fixative from the tissue, and the samples were dehydrated using a graded ethanol series. The paraffin blocks were cut (Leica Biosystems, Wetzlar, Germany) along the horizontal axis into 3 μm thick sections and stained with HE according to standard protocols. The samples were scanned using a NanoZoomer scanner (Hamamatsu, Sydney, Australia). The fiber diameter and cross-sectional area were calculated using an image analysis system (Image-Pro Plus, Media Cybernetics, Rockville, MD, United States) (Zhang et al., 2023).

Determination of meat quality in geese with large and small knobs

Tenderness determination

Tenderness was assessed using shear-force measurements. Based on previously selected fresh samples, portions of the chest and leg muscles were selected and sliced into elongated meat strips measuring 1.0 cm in width and 0.5 cm in thickness, aligned with the muscle fiber direction, and cleaned of any fascia or fat. Using a C-LM3 muscle tenderness instrument, meat samples were sliced along the vertical muscle fibers, and each sample was subjected to three cuts for shear-force measurement. The resulting shear force values were averaged to determine the tenderness (Geldenhuys, et al., 2014).

Determination of expressible water

The expressible water was determined as follows, A total of 1 g of tissue sample (m1) was dissected from the breast and leg muscls, using surgical scissors to trim them. The meat samples were sandwiched between 16 layers of filter paper, with an additional hard plastic backing plate placed on the outermost filter paper layer. Using a steel ring, a pressure of 35 kg was applied to the dilatometer platform for 5 min. After the application of pressure, the weight of the compressed meat samples (m2) was promptly recorded. The expressible water was calculated using the following formula: expressible water (%) = (m1−m2) × 100%/m1.

Meat color determination

Meat color was assessed at 24 h post-slaughter using a CR-400 colorimeter to measure the surface attributes of chest and leg muscles. In particular, values for redness (a*), yellowness (b*), and brightness (L*) were recorded, L* value was unaffected by bloom time; hue angle stabilized after 5 min, a* and b* values after 10 min and chroma after 20 min (Brewer et al., 2001; Weng et al., 2021).

pH determination

The procedure of pH measurement involved creating three incisions at distinct points along the cross-sectional surface of the chest and leg muscles of the carcass using a scalpel. Then, the electrode of a pen-type pH meter (specifically, a waterproof pH Spear test pen) was inserted to a penetration depth of 1 cm into the muscle tissue. The pH values of both breast and leg muscles were read three times immediately after slaughter, and the average value was calculated.

Determination of protein, fat, insoluble collagen, and water contents

The composition of thoracic and leg muscle samples was measured three times using a *Food Scan* rapid analyzer as described previously (Soren and Biswas, 2020). It primarily employs the near-infrared measurement principle, utilizing near-infrared spectroscopy technology to accurately quantify the content of the measured substance in meat products. This process avoids sample destruction or chemical treatment by analyzing signals within the spectral data associated with the absorption characteristics of the measured substance. Consequently, this method proves highly valuable for enhancing meat quality control and quality testing.

Egg production and fertilization rate of geese with large and small knobs

This article selected 50 female geese with varying knob sizes and paired them with 10 male geese of typical knob size. Our objective was to allow them to naturally mate, facilitating the production of fertilized eggs for our later experiments and observations. They were raised under identical feeding conditions until they reached 300 days of age, at which point we initiated measurements. These relevant performance evaluations continued until they reached 500 days of age.

Determination of hormone levels in geese with large and small knobs

To estimate hormone levels, blood was centrifuged, and serum was collected. The main hormones detected were the following: female geese, estrogen and growth hormone (GH) and male geese, androgen and GH levels by using commercially available enzymelinked immunosorbent assay (ELISA) kits (Wuhan EIAab Science Co., Ltd., Wuhan, Hubei, China). Operations were carried out according to the manufacturers' protocols. No cross-reaction with other structural analogues, and all the intra-assay and inter-assay coefficients of variation (CV) for each hormonal assay were less than 10% and 15%. Finally, the absorbance of 450 nm was read for each well by a microplate reader, Model 680 (BioRad, Hercules, CA, United States).

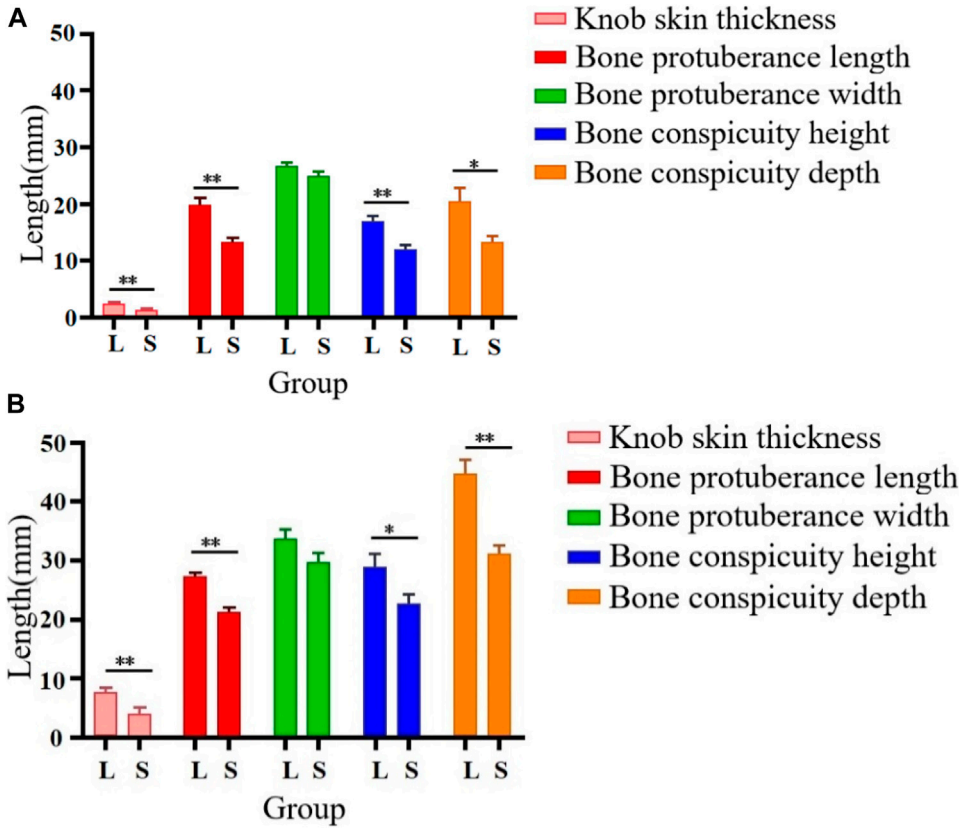

FIGURE 1
Knob skin thickness and bone characteristics in Yangzhou geese with large and small knobs. Heads of geese were collected to measure the knob skin thickness as well as the length, width, height, and depth of the bone protrusion. **(A)** 120 days of age. **(B)** 500 days of age. D, large-knob group; X, small-knob group.

Statistics and analysis

The test data were initially processed using Excel, and Student's t-test was used to analyze the significance of differences between knob size traits using SPSS 25.0. The results were expressed as the mean ± standard deviation.

Results

Knob skin thickness and bone protrusion parameters in geese with large and small knobs

As illustrated in Figure 1, we examined 120- and 500-day-old Yangzhou geese with large and small knobs. The criteria for classifying knob sizes were as follows: for the 120-day-old geese, large knobs were defined as volume (V) > 250,000 mm^3 and small knobs as V < 15,000 mm^3. For the 500-day-old geese, large knobs were defined as V > 40,000 mm^3 and small knobs as V < 25,000 mm^3. A comparative analysis was conducted on parameters such as the thickness of the overlying knob skin and the length, width, height, and depth of the bone protrusion. Bone protrusion length, height, and depth were significantly different in the groups of geese with large and small knobs ($p < 0.05$ in each case), whereas no significant knob-group effect was observed on the width of the bone protrusion ($p > 0.05$). Moreover, a noteworthy correlation was observed between knob size and bone protrusion size ($p < 0.05$), emphasizing that the dimensions of the bone protrusion hold a decisive role in determining knob size (Table 1).

Histological differences between large and small knobs

As illustrated in Figures 2, 3, there were some histological differences between the large and small knobs in Yangzhou geese. Specifically, among the 120-day-old geese, histological differences were predominantly observed within the epidermal layer. In the case of large knobs, both the horny and spinous cell layers were remarkably expanded compared to the dimensions of these layers in small knobs (Figures 2A1, B1, 3A; $p < 0.01$). Moreover, the epidermal layer in geese with large knobs was significantly more extensive than that in geese with small knobs (Figures 2A1, B1, 3A; $p < 0.05$). In 500-day-old geese, similar histological changes were detected. Large knobs were characterized by significantly augmented dimensions in the horny, acanthocyte, and reticular layers compared to those in small knobs (Figures 2A2, B2, 3B; $p < 0.01$).

TABLE 1 Correlation coefficients between knob and bone protuberance dimensions.

Parameter	Age	Knob length	Knob width	Knob height	Tumor skin thickness	Knob volume
Bone protuberance length	D120	0.964**	0.579	0.708*	0.835**	0.882**
	D500	0.844**	0.593	0.818**	0.897**	0.912**
Bone protuberance width	D120	0.438	0.35	0.537	0.179	0.543
	D500	0.466	0.955**	0.215	0.492	0.666*
Bone conspicuity height	D120	0.527	0.315	0.767**	0.739*	0.688*
	D500	0.300	0.145	0.833**	0.728*	0.456
Bone conspicuity depth	D120	0.548	0.503	0.794**	0.433	0.745*
	D500	0.706*	0.702*	0.765**	0.867**	0.861**
Bone conspicuity volume	D120	0.826**	0.519	0.809**	0.836**	0.873**
	D500	0.678*	0.623	0.852**	0.913**	0.842**

*$p < 0.05$, and **$p < 0.01$.

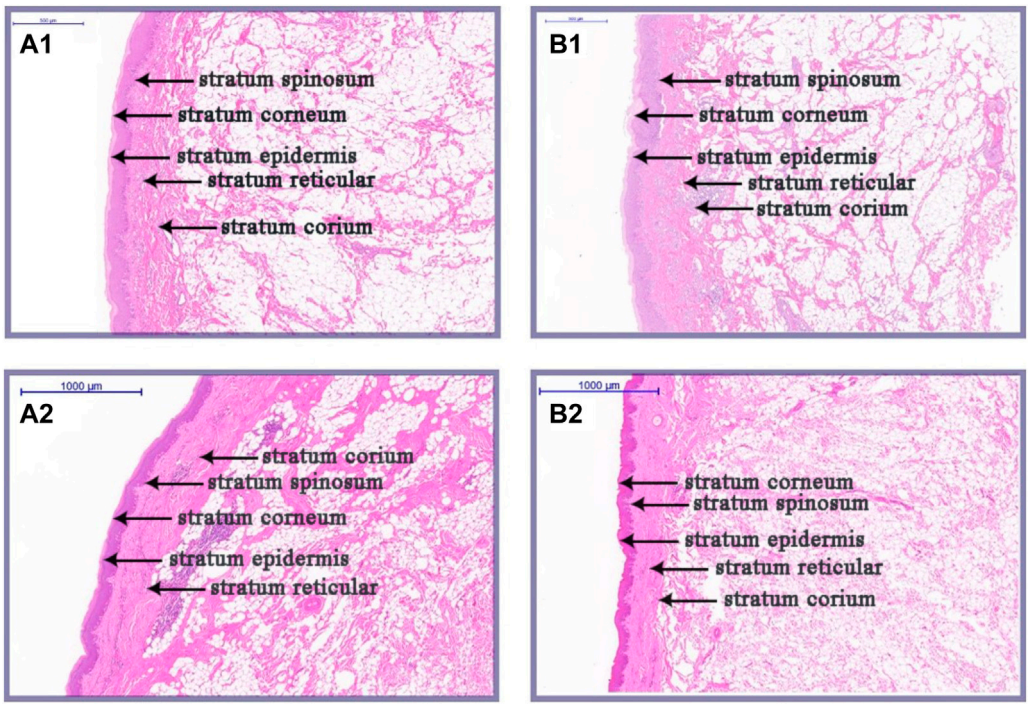

FIGURE 2
Histological images of large and small knobs in 120- and 500-day-old Yangzhou geese. Skin tissues of large and small knobs were collected from 120- and 500-day-old geese, fixed, embedded in paraffin and sectioned for staining with hematoxylin/eosin (magnification ×5). **(A1)** Large-knob skin tissue, 120 days. **(B)** Small-knob skin tissue, 120 days. **(A2)** Large-knob skin tissue, 500 days. **(B2)** Small-knob skin tissue, 500 days.

Anatomical characteristics and production performance in geese with large and small knobs

As indicated in Table 2, a comprehensive statistical analysis was conducted on the body weight, body size, and post-slaughter measurements of 120-day-old Yangzhou geese with large and small knobs. Notably, among the examined parameters, the chest depth of geese with small knobs was significantly larger than that of geese with large knobs ($p < 0.05$), whereas no significant differences were observed in other body dimensions (Table 3).

Meat quality indices of geese with small or large knobs

Table 4, 5 presents the results of the Meat quality indices of the geese with small or large knobs. Notably, we found that the leg

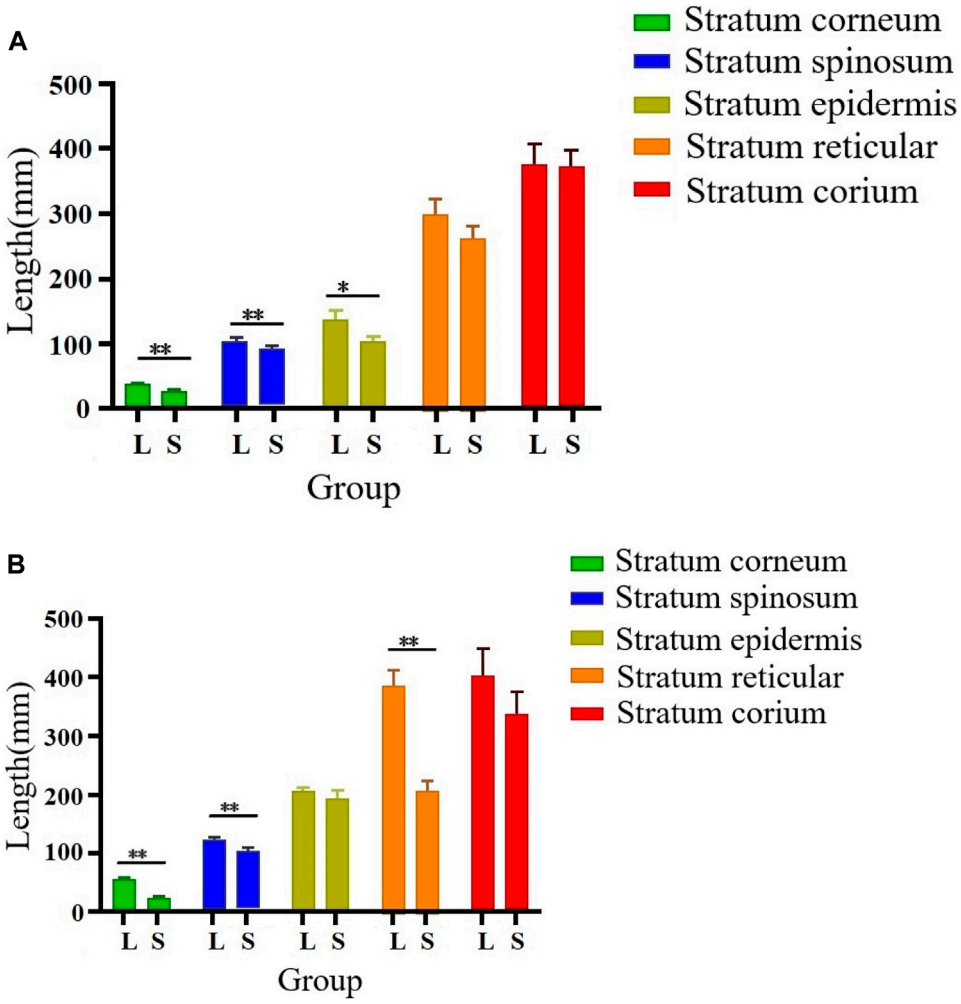

FIGURE 3
Morphometric comparison of large and small knobs in 120- and 500-day-old Yangzhou geese. Each layer in the knob skin tissue was carefully observed, and the length of each layer was measured. X-axis represents different layers; Y-axis represents layer length (in mm). **(A)** Histological differences in 120-day-old geese. **(B)** Histological differences in 500-day-old geese. L, large-knob group; S, small-knob group. Statistical significance of differences is illustrated as follows: $^{**}p < 0.01$, and $^{*}p < 0.05$.

TABLE 2 Production performance and carcass quality.

Parameter	Small knob	Large knob
Body weight, kg	4.42 ± 0.10	4.56 ± 0.11
Pec heavy, g	374.52 ± 17.03	407.35 ± 16.19
Leg muscle weight, g	350.14 ± 21.65	410.15 ± 22.60
Body slope length, cm	29.20 ± 1.31	29.23 ± 1.06
Chest width, cm	16.50 ± 0.22	16.00 ± 1.02
Chest depth, cm	14.20 ± 0.26[a]	12.45 ± 0.74[b]

muscles of geese with large knobs had a significantly higher hydraulic value compared to that of the leg muscles of geese with small knobs ($p < 0.05$). Similarly, the insoluble insoluble collagen content of the leg muscles in geese with large knobs was significantly higher than that in the leg muscles of geese with small knobs ($p < 0.05$). However, no other parameters were significantly different in geese with large and small knobs.

Egg production performance in geese with large and small knobs

A comparison between 300-day-old geese with large and small knobs showed interesting egg production patterns (Figure 4A). From October to January, the large-knob geese showed lower monthly egg production, whereas from February to April, egg production was higher than that in the small-knob geese. Notably, the small-knob geese began egg-laying earlier and achieved peak egg production in January. In contrast, the large-knob geese began laying eggs later and reached peak egg production in February. As illustrated in Figure 4B, of the 650 eggs collected from large-knob geese, 564 were fertilized, indicating a fertilization rate of 86.77%. In the small-knob group, 528 of 650 eggs were

TABLE 3 Effects of different knob sizes on goose performance.

Character	Small knob	Large knob
Sternum length, cm	17.62 ± 0.43	18.03 ± 0.94
Back width, cm	6.40 ± 0.43	6.70 ± 0.99
Tibial length, cm	9.36 ± 0.19	9.25 ± 0.32
Tibial circumference, cm	5.10 ± 0.19	5.20 ± 0.12
Half-submerged length, cm	81.20 ± 1.90	81.75 ± 1.30
Neck length, cm	29.40 ± 0.91	29.88 ± 1.42

Values in the same column marked by different uppercase letters are significantly different ($p < 0.05$). $n = 30$.

TABLE 4 Meat quality indices of the geese with small or large knobs.

Parameter		Flesh			PH	Shear force	Expressible water
		L*	a*	b*			
Small knob	pectoralis	39.35 ± 2.42	15.49 ± 2.14	5.50 ± 1.55	6.26 ± 0.05	6.26 ± 8.26	0.31 ± 0.06[a]
	leg muscles	42.23 ± 5.24	17.25 ± 2.16	8.77 ± 1.21	6.13 ± 0.07	46.50 ± 9.92	0.21 ± 0.02[b]
Large knob	pectoralis	39.14 ± 0.81	13.74 ± 0.34	4.67 ± 0.6	6.12 ± 0.15	6.12 ± 10.61	0.33 ± 0.02[a]
	leg muscles	43.21 ± 5.62	14.14 ± 3.58	5.04 ± 1.45	6.13 ± 0.16	38.15 ± 4.53	0.32 ± 0.01[a]

TABLE 5 Meat quality indices of the geese with small or large knobs.

Character	Small knob		Large knob	
	Pectoralis	Leg muscles	Pectoralis	Leg muscles
Moisture	69.46 ± 0.26	71.41 ± 0.50	69.65 ± 0.47	71.20 ± 0.59
Protein	22.15 ± 0.22	21.32 ± 0.16	22.58 ± 0.38	21.51 ± 0.26
Fat	22.15 ± 0.34	4.15 ± 0.29	22.58 ± 0.52	3.36 ± 0.72
Insoluble collagen	0.22 ± 0.05	0.32 ± 0.05[b]	0.41 ± 0.07	0.50 ± 0.04[a]

a*, redness; b*, yellowness; L*, brightness. There is no significant difference between values with the same letters in the same column ($p > 0.05$), and the difference between values with different letters in the same column is significant ($p < 0.05$).

fertilized, with a fertilization rate of 81.23%. Although hens with large knobs showed nominally higher fertilization rates than those in small-knob hens, the observed difference did not reach the set level of statistical significance ($p > 0.05$).

In vivo hormone concentrations in geese with large and small knobs

Female geese with small knobs had significantly lower estrogen concentrations than their larger knob counterparts (Figure 5A; $p < 0.05$). Similarly, testosterone concentrations were significantly lower in male geese with small knobs than in those with large knobs (Figure 5B; $p < 0.05$). Growth hormone levels were relatively consistent between male and female geese, with females having lower levels than males. Notably, geese with small knobs had significantly lower growth hormone levels compared to those in geese with large knobs (Figure 5C; $p < 0.05$).

Discussion

In this study, we aimed to uncover and analyze the anatomical and histological differences between geese with large and small knobs at the ages of 120 and 500 days. In particular, we explored differences in meat quality parameters, body dimensions, egg-laying performance, hormone levels, and knob dimensions in birds with large and small knobs (Djermanovic et al., 2021). We observed that knob thickness as well as bone protrusion length, height, and depth were significantly different between large and small knobs. The histological discrepancies observed in Yangzhou geese with varying knob sizes underscore noteworthy distinctions. Irrespective of age (either 120 or 500 days), both the horny and spinous cell layers of large knobs were significantly thicker than those of small knobs ($p < 0.05$). Remarkably, in the case of 500-day-old geese, the reticular layer thickness in large knobs was significantly thicker than that in small knobs ($p < 0.05$). This layer predominantly consists of robust insoluble collagen and elastic fiber bundles that could conceivably

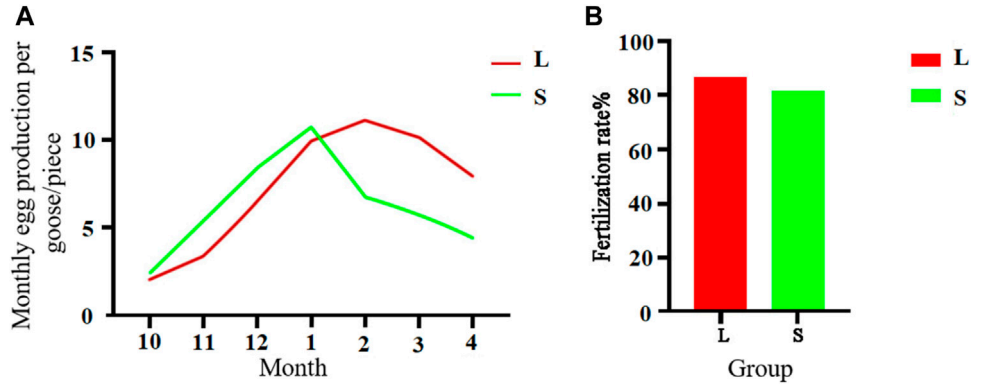

FIGURE 4
Monthly egg production **(A)** and egg fertilization rates **(B)** of female geese with different knob sizes. Monthly egg production in October–April. Overall egg fertilization rate. L, large-knob group; S, small-knob group.

FIGURE 5
Hormone concentrations in 500-day-old geese with large and small knobs. Yangzhou geese at the age of 500 days were divided into groups of large-knob males, small-knob males, large-knob females, and small-knob females. Serum was collected, and estrogen (E2) and growth hormone (GH) levels in female geese and testosterone (T) and GH levels in male geese were measured. **(A)** Serum E2 levels in 300-day-old female geese. **(B)** Serum T levels in 300-day old male geese. **(C)** Serum GH levels in 300-day-old geese plotted separately for males and females. Statistical significance of differences is illustrated as follows: $^{**}p < 0.01$, and $^{*}p < 0.05$.

influence the resilience of the skin overlying the knob. The insoluble collagen fibers within the reticular layer are interconnected with the subcutaneous tissues, suggesting that the thickness of this layer may also affect the thickness of the subcutaneous tissue.

Goose meat has exceptional nutritional value, with a rich protein content and minimal fat level. Consumers tend to be highly sensitive to meat quality while being less influenced by price fluctuations. Therefore, carcass quality is a pivotal criterion in goose breeding. In this study, we showed that at 120 days of age, male geese with larger knobs had significantly smaller breast depths than their small knob counterparts ($p < 0.05$). In this experiment, this paper used near-infrared FOODSCAN equipment for measurement. Near-infrared spectroscopy technology can quantitatively determine the insoluble collagen content in meat products without destroying the sample or chemically processing it by analyzing signals in the spectral data that are related to the absorption properties of insoluble collagen. Insoluble collagen plays an essential role in the connective tissue, serving as an irreplaceable factor in supporting the functions of both muscles and bones (Weston et al., 2002). The moisture content within the muscle tissue directly affects its succulence and palatability. Rearing geese under full grazing conditions enhances the moisture content of goose meat (Liu et al., 2011; Song et al., 2017). The findings of this study revealed that the leg muscles of geese with large knobs had higher moisture content, suggesting that selecting geese with larger knobs could indirectly foster the development of geese with heightened meat moisture content (Weng et al., 2021).

Conclusion

This study delved into the histological and anatomical characteristics of Yangzhou geese at two different ages, 500 days and 120 days, encompassing both those with large and small knobs. Our findings unveiled a crucial connection between bone protrusion size and knob size, indicating that the former can significantly influence the latter. Furthermore, Geese possessing larger knobs exhibited traits associated with precocial puberty.

Data availability statement

The original contributions presented in the study are included in the article/supplementary material, further inquiries can be directed to the corresponding author.

Ethics statement

All animal experiments were approved by the Animal Care and Use Committee of the Yangzhou University (approval number: 132-2020). The study was conducted in accordance with the local legislation and institutional requirements.

Author contributions

YaZ: Writing–original draft, Writing–review and editing. XX: Writing–original draft, Writing–review and editing. WJ: Data curation, Writing–review and editing. SQ: Writing–review and editing. QB: Writing–review and editing. ZC: Writing–review and editing. WL: Writing–review and editing. YoZ: Writing–review and editing. YuZ: Writing–review and editing. QX: Writing–review and editing. GC: Writing–review and editing.

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Conflict of interest

Author YoZ was employed by Yangzhou Tiangge Goose Industry Development Company Limited.

The remaining authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

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Expression of miRNAs in turkey muscle satellite cells and differential response to thermal challenge

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Thermal stress alters the transcriptome and subsequent tissue physiology of poultry; thus, it can negatively impact poultry production through reduced meat quality, egg production, and health and wellbeing. The modulation of gene expression is critical to embryonic development and cell proliferation, and growing evidence suggests the role of non-coding RNAs (RNA:RNA interaction) in response to thermal stress in animals. MicroRNAs (miRNAs) comprise a class of small regulatory RNAs that modulate gene expression through posttranscriptional interactions and regulate mRNAs, potentially altering numerous cellular processes. This study was designed to identify and characterize the differential expression of miRNAs in satellite cells (SCs) from the turkey *pectoralis major* muscle and predict important miRNA:mRNA interactions in these developing SCs under a thermal challenge. Small RNA sequencing was performed on RNA libraries prepared from SCs cultured from 1-week-old male Nicholas commercial turkeys (NCTs) and non-selected Randombred Control Line 2 turkeys during proliferation and differentiation at the control temperature (38°C) or under a thermal challenge (33°C or 43°C). A total of 353 miRNAs (161 known and 192 novel) were detected across the sequenced libraries. Expression analysis found fewer differentially expressed miRNAs in the SCs of NCT birds, suggesting that the miRNA response to heat stress has been altered in birds selected for their modern commercial growth traits. Differentially expressed miRNAs, including those with described roles in muscle development, were detected both among temperature treatments and between genetic lines. A prominent differential expression of miR-206 was found in proliferating turkey SCs with a significant response to thermal challenges in both lines. In differentiating SCs, isoforms of miR-1 had significant differential responses, with the expression of miR-206 being mainly affected only by cold treatment. Target gene predictions and Gene Ontology analysis suggest that the differential expression of miRNAs during thermal stress could significantly affect cellular proliferation and differentiation.

KEYWORDS

microRNA, satellite cell, skeletal muscle, growth selection, turkey

Introduction

Thermal stress can have a negative impact on poultry production. Both heat and cold stress have been shown to reduce meat quality, egg production, and wellbeing, including the overall health and quality of life in production birds (Henrikson et al., 2018; Barnes et al., 2019; Patael et al., 2019; Ouchi et al., 2021). Previous studies have shown that thermal challenges can systemically create changes in live birds at the transcriptomic and physiological levels, both in specific tissues, including the important food quality tissues and muscles (Reed et al., 2017a; Reed et al., 2017b; Al-Zghoul et al., 2019; Barnes et al., 2019; Xu et al., 2021a; Reed et al., 2022a; Reed et al., 2022b). Some of these changes are tissue-specific shifts in, for example, lipid synthesis and degradation pathways and the upregulation of protein degradation mRNAs and other stress response pathways (Barnes et al., 2019; Xu et al., 2021b). Comparisons of muscles from diverse genetic lines of poultry, specifically breast muscle (*pectoralis major*) stem cells (satellite cells, SCs), have shown differences in their response to thermal challenges (Wilson et al., 1975; Li et al., 2018; Xu et al., 2021a; Xu et al., 2021b). As self-renewing mesenchymal cells, SCs enable muscle hypertrophy, maintenance, and damage repair. Avian SCs are highly active in the early post-hatch period (Halevy et al., 2000; Mozdziak et al., 2002), and their activity can be altered by environmental stimuli with potential long-lasting effects on skeletal muscle growth (Piestun et al., 2013; Loyau et al., 2014). A better understanding of such responses could allow for targeted selection in breeding for creating more resilient birds.

Growing evidence suggests the role of non-coding RNAs, such as microRNAs (miRNAs), in the regulation of muscle growth and development and their response to thermal stress in animals (Andreote et al., 2014; Fu et al., 2018; Nawab et al., 2018; Sengar et al., 2018; Lang et al., 2019; Raza et al., 2021). MicroRNAs are 18–25 nt single-stranded RNAs that are thought to function primarily in posttranscriptional gene silencing by base pairing with target mRNAs, leading to destabilization, mRNA cleavage, or translational repression (Saliminejad et al., 2019). Gene silencing mediated by miRNAs plays an important role in animal development and disease (Kloosterman and Plasterk, 2006), with tissue-specific expressions being common in vertebrate development (Wienholds et al., 2005; Ason et al., 2006). Several studies have examined miRNA involvement in the skeletal muscle of poultry (Li et al., 2011; Andreote et al., 2014; Harding and Velleman, 2016; Velleman and Harding, 2017; Jebessa et al., 2018). Studies in chicken have shown that some miRNAs that are commonly differentially expressed in human muscle disorders are also differentially expressed in chicken muscle-development disorders (Shu et al., 2021). Other studies on chicken breast muscles have shown that miRNAs appear to be important during muscle development and the deposition of intramuscular fat, both of which can impact the final meat quality (Fu et al., 2018; Liu et al., 2021).

Little is known about miRNA expression and function in turkeys. A previous work by our group examined the role of three miRNAs (miR-16, miR-24, and miR-128) in the expression of genes essential to satellite cell function in turkeys. The inhibition of these miRNAs differentially affected the expression of syndecan-4, glypican-1, and myogenic regulatory factors, myogenic differentiation 1 (*myoD*) and myogenin (*MYOG*) (Harding and Velleman, 2016). Two of the miRNAs (miR-24 and miR-128) also played a role in myogenic satellite cell migration (Velleman and Harding, 2017). Further investigation of the miRNA expression and their functional interactions with mRNAs is needed to create a more complete picture of muscle development in production turkeys, particularly in regards to thermal stress. A critical initial step in identifying miRNA:mRNA target interactions is through miRNA characterization and computational prediction.

We have previously observed statistically significant differences in the gene expression (mRNA) of turkey *p. major* muscle SCs between growth-selected and non-selected birds and in response to thermal challenges (Reed et al., 2017a; Reed et al., 2017b; Reed et al., 2022a; Reed et al., 2022b). The current study was designed to identify miRNAs expressed in *p. major* muscle SCs, to identify promising candidates for further investigation, to characterize the differential expression of miRNAs, and to predict important miRNA:mRNA interactions in developing turkey skeletal muscle SCs. The experimental design of this study is novel, showing that the use of muscle satellite cells allows the delineation of their contribution independently from other cell types in the muscle tissue, whereby these mechanisms can be more clearly linked to satellite cell function. We hypothesized that the expression of miRNAs in turkey muscle SCs would be significantly altered by thermal challenges and would vary in cells from commercial growth-selected birds compared to non-selected birds.

Materials and methods

RNA for this study was obtained from cultured SCs previously isolated from the *p. major* muscles of 1-week-old male Nicholas commercial turkeys (NCTs) and Randombred Control Line 2 (RBC2, representing commercial turkeys of 1966) turkeys. RBC2 turkeys were initiated in 1966 and maintained at the Poultry Research Center of The Ohio State University, Wooster, OH, without the conscious selection of any trait and were used as an important control in studies of select lines (Nestor et al., 1969). NCTs are modern meat-type turkeys obtained from Nicholas turkeys (Aviagen Group, Lewisburg, WV).

Pooled turkey SCs were cultured as described by Reed et al. (2017a) and Reed et al. (2022a). In brief, the SCs from both lines were plated in 0.1% gelatin (Sigma-Aldrich, St. Louis, MO)-coated 24-well plates (Greiner Bio-One, Monroe, NC) with 15,000 cells per well in Dulbecco's Modified Eagle's Medium (DMEM, Sigma-Aldrich) plating medium containing 10% chicken serum (Gemini Bio-Products, West Sacramento, CA), 5% horse serum (Gemini Bio-Products), 1% antibiotics–antimycotics (Gemini Bio-Products), and 0.1% gentamicin (Gemini Bio-Products). Satellite cells were incubated in a 95% air/5% CO_2 incubator (Thermo Fisher Scientific, Waltham, MA) at 38°C for 24 h. After 24 h of attachment, the plating medium was replaced with McCoy's 5A (Sigma-Aldrich) growth medium containing 10% chicken serum (Gemini Bio-Products, West Sacramento, CA), 5% horse serum (Gemini Bio-Products), 1% antibiotics–antimycotics (Gemini Bio-

Products), and 0.1% gentamicin (Gemini Bio-Products) for 72 h. The growth medium was refreshed every 24 h for 72 h. After 72 h of proliferation, the growth medium was replaced with a DMEM differentiation medium containing 3% horse serum, 1% antibiotics–antimycotics, 0.1% gentamicin, 0.1% gelatin, and 1 mg/mL bovine serum albumin (BSA, Sigma-Aldrich) for 72 h of differentiation. The differentiation medium was changed every 24 h for 72 h.

Proliferation experiment

After 24 h in the plating medium, the cells fed the growth medium and within the treatment were replicate-cultured at an experimental temperature (33°, 38°, or 43°C) for 72 h with the medium being replaced every 24 h. The control temperature of 38°C is the approximate temperature measured in newly hatched poults (38.0°C–38.5°C, G. Strasburg, unpublished data), and heat and cold treatments (43°C and 33°C, respectively) deviate from the approximate body temperature of mature turkeys (41.5°C). These temperatures have been shown to produce significant effects on satellite cell proliferation (Clark et al., 2016; Xu et al., 2021a). At harvest, SCs were collected using RNAzol RT (Sigma-Aldrich) and stored at −80°C until RNA isolation.

Differentiation experiment

For differentiation, the cells were cultured as previously described by Reed et al. (2017b) and Reed et al. (2022b). The cells within the treatment were replicate-plated at 38°C (control) or at one of the challenge temperatures (33°C or 43°C), and the medium was changed every 24 h for the 72 h of differentiation. The cells were harvested as mentioned previously.

RNA isolation and sequencing

Total RNA was isolated from each sample by RNAzol RT (Sigma-Aldrich) extraction, DNase-treated (TURBO DNA-free TM Kit, Ambion, Inc.), and stored at −80°C. The initial RNA concentration and quality were assessed by spectrophotometry (NanoDrop 1000), and the samples were submitted for library preparation and sequencing at the University of Minnesota Genomics Center. Each sample was further quantified by the RiboGreen assay (Invitrogen Corp.) using the 2100 Bioanalyzer (Agilent Technologies). Due to poor cell growth during proliferation at 33°C, the RNA quantity for the RBC2 treatment group was insufficient, and this group was excluded from further analysis. For each of the remaining treatment groups, the replicate samples were prepared for sequencing (two biological replicates per treatment group). Indexed libraries (n = 22) were constructed using the Takara Bio smRNA Library Preparation Kit and sizes selected for approximately 170 bp inserts. The libraries were multiplexed and sequenced on the NovaSeq SP platform using v1.5 chemistry (Illumina, Inc.) to produce 51-bp paired-end reads (data accessioned as part of the NCBI SRA BioProject PRJNA842679).

Illumina sequence data handling

Illumina sequencing reads were screened for low-quality bases and adapter contamination with FastQC 0.11.9 (https://www.bioinformatics.babraham.ac.uk/projects/fastqc/). Per-library FastQC reports were aggregated into a joint report for easy browsing using MultiQC 1.13 (Ewels et al., 2016). For each sequenced library, the "forward" (R1) read was used for downstream analyses. Sequencing adapters were removed from the reads using cutadapt 4.2 (Martin, 2011). The first three bases were additionally removed during trimming to remove non-biological bases added during library preparation. Reads were removed if their lengths were shorter than 15 nt after trimming. Trimmed reads were then cleaned of ribosomal sequences using BBDuk 39.01 (https://sourceforge.net/projects/bbmap/). The reference sequences used for ribosomal depletion were large subunit and small subunit ribosomal sequences retrieved from SILVA release 132 (Quast et al., 2013). Reads were removed if they had an exact match of at least 15 nt to one of the reference sequences from SILVA. Reads that were trimmed of adapters and depleted of ribosomal sequences were used for downstream analyses.

miRNA prediction

Cleaned reads from all libraries were combined into a single file for the prediction of novel miRNAs against the turkey genome. The turkey genome assembly (GCA_943295565.1) was prepared for mapping using Bowtie 1.3.1 (Langmead et al., 2009). Characterized mature miRNAs from chicken (*Gallus gallus*) were downloaded from miRBase release 22.1 to use as previously known miRNAs. Novel miRNAs were predicted in the combined sequencing libraries using miRDeep2 0.1.2 (Friedländer et al., 2012), and miRNA sequences were retained if their miRDeep2 score was >0.

miRNA expression profiling

Cleaned reads from each library were separately mapped to the turkey genome assembly using Bowtie 1.3.1 (Langmead et al., 2009). The options used were "-n 0 -e 80 -l 15 -m 5 --best--strata" to recreate the same parameters that were used for miRNA discovery using miRDeep2. SAM files were converted to BAM files using SAMtools 1.14 (Danecek et al., 2021). Processing of alignment files was performed in parallel with GNU parallel version 20210822 (Tange, 2018). miRNA regions identified using miRDeep2 were converted to SAF files for expression quantification. miRNA expression was quantified using "featureCounts" version 2.0.3 (Liao et al., 2014), requiring a minimum mapping quality of 10 for a read to be counted. To identify differentially expressed miRNAs (DEMs), expression values were first normalized by the library size and multiplied by a factor of 1×10^6, corresponding to counts per million (CPM) mapped miRNA reads, where the library size is the total number of reads mapped to miRNA precursors. The counts matrix from featureCounts was analyzed using the "edgeR" package (Robinson et al., 2010) in the R statistical computing

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TABLE 1 Summary of RNA-seq data used for miRNA discovery and expression analyses[a].

Experiment	Line	Temperature (°C)	Replicate	PF cluster	Yield (Mb)	% ≥Q30 bases	Mean Q score
Proliferation	NCT	33	A	16,185,368	1,651	77.54	32.40
	NCT	33	B	18,663,090	1,904	77.35	32.37
	NCT	38	A	18,513,867	1,888	77.07	32.31
	NCT	38	B	15,089,576	1,539	77.49	32.38
	NCT	43	A	19,911,181	2,031	77.38	32.35
	NCT	43	B	20,772,409	2,119	77.73	32.44
	RBC2	38	A	15,555,080	1,587	77.08	32.32
	RBC2	38	B	18,487,011	1,886	77.49	32.39
	RBC2	43	A	19,835,488	2,023	77.73	32.44
	RBC2	43	B	19,131,708	1,951	77.34	32.37
Differentiation	NCT	33	A	21,728,024	2,216	77.68	32.41
	NCT	33	B	21,552,098	2,198	77.84	32.46
	NCT	38	A	20,274,703	2,068	77.87	32.47
	NCT	38	B	20,879,610	2,130	77.76	32.43
	NCT	43	A	30,452,556	3,106	78.65	32.63
	NCT	43	B	16,756,472	1,709	77.48	32.38
	RBC2	33	A	16,405,230	1,673	77.48	32.36
	RBC2	33	B	23,417,907	2,389	77.88	32.46
	RBC2	38	A	16,314,326	1,664	77.80	32.44
	RBC2	38	B	22,244,407	2,269	77.94	32.47
	RBC2	43	A	21,406,067	2,183	77.65	32.42
	RBC2	43	B	15,893,508	1,621	77.87	32.46

[a]For each library, the total number of PFs, clusters (the number of reads passing filter in millions per lane), yield (Mbases), percentage of bases with the quality score (Q) ≥30, and mean Q score are given.

environment, version 4.2.2 (R Core Team, 2022). miRNAs with a low expression were filtered by removing those that did not have at least three assigned reads in at least two libraries. Global patterns in miRNA expression were assessed with principal component analysis using the prcomp function in R. Variance partitioning analyses were conducted using the "variancePartition" package (Hoffman and Schadt, 2016) in R, estimating the contributions of the incubation temperature, genotype, and an interaction between the incubation temperature and the genotypic variance in miRNA expression. Differential expression analyses were carried out with the quasi-likelihood F-test using edgeR (Chen et al., 2016). Differences were evaluated for the fold change ($\log_2$FC) and were considered significant at p <0.05. BioVenn (Hulsen et al. (2008) was used to create Venn diagrams.

miRNA target prediction

Potential miRNA target genes were initially predicted using TargetScan 8.0 (McGeary et al., 2019) and miRDB (Chen and Wang, 2020) using the chicken miRNA database. The given sequence differences between turkey and chicken genomes and potential binding sites were subsequently computationally predicted using miRanda v3.3a (Enright et al., 2003). Although the trio-based turkey genome assembly (GCA_943295565.1) offers improved coverage and assembly quality, at the time of this study, it had not been annotated in either the Ensembl or NCBI databases. Therefore, to identify the genes associated with the predicted turkey miRNAs, we used the reference UMD-5.1 assembly to align the miRDeep2-predicted consensus precursor sequences via BLAST. Gene targets in turkey were predicted by aligning the miRNA sequences against all RNA transcripts in the annotated UMD-5.1 genome build (NCBI annotation 104) with position-weighted scoring, an alignment score of >150, and | energy-kcal/mol| >7.0. Enrichment tests for target genes were performed using the PANTHER Overrepresentation Test [GO Consortium release 20150430 (Mi et al., 2013); http://geneontology.org/]. GO analysis utilized the chicken (*G. gallus*) reference gene list with ~66% of turkey loci (Annotation 105) having ID homologs. The differences were considered significant at p <0.05.

10.3389/fphys.2023.1293264

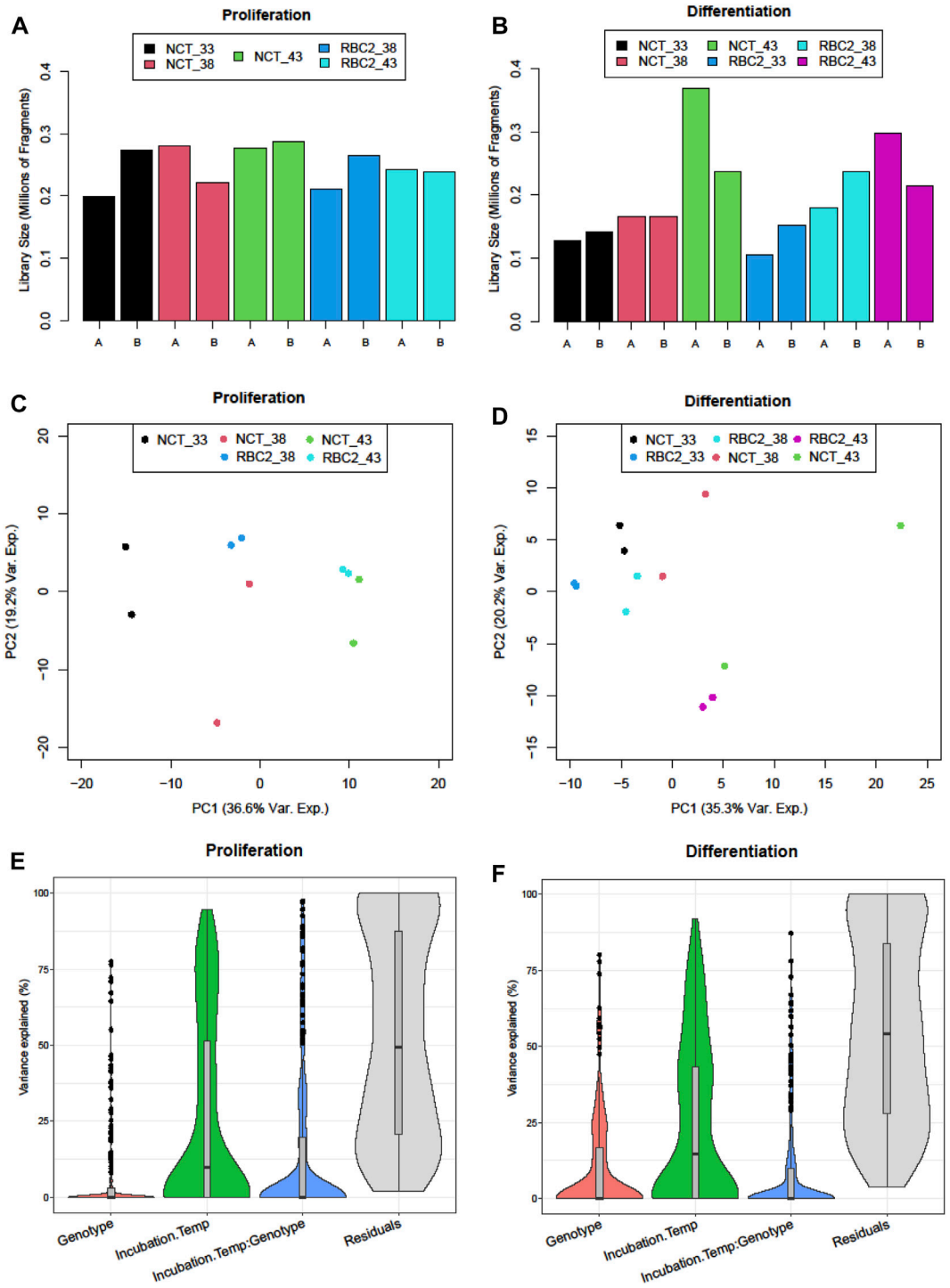

FIGURE 1
Distribution of sequencing reads (millions of fragments) in sequencing libraries of treatment groups of **(A)** proliferating and **(B)** differentiating muscle satellite cells. PCA plots of normalized read counts for **(C)** proliferating and **(D)** differentiating satellite cells. Sample-to-sample distances (within and between treatments) are illustrated for each treatment sample on the first two principal components. The samples are plotted according to the treatment. The distribution of sample variance by the treatment factor: genotype (line), temperature, interaction, and residual for **(E)** proliferating and **(F)** differentiating satellite cells.

Workflow availability

All scripts needed to recreate the analyses described previously are available in a GitHub repository at https://github.com/TomJKono/Turkey_MSC_miRNA.

Results

Small RNA sequencing

The results for the sequencing of the small RNAs are summarized in Table 1. The number of PF (passing filter) clusters averaged 19.52M reads encompassing an average 1,991 Mb per library. The read quality was consistently high with an average mean Q score of 32.4. Library sizes from the proliferation experiment were very similar (Figure 1A) and were averaged higher than those from the differentiation experiment where the numbers of reads were more variable among libraries (Figure 1B). Here, the number of reads was the lowest in the 33°C treatment replicates and the highest in the 43°C replicates.

Identification and the expression of conserved and novel miRNAs

Clean reads obtained from all sequencing libraries in both experiments were used for the detection of expressed miRNAs in the SCs using miRDeep2. The performance of miRDeep2 in the detection of known miRNAs (those identified based on the sequence comparison of their miRNA precursors with the miRBase dataset of *G. gallus*) and novel miRNAs is presented in Supplementary Table S1. In this study, a total of 353 miRNAs (161 known and 192 novel) were detected. The expression of putative novel miRNAs was lower than that of the known miRNAs.

Novel miRNAs were considered high-confidence if both the putative mature and star miRNAs (miRNA corresponding to the other side of the hairpin) reported by miRDeep2 were detected in at least two independent samples, having the exact same 5′- and 3′-ends and allowing no mismatches. The cutoff values for confidence are somewhat arbitrary for novel miRNA predictions, but some studies suggest that a miRDeep score >1, significant RNAfold *p*-value, and mature reads >10 can be used as minimum values. Based on these criteria, 118 of the 192 detected novel miRNAs (61.4%) were considered high-confidence. In addition, sequencing reads were found to map to various miRBase gga-miRNAs that were not included within the "known" category. These "known miRNAs not detected using miRDeep2" were observed due to unusual precursor structures that do not fit the assumed biogenesis model of miRDeep2. The gga-miRNAs classified as "not detected" included 14 gga-miRNAs with mapped read counts >100 among the libraries (Supplementary Date Sheet S1). Although these were not included in subsequent analyses, the expression of three of these (gga-let-7b [140,731 mapped reads], gga-let-7l-2 [19,029], and gga-mir-210a [15,620]) may warrant further investigation. Using the UMD-5.1 genome assembly, 150 known and 130 novel miRNAs were uniquely mapped to the turkey genome (Supplementary File 1). Based on the NCBI UMD-5.1 annotation (v104), 181 (51.3%) of the precursor sequences (92 and 89 of the known and novel miRNAs, respectively) occurred within annotated genes.

Thermal challenge of proliferating satellite cells

Distributions of the expressed miRNAs are summarized in Figure 2. In the observed expression of 294 miRNAs, 170 were common in all treatments in both experiments (proliferation and differentiation). The expression of 39 miRNAs was low (average of 7.5 reads/treatment) and was limited to proliferating SCs. Only four miRNAs were uniquely expressed in single-treatment groups in the proliferation experiment (one each in the NCT 33°C, NCT 38°C, RBC2 38°C, and RBC2 43°C treatments), and all of them were novel miRNAs with a low expression (average of 2.6 reads/treatment).

Variation in the expression among treatment groups was visualized by the principal component analysis (PCA). In the proliferation experiment, treatment groups clustered distinctly along the first principal component (PCA1) (Figure 1C) with replicate treatment pairs occurring as nearest neighbors within the PCA space. The greatest within-treatment separation was seen for the NCT samples in the control (38°C) treatment along the PCA2. Variance partitioning was used to estimate the contributions of the incubation temperature, genotype, and temperature × genotype interaction and found that the incubation temperature explained a greater proportion of the variation than genetic background (Figure 1E).

Identification of differentially expressed miRNAs

Normalization by the library size resulted in a counts matrix of 271 miRNAs (151 known and 120 novel) for analyses with EdgeR (Supplementary Table S2). With heat treatment (43°C), only a single DEM (miR-206) was identified in NCT SCs in comparison to the control temperature (38°C). The expression of this miRNA was significantly elevated by heat treatment ($\log_2$FC = 3.74). In contrast, heat treatment of RBC2 SCs had a greater effect on the miRNA expression where 73 DEMs (44 known and 29 novel miRNAs) were identified in comparison to the control temperature (38°C) (Supplementary Table S3). Of these 73 DEMs, 34 were upregulated and 39 downregulated by heat treatment. Twenty nine of the 73 DEMs had $|\log_2FC|$ >1.0, and 12 had $|\log_2FC|$ >2.0. The greatest upregulation was observed for miR-206 ($\log_2$FC = 4.41), miR-N145 (3.69), and miR-N34 (2.75). The greatest downregulation was observed for the novel miRNAs miR-N77 ($\log_2$FC = −4.0), miR-N23 (−3.52), miR-N54 (−3.31), and miR-N82 (−2.96). Libraries for SCs proliferating at 33°C were only sequenced for the NCT line, and no DEMs were identified in comparison to these with the control temperature.

The response of the two turkey lines was dramatically different at the control and heat treatment temperatures. At the control temperature (38°C), four miRNAs were found to be differentially expressed between the SCs of commercial birds (NCT) and the RBC2 line (Figure 3; Supplementary Table S3). These included the known miRNAs, miR-206 and miR-184-5p, where the expression was lower in RBC2 SCs ($\log_2$FC = −2.29 and −1.58, respectively). The novel DEMs, miR-N96 and miR-N173, had higher expressions of RBC2 SCs ($\log_2$FC = 1.34 and 2.57, respectively).

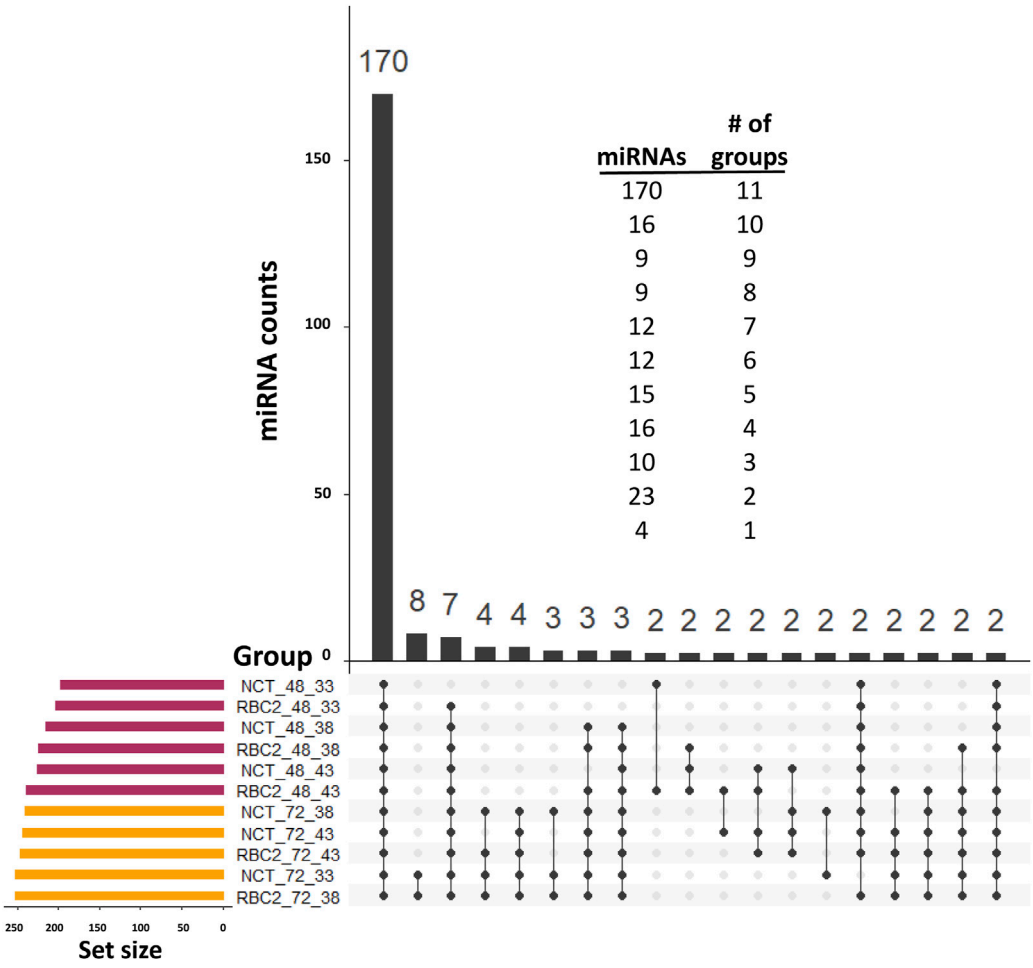

FIGURE 2
UpSet plot (Conway et al., 2017) of the expressed miRNAs. For inclusion, miRNAs must first have at least three assigned reads in at least two libraries and a treatment group average number of reads of >2.0. The horizontal bars on the left indicate the number of miRNAs expressed in each treatment. Individual points in the matrix represent miRNAs specific to each treatment, and the lines between points represent the miRNAs common to different groups. We excluded 70 single miRNAs that individually had unique group distributions. The vertical bars above indicate the number of miRNAs specific to or common to different treatments. The distribution of miRNAs by the number of treatment groups in which they were included is given above.

At the elevated temperature (43°C), line comparison of RBC2 and NCT SCs found eight DEMs (Figure 3; Supplementary Table S3). These included six known miRNAs, miR-129-5p, miR-146b-5p, miR-206, miR-1416-5p, and two isoforms of miR-2954. Log_2FC for these DEGs was generally low (log_2FC = −0.91 to 0.84), with the exception of miR-206 (−1.63), where the expression in RBC2 cells was significantly lower compared to NCT SCs, similar to those seen at the control temperature. The two novel DEMs, miR-N154 and miR-N185, were both upregulated in RBC2 cells compared to NCT (log_2FC = 0.78 and 1.39, respectively).

Thermal challenge of differentiating SCs

A total of 255 predicted miRNAs were observed in SCs during differentiation, with only eight being uniquely expressed in the differentiating cells (Figure 2). The unique transcripts were a mix of known (six) and novel (two) miRNAs with a low average expression (3.7 reads/treatment group). Treatment groups clustered distinctly within the first two principal components (Figure 1D) and replicate samples generally clustered together with the exception of NCT 43°C samples, which had the largest separation within the PCA space. Variance partitioning found the incubation temperature to explain the largest proportion of the variation (Figure 1F), and the genotype had a greater variance component in differentiating SCs when compared to proliferating SCs.

Differentially expressed miRNAs following heat treatment

The counts matrix for the differentiation experiment included 213 miRNAs (148 known and 65 novel) (Supplementary Table S4). Several DEMs were found in within-line comparisons between the 43°C and 38°C treatments (Figure 4, Supplementary Table S5). In NCT SCs, six DEMs were identified including the known miR-1559-5p and five novel miRNAs (miR-N29, miR-N105, miR-N140, miR-N157, and miR-N183). Directional regulation was split, with three

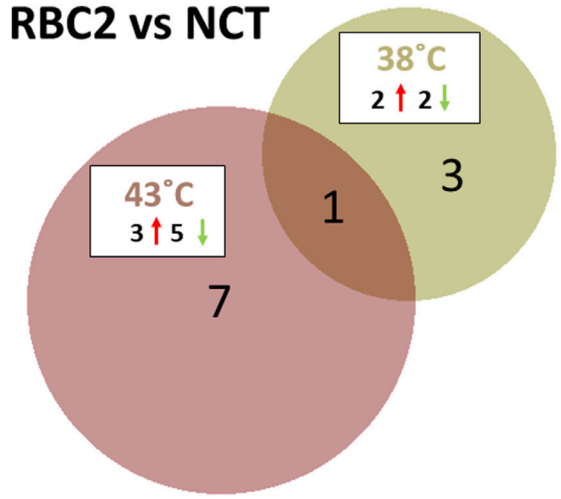

FIGURE 3
Distribution of DEMs during the proliferation of cultured turkey *p. major* SCs. For each temperature comparison, the DEMs with FDR *p*-value <0.05 that were shared or unique to each line (RBC2 and NCT) are indicated in the Venn diagram. The circle size is proportional to the number of DEMs.

miRNAs (miR-N105, miR-N140, and miR-N183) being upregulated in NCT SCs (log_2FC = 6.22, 2.62, and 2.12, respectively) at 43°C and three (miR-N29, miR-1559-5p, and miR-N157) being downregulated (log_2FC = −5.02, −2.44, and −2.19, respectively).

The response to heat treatment was more significant in RBC2 SCs than that in NCTs. In the RBC2 within-line comparison (43°C vs. 38°C), 62 DEMs were identified (Figure 4; Supplementary Table S5), including 39 known and 23 novel miRNAs, with 31 being upregulated and 31 downregulated with heat treatment. The log_2FC of these DEMs ranged from 6.98 to −3.19 with 35 having $|log_2FC|$ >1.0 and 13 with $|log_2FC|$ >2.0. The greatest upregulation was observed for miR-205b (log_2FC = 6.98) and miR-N30 (4.17), and the largest downregulation was observed for miR-N54 (log_2FC = −3.19) and miR-460b-5p (−2.25).

Five DEMs were shared between the NCT and RBC2, 43°C vs. 38°C, comparisons. The expression of miR-N105, miR-N140, and miR-N183 was significantly higher in heat-treated cells than that in the controls in both lines. In contrast, miR-N157 and miR-1559-5p were significantly downregulated in heat-treated cells compared to controls.

DE analysis found no significant differences in miRNA expression between RBC2 and NCT SCs at either the control temperature (38°C) or heat treatment (43°C).

Differentially expressed miRNAs following cold treatment

A within-line comparison found six DEMs in the NCT SCs being incubated at 33°C relative to controls (38°C). These included three isoforms of miR-1 and three novel miRNAs (miR-N183, miR-N140, and miR-N30) (Figure 4; Supplementary Table S5). Each of the DEMs showed higher levels of expression at 38°C compared to 33°C with average log_2FC >3.0.

As seen for heat treatment, the proliferating RBC2 SCs showed a greater response to cold treatment than the NCT SCs (Figure 4; Supplementary Table S5). The comparison of the 33°C-treated cells to the control identified 37 DEMs, including 24 known and 13 novel miRNAs, with 25 being upregulated and 12 downregulated. Overall, log_2FC ranged from 4.83 to −3.18 with 20 DEMs having $|log_2FC|$ >1.0 and eight with $|log_2FC|$ >2.0. The greatest fold change was observed for two predicted novel miRNAs (miR-N29 and miR-102; log_2FC = 4.83 and 2.89, respectively), with a higher expression at 38°C. The novel miRNA, miR-N68, showed the greatest expression change at 33°C (log_2FC = −3.18). Five DEMs were shared between NCT and RBC2, 33°C vs. 38°C, comparisons. In SCs, from both lines, the expression of miR-1a-1-5p, miR-1a-2-5p, miR-1b-5p, miR-N140, and miR-N183 was significantly lower in cold-treated cells compared to controls.

Significant differences in miRNA expression were observed between the lines with cold treatment (33°C), and 33 miRNAs were differentially expressed between RBC2 and NCT SCs

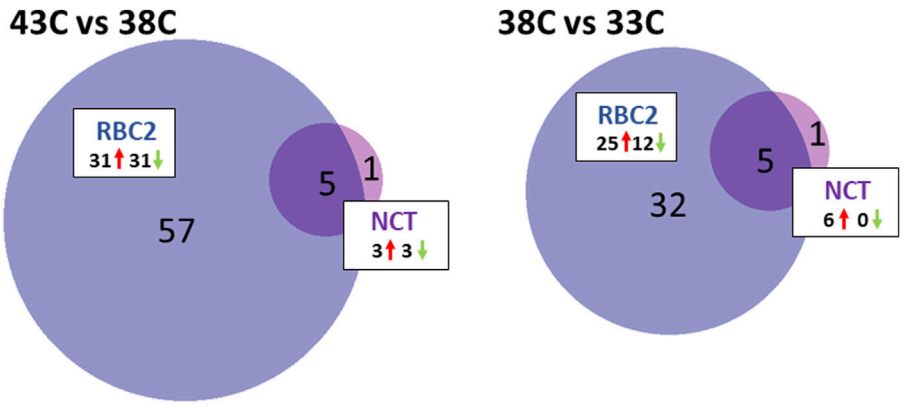

FIGURE 4
Distribution of DEMs during the differentiation of cultured turkey *p. major* SCs. For each temperature comparison, the DEMs with FDR *p*-value <0.05 that were shared or unique to each line (RBC2 and NCT) are indicated in the Venn diagram. The circle size is proportional to the number of DEMs.

(Supplementary Table S5). This group of DEMs was primarily comprised of known miRNAs (29) but with a relatively low overall fold change (average $\log_2$FC = 0.106). The greatest upregulation was observed for miR-N72 and two isoforms of miR-1677-5p ($\log_2$FC = 2.69, 1.87, and 1.87, respectively). A significant downregulation was the greatest for miR-N63, miR-206, and miR-1416-5p ($\log_2$FC = −1.81, −1.25, and −1.11, respectively).

miRNA target predictions

Target predictions used sequences of each of the DEMs ($|\log_2 FC| > 1.0$) to query the annotated transcript sequences in the turkey genome for potential miRNA target sites.

Genes targeted by DEMs in proliferating cells

Target predictions for the four DEMs identified in the RBC2 vs. NCT (38°C) line comparison averaged 1,515 target sites within 751 genes. Genes containing predicted sites with the highest alignment score are summarized in Supplementary Table S6. For the downregulated DEMs (miR-184 and miR-206), these included genes such as *ATG16L1* (autophagy-related 16-like 1), *KLHL7* (kelch-like family member 7), *KCNT1* (potassium- and sodium-activated channel subfamily T member 1), *MILR1* (mast cell immunoglobulin-like receptor 1), *HLCS* (holocarboxylase synthetase), *CPEB1* (cytoplasmic polyadenylation element-binding protein 1), and an uncharacterized locus (*LOC104914368*). The downregulation of the expression of these miRNAs could predictably increase the translation of these target genes. GO analysis of the suite of predicted targets of the two downregulated miRNAs (miR-184 and miR-206) showed significant enrichment (1.42×; p = 1.52E-02) for localization (GO: 0051179). For the upregulated novel DEMs (miR-N96 and miR-N173), target genes included *LOC100541175* (keratin type-I cytoskeletal 13-like), *PDE6C* (phosphodiesterase 6C), *CRAT* (carnitine O-acetyltransferase), *EPHX1* (epoxide hydrolase 1), *EXD2* (exonuclease 3′–5′ domain-containing 2), and *USP47* (ubiquitin-specific peptidase 47). The upregulation of the expression of these miRNAs could predictably decrease the translation of these target genes. For these upregulated miRNA targets, GO molecular function terms for protein binding (GO: 0005515; 1.29×; p = 4.05E-03) and ion binding (GO:0043167; 1.26×; p = 1.24E-02), and GO biological process terms for the apoptotic signaling pathway in response to DNA damage (GO:0008630; 5.71×; p = 2.24E-02) and cellular localization (GO:0051641; 1.51×; p = 1.04E-02) were significantly enriched.

In the heat-treated cells (43°C), only two of the eight DEMs had $|\log_2 FC| > 1.0$. These include miR-206 (downregulated in RBC2 relative to NCTs, −1.631) and miR-N185 (upregulated in RBC2, 1.392). Targets for miR-206 are as presented previously (Supplementary Table S6), and the top targets for miR-N185 included *HSPB7* (heat-shock protein family B (small) member 7) and *SCN2B* (sodium voltage-gated channel beta subunit 2). Given the increased number of DEMs identified in RBC2 cells under heat treatment (73), the number of potential miRNA interaction sites is significantly increased compared to those in NCT cells. Of the 73 DEMs, 29 had $|\log_2 FC| > 1.0$, and target site prediction identified nearly 2,500 predicted target sites within an average 1,065 genes per miRNA. Targets for the top 10 genes per miRNA are given in Supplementary Table S7.

GO analysis of the suite of predicted targets for miRNAs downregulated by heat treatment found the highest significant enrichment for the GO biological process term ubiquitin-dependent protein catabolic process (GO:0006511; 1.54×; p = 2.18E-02), protein catabolic process (GO:0030163; 1.49×; p = 1.00E-02), proteolysis involved in the protein catabolic process (GO:0051603; 1.48×; p = 4.65E-02), and cellular response to stress (GO:0033554; 1.47×; p = 3.42E-05) and for GO molecular function term identical protein binding (GO:0042802; 1.55×; p = 3.45E-04) and enzyme binding (GO:0019899; 1.41×; p = 1.13E-03). Analysis of targets for upregulated miRNAs found the highest significant enrichment for the GO biological process term regulation of cell differentiation (GO:0045595; 1.52×; p = 5.57E-03), regulation of the developmental process (GO:0050793; 1.48×; p = 3.47E-05), and cellular response to stress (GO:0033554; 1.46×; p = 2.67E-04) and for the GO molecular function term identical protein binding (GO:0042802; 1.5×; p = 1.10E-02).

Genes targeted by DEMs in differentiating cells

In the heat-treated cells, no DEMs were observed between the cell lines (NCT vs. RBC2), and fewer expression differences were observed within NCT lines when comparing the heat treatment group (43°C) to the control (38°C). In the NCT comparison, six DEMs had $|\log_2 FC| > 1.0$, with an average of 943 targets identified among an average of 548.3 genes (Supplementary Table S8). Among the downregulated DEMs (miR-1559-5p, miR-N29, and miR-N157), the top gene targets included *HNRNPH3* (heterogeneous nuclear ribonucleoprotein H3), *KIF9* (kinesin family member 9), *KAT14* (lysine acetyltransferase 14), *AGRN* (agrin), *BCL11B* (BAF chromatin remodeling complex subunit BCL11B), and *HACD1* (3-hydroxyacyl-CoA dehydratase 1). Predicted targets for these three miRNAs were downregulated by cold treatment and found significant enrichment for the GO biological process term regulation of the cellular process (1.27×; p = 5.87E-07). Among the upregulated DEMs (miR-N105, miR-N140, and miR-N183), the top gene targets included *PCSK7* (proprotein convertase subtilisin/kexin type 7), *COL18A1* (collagen type XVIII alpha 1 chain), *ZBTB17* (zinc finger and BTB domain-containing 17), *JPH2* (junctophilin 2), *CAMTA1* (calmodulin-binding transcription activator 1), *FGFRL1* (fibroblast growth factor receptor-like 1), and *TMEM132A* (transmembrane protein 132A). Biological processes of secretion (GO:0046903; 2.39×; p = 1.52E-06), organic acid transport (GO:0015849; 2.36×; p = 3.04E-05), carboxylic acid transport (GO:0046942; 2.30×; p = 5.24E-05), and secretion by cell (GO:0032940; 2.28×; p = 2.48E-05) showed the greatest fold enrichment.

Similar to the proliferating SCs, an increased number of DEMs (62) was observed in the differentiating RBC2 cells under heat treatment, significantly increasing the number of potential miRNA interaction sites as compared to the NCT cells. Of the 62 DEMs, 35 had $|\log_2 FC| > 1.0$, and target site prediction identified an average of 1888.4 predicted target sites within an average of 912.6 genes per miRNA. Targets for the top 10 genes for each miRNA are given in Supplementary Table S9. GO analysis of the 7,104 potential target genes for the 17 downregulated miRNAs

(|$\log_2$FC| >1.0) found the greatest enrichment for the biological process term apoptotic signaling pathway (GO:0097190; 1.88×; p = 3.19E-04), multicellular organismal-level homeostasis (GO: 0048871; 1.81×; p = 1.43E-04), and macroautophagy (GO: 0016236; 1.81×; p = 6.45E-04). GO analysis of the 6,980 potential target genes for the 18 upregulated miRNAs (|$\log_2$FC| >1.0) found the biological process term cellular component disassembly (GO: 0022411; 1.75×; p = 2.75E-04), extracellular matrix organization (GO:0030198; 1.69×; p = 7.02E-04), and programmed cell death (GO:0012501; 1.59×; p = 1.95E-05) were significantly overrepresented.

In cold-treated cells (33°C), target predictions for the eight DEMs (|$\log_2$FC| >1.0) identified in the line comparison (RBC2 vs. NCT) averaged 1837.7 target sites within 850 genes. Genes containing predicted target sites with the highest alignment score are summarized in Supplementary Table S10. For the downregulated DEMs (miR-206, miR-1416-5p, miR-2954 (two isoforms), and miR-N63), these included genes such as *MILR1* (mast cell immunoglobulin-like receptor 1), *CACNA1E* (calcium voltage-gated channel subunit alpha 1E), *PLEKHM2* (pleckstrin homology and RUN domain-containing M2), *HLCS* (holocarboxylase synthetase), *UTP18* (UTP18 small subunit processome component), *CPEB1* (cytoplasmic polyadenylation element-binding protein 1), *LMX1A* (LIM-homeobox transcription factor 1 alpha), *TMEM109* (transmembrane protein 109), *TYW5* (tRNA–yW synthesizing protein 5), *LOC104913138* (protein ABHD14B-like), and an uncharacterized locus (*LOC104914368*). The downregulation of the expression of these miRNAs could predictably increase the translation of these target genes. The overrepresentation test found significant enrichment for the molecular function protein homodimerization activity (GO: 0042803; 2.11×; p = 4.79E-05). For the upregulated DEMs (miR-1677 (two isoforms) and miR-N72), the targets included genes such as *BSDC1* (BSD domain-containing 1), *AGTR1* (angiotensin II receptor type 1), *CKB* (creatine kinase B), *LOC104911408* (N-acetylneuraminate 9-O-acetyltransferase-like), *MYCL* (MYCL proto-oncogene), bHLH (transcription factor), and *KCNJ13* (potassium inwardly rectifying channel subfamily J member 13). Overrepresentation associated with the predicted target genes included the biological process negative regulation of the cellular process (GO:0048523; 1.42×; p = 5.12E-05).

As seen in the proliferating cells, NCT SCs showed that fewer miRNAs were significantly affected by cold treatment. In NCT cells, all six DEMs were upregulated and had |$\log_2$FC| >1.0, and target predictions for these miRNAs averaged 1,591.2 targets within an average of 759.8 genes (Supplementary Table S11). The highest target alignment scores for these upregulated miRNAs (miR-1, three isoforms: miR-N30, miR-N140, and miR-N183) included *FSD2* (fibronectin type III and SPRY domain containing 2), *CSRNP1* (cysteine and serine rich nuclear protein 1), *TMEM132A* (transmembrane protein 132A), and the uncharacterized *LOC104914368*. GO analysis implicates the regulation of blood circulation (GO:1903522; 2.72×; p = 8.61E-05) as an overrepresented biological process.

In RBC2 cells, 20 of the 37 DEMs had |$\log_2$FC| >1.0, and target predictions for these miRNAs averaged 1800.1 targets in 830.9 genes (Supplementary Table S12). Among the 17 upregulated DEMs, the genes with the highest target scores included *SCAP* (SREBF chaperone), *KAT14* (lysine acetyltransferase 14), *ATP10A* (ATPase phospholipid transporting 10A (putative)), *CSRNP1* (cysteine- and serine-rich nuclear protein 1), *PCSK7* (proprotein convertase subtilisin/kexin type 7), *ABCC2* (ATP-binding cassette subfamily C member 2), *CACNA1E* (calcium voltage-gated channel subunit alpha 1E), *TIMM44* (translocase of inner mitochondrial membrane 44), *OSBP2* (oxysterol binding protein 2), *ACSL6* (acyl-CoA synthetase long-chain family member 6), *TMEM132A* (transmembrane protein 132A), BCL2L1 (BCL2-like 1), *ACTN1* (actinin alpha 1), and the uncharacterized locus *LOC104914368*. The significant enrichment for the target genes include the biological process of multicellular organismal-level homeostasis (GO:0048871; 1.74×; p = 5.28E-04) and the molecular function term helicase activity (GO:0004386; 1.85×; p = 3.18E-04). For the two downregulated DEM (miR-N56 and miR-N68) genes with the top target alignment scores, we have the following *RBBP8NL* (RBBP8 N-terminal like), *RFX7* (regulatory factor X7), *AANAT* (aralkylamine N-acetyltransferase), *LOC100545461* (antigen-presenting glycoprotein CD1d-like), *LOC100539021* (T-cell surface glycoprotein CD1b-3), and *CADM3* (cell adhesion molecule 3). GO analysis of the suite of predicted targets include the biological function term monoatomic ion transport (GO: 0006811; 2.33×; p = 6.31E-06) and molecular function terms, such as lipid kinase activity (GO:0001727; 8.42×; p = 4.60E-05), active monoatomic ion transmembrane transporter activity (GO: 0022853; 3.78×; p = 4.40E-05), and active transmembrane transporter activity (GO:0022804; 3.03×; p = 2.30E-05).

Discussion

MicroRNAs are a class of small regulatory RNAs found in almost all animal species that play an important role in controlling the abundance of transcripts in the vertebrate transcriptome (Moran et al., 2017). These short RNA molecules predominantly recognize target sites in the 3′UTRs of mRNAs, typically leading to posttranscriptional repression as a means of modulating the gene expression (Simkin et al., 2020). Posttranscriptional downregulation by miRNAs can have a physiological stimulatory effect, as in the example of the Texel sheep breed where a sequence mutation produced an miR-1/206 binding site, leading to a muscle growth phenotype through the suppression of myostatin (Clop et al., 2006). The modulation of gene expression is critical for embryonic development and cell proliferation in poultry, and miRNAs have been reported to play important roles in these processes (Glazov et al., 2008; Hicks et al., 2008; Harding and Velleman, 2016; Velleman and Harding, 2017; Jebessa et al., 2018). The differential expression of miRNAs associated with growth traits (Li et al., 2011; Andreote et al., 2014; Ouyang et al., 2015) has been reported in chickens. In this study, an extensive set of miRNAs was characterized by small RNA sequencing of turkey *p. major* muscle SCs, identifying a total of 353 miRNAs (161 known and 192 novel). The presence of the known miRNA transcripts in the turkey SCs was consistent with the most abundant miRNAs observed in surveys of chicken skeletal muscles (Li et al., 2011; Ouyang et al., 2015; Khatri et al., 2018).

An expression unique to a limited set of tissues is indicative of highly specific miRNA interactions with a small set of target genes

(Bassett et al., 2014). The tissue-specific expression of miRNAs may serve to broadly control translation in specific cells or developmental stages and perhaps modulate developmental fluctuation caused by the environment (Li et al., 2009). The expression of miRNAs is often elevated in specific tissues, and there is strong evidence for the action of specific miRNAs in muscle growth and development (Goljanek-Whysall et al., 2012). For example, miR-206 and closely related members of the miR-1 family are specifically expressed in mammalian muscles (Sempere et al., 2004; McCarthy, 2008; Townley-Tilson et al., 2010) and are required for proper morphogenesis during early embryonic development (Kim et al., 2006; O'Rourke et al., 2007; Ma et al., 2015). The expression of this miRNA family may also be muscle specific in poultry (Li et al., 2011).

The expression of miR-206 in mammals and chicken is enhanced by muscle transcription factors MyoD, MYOG, and myocyte enhancer factor-2 (Mef2) (Rao et al., 2006; Sweetman et al., 2008). However, its expression in mammals is inhibited by transforming growth factor-β (TGF-β) (Winbanks et al., 2011). In bovids, the inhibition of miR-206 and miR-1 was found to enhance SC proliferation (Dai et al., 2016). The downregulation of genes targeted by miR-206 is required for the transition of SCs in mice from proliferation to differentiation (Chen et al., 2006; Chen et al., 2010; Dey et al., 2011). The differential expression of miR-206 in turkeys in proliferating SCs and miR-1 isoforms in differentiating SCs suggests a significant response in SC development resulting from a thermal challenge. However, it is important to note that the functionality of this miRNA may be different in poultry. Associations between the miR-206 expression and general growth (Xu et al., 2013) and more defined traits such as birthweight (Jia et al., 2016), embryo myogenesis (Goljanek-Whysall et al., 2014), and muscle growth (Li et al., 2011) have been reported in chicken. However, few studies have characterized gene interactions with this miRNA. Search for target sites for gga-miR-206 in miRDB identified 675 predicted targets and 356 transcripts with conserved sites predicted using TargetScan. The comparison of these chicken targets with the 529 genes predicted to be targeted by miRanda in turkey found only 12 genes common to all three groups including *ADPGK* (ATP-dependent glucokinase), *COL19A1* (collagen type XIX alpha 1 chain), *FAM91A1* (family with sequence similarity 91 member A1), *KTN1* (kinesin receptor), *MEIS1* (Meis homeobox 1), *NET1* (neuroepithelial cell-transforming 1), *RAPGEF2* (rap guanine nucleotide exchange factor 2), *RNF111* (ring finger protein 111), *SMG7* (nonsense-mediated mRNA decay factor), *TNPO1* (transportin-1), *TRIM2* (tripartite motif-containing 2), and *ZNF827* (zinc finger protein 827), which have various predicted cellular processes but without any notable ties to the SC function or muscle development.

Analysis of miRNAs in turkey SCs found a significant differential expression of known and novel miRNAs, both between genetic lines (RBC2 and NCT) and in response to a thermal challenge. The larger variance component attributed to temperature treatment is expected as a level of physiological response common between the genetic lines and unchanged by selection would be hypothesized. The greater number of DEMs observed in proliferating and differentiating SCs of the RBC2 line compared to the NCT suggests that miRNA response to heat stress has been altered in birds selected for their modern commercial growth traits. Previous RNA-seq studies of mRNA expression within an identical experimental system suggest that growth selection in turkeys has altered the developmental potential of SCs in commercial birds. In proliferating SCs, a greater number of differentially expressed mRNAs were observed from the growth-selected NCT birds, and a pathway analysis indicated a shift toward early myogenesis (Reed et al., 2022a). In differentiating SCs, cold treatment produced expression changes in genes involved in the regulation of skeletal muscle tissue regeneration and sarcomere organization, whereas heat treatment increased the expression of genes regulating myoblast differentiation and survival, particularly in the NCT line (Reed et al., 2022b).

The function of miRNAs in gene regulation is defined by the gene or a group of genes that they target. Target predictions are important in attributing a functional consequence to miRNA differential expression. However, relying on predictions based on comparative datasets (human or chicken) is necessarily biased and highly sensitive to sequence variation due to the small interacting target sequences of miRNAs. Predictions based on the turkey genome and gene set offer a more reliable prediction and sequences. Target prediction algorithms suggest that many miRNAs may interact with a large group of genes, and this is supported by our target predictions. However, the degree to which prediction algorithms identify false positives is a concern (Pinzón et al., 2017; Fridrich et al., 2019). Therefore, the target and pathway predictions resulting from this study necessitate future validation studies to confirm miRNA-specific targets and their functions. Interestingly, three miRNAs (miR-16, miR-24, and miR-128) predicted in an earlier study (Harding and Velleman, 2016), for interacting with genes essential to the SC function (syndecan-4 and glypican-1), were expressed in the present study but were not included among the DEMs.

The interaction of miRNAs with gene targets is a function of the sequence match and accessibility of the target site, as mediated by the secondary structure of target mRNAs (Kertesz et al., 2007). Target sites for miRNAs are also subjected to variable rates of selection, and the sequence conservation of sites is a useful predictor of functionality (Krek et al., 2005). MicroRNAs appear to be under variable selective pressure ranging from strong selection acting on targets of some miRNAs to weak selection for other miRNAs that have many targets (Simkin et al., 2020). While some miRNAs and their targets are highly conserved (Chen and Rajewsky, 2006), others are genus- or species-specific (Kozomara and Griffiths-Jones, 2014). Comparative studies have shown that ancient miRNAs, those highly conserved among divergent taxa, are under stronger selection and are more broadly expressed (Simkin et al., 2020).

Studies have demonstrated that the thermal challenge affects the growth and subsequent structure of poultry breast muscles (Halevy et al., 2001; Piestun et al., 2017; Patael et al., 2019) with downstream effects on the meat quality. A thermal challenge has significant effects on SC proliferation, differentiation, and adipogenic potential with a differential impact on growth-selected lines of turkeys (Clark et al., 2016; Reed et al., 2017a; Reed et al., 2017b; Xu et al., 2021a; Xu et al., 2021b; Reed et al., 2022a; Reed et al., 2022b). The activation and proliferation of SCs is modulated by signaling molecules which direct myogenesis through signaling pathways. These processes are

modulated by fine tuning gene expression, likely through RNA/RNA interactions, such as those involving miRNAs. In addition to miRNAs, we also characterize the expression of circular RNAs (circRNAs) in this same experimental system. CircRNAs are novel, single-stranded RNAs that are generated through the splicing of exonic/intronic sequences and are hypothesized to act as miRNA sinks (Wilusz, 2018).

The identification of non-coding RNA molecules provides further insight into the biological response to a thermal challenge and how selection for growth and increased muscle mass has altered this response. Our analyses identified a large number of genes and gene pathways potentially targeted by miRNAs in the turkey SCs available for future studies. The DEMs identified in this study of turkey SCs appear to be related to processes of muscle growth and development similar to their mammalian counterparts, and GO analysis suggests that the differential expression of miRNAs during a thermal challenge significantly affects cellular proliferation and differentiation. We caution that, to date, few studies have directly confirmed molecular miRNA/mRNA interactions in poultry (Velleman and Harding, 2017; Wu et al., 2019; Zhang et al., 2022), and most of the predicted gene interactions are currently based on the assumption that these RNA interactions in bird cells are similar to those observed in mammals (Goljanek-Whysall et al., 2012). There is, however, reason to assume that homologous miRNA:mRNA interactions do exist as target sites for miRNAs are amongst the most highly conserved motifs within mRNA 3′UTRs (Simkin et al., 2020).

This study identified miRNAs expressed in turkey muscle SCs, characterized their differential expression, and predicted important miRNA:mRNA interactions in turkey skeletal muscle SCs. Target gene predictions and Gene Ontology analysis suggest that the differential expression of miRNAs during a thermal challenge could significantly affect SC proliferation and differentiation. The distribution of DEMs suggests that selection for commercial production traits has altered the miRNA expression, providing new hypotheses for future research.

Data availability statement

The datasets presented in this study can be found in online repositories. The names of the repository/repositories and accession number(s) can be found at: https://www.ncbi.nlm.nih.gov/; PRJNA842679.

Ethics statement

Ethical approval was not required for the study involving animals in accordance with the local legislation and institutional requirements because this study used previously established cell lines.

Author contributions

KR: conceptualization, formal analysis, funding acquisition, investigation, methodology, writing–original draft, and writing–review and editing. KM: formal analysis and writing–review and editing. TK: formal analysis and writing–review and editing. AP: formal analysis, visualization, and writing–original draft. GS: conceptualization, funding acquisition, project administration, and writing–review and editing. SV: conceptualization, funding acquisition, investigation, resources, and writing–review and editing.

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Conflict of interest

The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

Publisher's note

Supplementary material

The Supplementary Material for this article can be found online at: https://www.frontiersin.org/articles/10.3389/fphys.2023.1293264/full#supplementary-material

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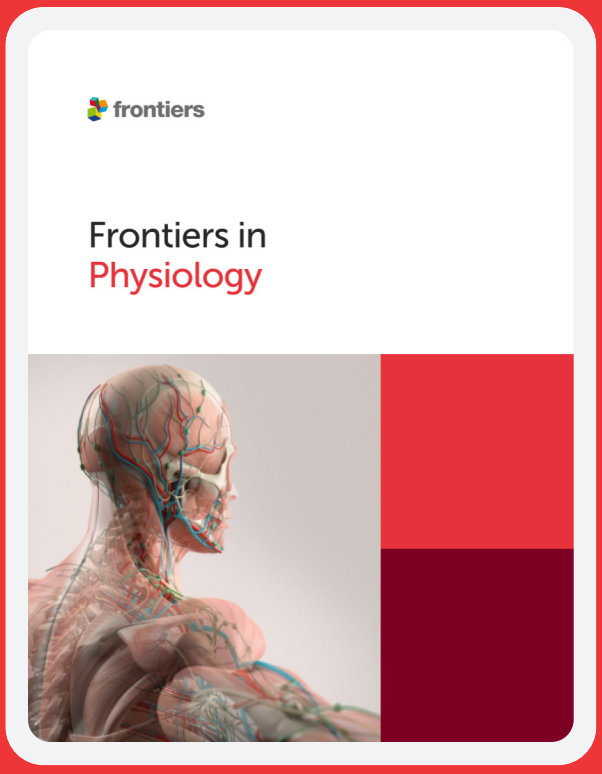

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