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Nutritional impacts the health and physiology of the avian gastro-intestinal tract

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Published in
Frontiers in Physiology

ISSN 1664-8714
ISBN 978-2-8325-5388-6
DOI 10.3389/978-2-8325-5388-6

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Nutritional impacts the health and physiology of the avian gastro-intestinal tract

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Citation

Pierzchała-Koziec, K., Assadi Soumeh, E., Scanes, C. G., Selvaraj, R., Shanmugasundaram, R., Olukosi, O., eds. (2024). *Nutritional impacts the health and physiology of the avian gastro-intestinal tract*. Lausanne: Frontiers Media SA. doi: 10.3389/978-2-8325-5388-6

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frontiers | Frontiers in Physiology

TYPE Editorial
PUBLISHED 21 August 2024
DOI 10.3389/fphys.2024.1470024

OPEN ACCESS

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RECEIVED 24 July 2024
ACCEPTED 29 July 2024
PUBLISHED 21 August 2024

CITATION
Pierzchała-Koziec K, Olukosi O, Selvaraj R, Shanmugasundaram R, Assadi Soumeh E and Scanes CG (2024) Editorial: Nutritional impacts the health and physiology of the avian gastro-intestinal tract.
Front. Physiol. 15:1470024.
doi: 10.3389/fphys.2024.1470024

Editorial: Nutritional impacts the health and physiology of the avian gastro-intestinal tract

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KEYWORDS

nutrition, gastrointestinal tract, microbiome, birds, health, botanicals 1, nutritional

Editorial on the Research Topic
Nutritional impacts the health and physiology of the avian gastro-intestinal tract

1 Introduction

There is a considerable body of information on the nutritional requirements of poultry and other avian species. Moreover, the avian gastro-intestinal tract marked similarities to that of mammals. Kuzmina et al. contribute an Opinion article on activities of intestinal enzymes. Unique features of the physiology of the avian gastro-intestinal tract include the separate glandular and muscular regions of the stomach, names the proventriculus and gizzard, the presence of a crop, a feed storage organ extending from the esophagus and the presence of two ceca. In addition, there are developmental differences between birds and viviparous mammals with bird embryos growing in large yolky eggs. In the present topic, Kuzmina discusses the importance of the embryonic yolk sac in birds in comparison with other vertebrates.

Research on avian nutritional physiology and health is increasingly employing anti-oxidative and molecular parameters together with traditionally employed parameters including body weight, feed efficiency and organ weights.

Among the objectives of this topic are the following:

1. To address both unique aspects of avian nutritional physiology and some poultry specific management techniques.
2. To determine effects of pathogens on the functioning of avian gastro-intestinal tract and how adverse effects be mitigated.
3. To understand the nutritional impacts on gut microbiota and the cross-talks between the microbes and the host.

4. To develop nutritional and other strategies (botanics, probiotics etc.) to reduce food borne colonization in poultry and reduce prevalence of food borne pathogens in poultry products.

2 Discussion

The importance of dietary arginine is reviewed (Fathima et al.). Not only is arginine required for protein synthesis but also is necessary to production of nitric oxide (NO), functioning of the immune functioning, gut microbiota and regulating mammalian target of rapamycin (mTORC) (Fathima et al.).

There were no effects of either dietary calcium and/or lipopolysaccharide (LPS) challenges on serum concentrations of ionized calcium and inorganic phosphate in hens towards the end of their production cycle (Li et al.). However, serum concentrations of alkaline phosphatase (ALP) were markedly elevated in hens receiving a low calcium feed but depressed those in hens challenged with LPS (Li et al.). Moreover, bone concentrations of calcium were increased in hens receiving LPS challenges (Li et al.). Splenic expression of inflammatory cytokines, TNF-α, interleukin (IL)-1β and IL-6, IL-10, IL-17 and interferon γ, were increased in hens receiving LPS challenges but unaffected by calcium level in the feed (Li et al.). Moreover, tibial expression of TNF-α, IL-1β, IL-6, IL-10, IL-17 and interferon γ were increased in hens receiving LPS challenges but, with the exception of TNF-α, unaffected by calcium level in the feed (Li et al.). Tibial expression of osteoblast metabolism-related genes: *ALP* and *Ocn* was influenced by calcium level or injection of LPS. Unexpectedly, mRNA gene expression of *FGF23* was not changed by the treatments (Li et al.). Expression of ALP was increased in hens receiving a low calcium feed but decreased following challenge with LPS (Li et al.). Expression of osteocalcin was increased in hens on a low calcium feed or following LPS challenge. The effect of LPS were greater in hens on a low calcium diet (Li et al.). The increase in tibial expression of TNF-α was greater in hens on a low compared to recommended levels of calcium (Li et al.).

Increasing numbers of poultry are raised without antibiotics. There is growing attention to using dietary supplements to replace antibiotics, to alter intestinal microbial populations, to stimulate antioxidative and immune systems and to improve overall intestinal health. Plant based and other dietary microingredients have been increasing considered as dietary supplements for humans, livestock, companion animals and poultry. These botanics can have beneficial effects including "*anti-inflammatory, anti-oxidative and pro-gut health*" (reviewed: Sulaiman et al.).

Feeding hens and/or pullets a flaxseed enriched diet increased the yolk concentrations of the following long-chain omega-3 polyunsaturated fatty acids (n-3 FAs): alpha-linolenic acid (C18: 3 n-3), eicosapentaenoic acid (C20:5 n-3), and docosapentaenoic acid (C22:5 n-3) (Whittle et al.). Moreover, there was an increase in brain weight but a decrease in brain concentrations of alpha-linolenic acid (C18:3 n-3) (Whittle et al.).

Both intraperitoneal and intracerebroventricular administration of the polyphenolic phytocompound, oleuropein, reduce feed intake in 4-day-old chickens (Sulaiman et al.). Moreover, plasma concentrations of glucose were depressed following administration of oleuropein (Sulaiman et al.).

Addition of a mixture of wheat germ, hops and grape seed extract was followed by increased growth rate in broiler chickens and improved feed efficiencies (Zou et al.). Based on fecal microorganisms, there were shifts in the gastro-intestinal microbiomes with decreases in numbers of both *Salmonella* and *E. coli* but increases in those of *Lactobacillus* (You et al., 2023).

What is unique in this study was the measurement of release of ammonia and hydrogen sulfide from manure; these being environmental pollutants released by poultry and livestock production (You et al., 2023).

Addition of pretreated Chinese herbal medicine to feed was followed by increased rates of egg laying and improved feed efficiency in laying hens towards the end of their production cycle (Zou et al.). Accompanying this were shifts in antioxidative systems including both enzyme activity and gene expression in plasma, liver, magna of oviduct and uterus (a region of the oviduct) (Zou et al.).

Sweet wormwood (*Artemisia annua*) has been employed in Chinese traditional medicine to treat malaria and consequently against the intracellular Plasmodium. What is not clear whether sweet wormwood *per se* has positive or negative effects on chickens. One study in the present topic addresses the ability of sweet wormwood leaf powder to ameliorate effects of a challenge with mixed *Eimeria* spp. in laying hens (Sharma et al.). This builds on the work of Jiao et al. (2018) As would be expected, egg production was decreased by a mixed *Eimeria* spp. Challenge (Sharma et al.). The effect was partially overcome by dietary supplementation with sweet wormwood leaf powder (Sharma et al.). While a challenge with mixed *Eimeria* spp. influenced the expression of a series of genes for intestinal proteins including mucin 2, tight junction proteins and transporter proteins and both small intestine villus height and crypt depth, there were no effects of supplementation with sweet wormwood leaf powder (Sharma et al.). Similarly, *Eimeria* challenge influenced anti-oxidative system but the effects were no ameliorated by supplementation with sweet wormwood leaf powder did not (Sharma et al.).

Ducks and geese are force-fed to produce those fatty livers needed for "Bloc foie gras" and "Pâté de foie gras". There are increases in both liver weight and lipid percentage fat in *force fed mule ducks* (Caïrina moschata x *Anas platyrhynchos*) (Atallah et al.). There is hepatic steatosis with concentrations of lipids rising from about five percent to about 60% with increasing bouts of force feeding (Atallah et al.). There are other novel shifts in hepatic physiology such as with the anti-oxidant status. In both male and female mule ducks, hepatic activities (expressed per unit protein) of superoxide dismutase were increased while those of catalase were decreased with increasing bouts of force feeding (Atallah et al.). Moreover, there were decreases in glutathione (total, oxidized and reduced expressed per unit protein) but increases in hypoxia-inducible factors 1 and 2 (Atallah et al.). Atallah and colleagues (2024) also report in the effects of force feed on expression of a comprehensive series of gene. There were little changes in inflammatory related genes, interleukin 18 and tumor necrosis factor-α (TNF-α) or metabolic related genes (Atallah et al.). However, expression of fatty acid binding protein greater than eighty-fold with 21 bouts of force feeding (females: 121-fold, males 87-fold increase).

Fecal metabolites were quantified by H^1 Nuclear Magnetic Resonance (NMR) spectroscopy in wild ducks (mallards) subjected to feed restriction (Murray and Machin). There were decreased fecal concentrations of O-phosphocholine, serine, taurine, caprate, ascorbate and increased fecal concentrations of 3-hydroxybutyrate, creatine, methyl amine, arabinitol, glutamic acid, glucuronate, trehalose, glucose and trimethylamine (Murray

and Machin). This provides a tool to investigate nutritional status of mallards in the wild based on fecal samples.

Author contributions

K-PK: Conceptualization, Writing–original draft, Writing–review and editing. OO: Conceptualization, Writing–review and editing. RSe: Conceptualization, Writing–review and editing. RSh: Conceptualization, Writing–review and editing. EA: Conceptualization, Writing–review and editing. CS: Conceptualization, Writing–original draft, Writing–review and editing.

Funding

The author(s) declare that no financial support was received for the research, authorship, and/or publication of this article.

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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.

The author(s) declared that they were an editorial board member of Frontiers, at the time of submission. This had no impact on the peer review process and the final decision.

Publisher's note

frontiers | Frontiers in Physiology

TYPE Original Research
PUBLISHED 20 January 2023
DOI 10.3389/fphys.2023.1110301

OPEN ACCESS

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SPECIALTY SECTION
This article was submitted to Avian Physiology,
a section of the journal
Frontiers in Physiology

RECEIVED 28 November 2022
ACCEPTED 11 January 2023
PUBLISHED 20 January 2023

CITATION
Yu A-C, Wang M-A, Chen L, Long C, Guo Y, Sheng X-H, Wang X-G, Xing K, Xiao L-F, Ni H-M, Li J-T and Qi X-L (2023), Effects of dietary pretreated Chinese herbal medicine supplementation on production performance, egg quality, uterine histopathological changes, and antioxidant capacity in late-phase laying hens.
Front. Physiol. 14:1110301.
doi: 10.3389/fphys.2023.1110301

Effects of dietary pretreated Chinese herbal medicine supplementation on production performance, egg quality, uterine histopathological changes, and antioxidant capacity in late-phase laying hens

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Aims: The study aimed to evaluate the effects of pretreated Chinese herbal medicine (PCHM) on egg quality, production performance, histopathological changes in the uterus, antiox idant capacity, and antioxidant gene expression in late-phase layers.

Methods: Jinghong No.1 layers (n = 360, 68 weeks old) were assigned randomly to one of f our dietary interventions. Each treatment was replicated six times. Repeat 15 chickens per g roup. All birds were fed a diet composed of a corn-soybean meal-based diet supplemented with 0, 0.2, 0.4, or 0.8% PCHM for 6 weeks.

Results: Dietary PCHM supplementation had no significant effects on laying rate, feed con sumption, yolk color, and shape index. With increasing PCHM level the Haugh unit linearly increased ($P < 0.05$). Supplementation of 0.8% PCHM increased egg weight, compared with the control ($P < 0.05$). PCHM can effectively alleviated the pathological changes caused by aging in the uterus including hemorrhage, and many inflammatory cell infiltrations. Supplementation of 0.4% PCHM increased glutathione peroxidase (GSHPx) in liver, magnum, and plasm considerably, compared with the control ($P < 0.05$). Supplementation of PCHM decr ease in the liver, magnum, and uterus on malondialdehyde (MDA) content, compared with the control ($P < 0.05$). Compared with the control group, mRNA expressions of glutathione peroxidase 1 (GPX1), peroxidase 4 (GPX4), catalase (CAT), and nuclear factor E2-related factor 2 (Nrf2) in the magnum, liver, and uterus were dramatically rose in the 0.4% PCHM supplementation group ($P < 0.05$). In summary, dietary supplementation after PCHM increased egg weight and quality in late-phase laying hens.

Conclusion: Dietary PCHM increased the antioxidative capacity of late-phase laying hens, which could be associated with increased mRNA expression of antioxidant enzymes and Nrf2. These findings provide potential for using PCHM to increase the production performance in late-phase laying hens.

KEYWORDS

antioxidant capacity, egg quality, late-phase laying hens, pretreated Chinese herbal medicine, performance

Introduction

The final stage of the laying cycle is characterized by a sharp drop in both egg performance (Attia et al., 2020) and egg quality (Park and Sohn, 2018). Yolk synthesis and accumulation are both reduced as egg output performance (Zakaria et al., 1983). Parallelly, egg size or weight increases, and Haugh unit (HU) and eggshell breaking strength (EBS) decrease as hens age. It has been observed that laying hens' oviducal regression will directly affect egg production and quality (Gongruttananun et al., 2017). This may be related to the aging of oviducts. Age increases oxidant production from multiple sources, while antioxidant enzymes, the major lines of defense, decline (Zhang et al., 2015).

The activities of Chinese herbal medicines used as feed additives are extensive, including anti-apoptosis (Ibtisham et al., 2019), anti-inflammatory, and antioxidant ability (Xie et al., 2019). *Angelica* is a tonifying, nourishing, and edible herb with a long history of use, as well as for anti-inflammatory and antioxidant activity (Wei et al., 2016). Multiple live animal models of oxidative stress-related disease have shown that *Astragalus* extract offers substantial protection to the heart, kidneys, and intestines (Shahzad et al., 2016). *Epimedium* possesses multiple functions relevant in disease (Ma et al., 2011), including acting as an antioxidant (Yang et al., 2020). *Houttuynia cordata* also has medicinal properties (Wu et al., 2021), including acting as a free-radical scavenger and an anti-inflammatory (Ahn et al., 2017). Accumulating evidence has indicated that dietary herbs improve egg quality and production performance of laying hens. For example, Xie et al. (2019) found that the anti-inflammatory effect of Astragali can improve the albumen quality, and a blend of *Lonicera confusa* and *Astragali Radix* improved yolk color (Xie et al., 2019). *Epimedium* promotes follicular granulosa cell proliferation and differentiation, as well as hormone secretion and follicle development, which increases egg production rate (Guo et al., 2020a). Whole extracts or isolated compounds from *Angelica*, *Astragalus*, *Epimedium*, *Houttuynia*, and *Leonurus* can act as antioxidants. Currently, there are few studies on the five kinds of mixed Chinese herbal medicine in late-phase laying hens.

The objective of the current study was to investigate the effects of pretreated Chinese herbal medicine (PCHM) on production performance, egg quality, histopathological changes in the uterus, antioxidant capacity, and gene expression of antioxidant enzymes in late-phase laying hens.

TABLE 1 Main ingredients of the pretreated Chinese herbal medicine.

Item	Control group
Angelica	Angelica polysaccharide
Astragalus	Astragalus polysaccharide
Epimedium	Flavone
Houttuynia	Chlorogenic acid
Motherwort	Alkaloid

Materials and methods

Animal care and use

The Beijing University of Agriculture's Animal Care and Use Committee approved all experimental protocols (Approval ID: BUA-zc-20200073).

Experimental materials

Epimedium, *Astragalus*, *Angelica*, *Leonurus*, and *Houttuynia* were all purchased from Yiren Pharmaceutical Co., Ltd. (Baoding, Hebei, China). The test bacteria and enzymolysis enzymes were purchased from Beijing challenge Biotechnology Co., Ltd. (Beijing, China). The main components of *Angelica*, *Astragalus*, *Epimedium*, *Houttuynia*, and *Leonurus* are *Angelica* polysaccharide, *Astragalus* polysaccharide, flavone, chlorogenic acid, and alkaloid, respectively (Table 1). In this experiment, *Angelica* and *Astragalus* were treated by fermentation, *Leonurus* was treated by enzymatic hydrolysis, and *Houttuynia* and *Epimedium* were not treated. According to the pre-experiment, the main components of *Angelica* and *Astragalus* reach their highest level through fermentation, the main components of *Leonurus* through enzymatic hydrolysis, and the main components of *Houttuynia* and *Epimedium* through no treatment at all. According to the Chinese veterinary medicine code, the mixing ratio of *Epimedium*, *Astragalus*, *Angelica*, *Leonurus*, and *Houttuynia* after pretreatment is 1:2:2:1:2. After processing, the mixed herbs were called PCHM. Fermentation conditions: *Bacillus licheniformis*, *Bacillus coagulans*, *Aspergillus niger*, *Bacillus subtilis* (1:1:1:1), conditions: time: 5 days, temperature: 37°C, water content: 45%. Enzymatic hydrolysis conditions: complex enzyme (pectinase, cellulase, laccase), conditions: time: 4 h, temperature: 55°C, water content: 45%.

Birds and diets

A total of three hundred and sixty 68-week-old Jinghong No. 1 laying hens (initial body weights 1.65 ± 0.10 kg) with a similar weight and genetic background were used. A completely randomized design was used to divide the birds into the four treatment groups. Each treatment had six replicates with 15 birds each. All birds were fed a basal diet for 1 week before being assigned a diet containing maize-soybean meal containing 0, 0.2, 0.4, or 0.8% PCHM for 6 weeks. Table 2 displays the composition and nutritional levels of the diet based on maize-soybean meal. The experimental diet feeding period lasts for 6 weeks and the birds in the experiment were exposed to 16-h light cycles and had free access to water and the experimental diets at all times.

Sample collection

Every day, data on egg production and weight were logged. The number of eggs produced was expressed as an average hen-day production, which was derived by dividing the total number of hen-

TABLE 2 The composition and nutritional level of the basic diet (air-dried basis).

Ingredients	Content (%)	Nutrient level	Content (%)
Corn	62.0	Metabolizable energy, MJ·kg^{-1}	11.2
Soybean meal	24.0	Crude protein (%)	16.6
Soybean oil	1.00	Calcium (%)	3.26
Limestone	8.00	Available phosphorus (%)	0.40
Dicalcium phosphate	1.80	dl-Methionine (%)	0.34
Salt	0.35	l-Lysine (%)	0.82
dl-Methionine	0.10		
Premix[a]	2.75		
Total	100		

[a]The premix provided the following per kilogram of the diet: VA 12,500 IU, VD3 5,250 IU, VE 21.25 mg, VK3 4.375 mg, VB1 2.5 mg, VB2 11.25 mg, VB6 6.25 mg, VB12 3 mg, nicotinic acid 50 mg, D-pantothenic acid 40.75 mg, folic acid 6 mg, biotin 2.375 mg, Fe 87.5 mg, Zn 68 mg, Cu 9.5 mg, Mn 75 mg, I 1.5 mg, and Se 0.3 mg.

TABLE 3 Primer sequences used for quantitative real-time PCR.

Gene	Primer sequence (5′-3′)	Fragment size (bp)	Accession number
CAT	Forward: ACCAAGTACTGCAAGGCGAAAGT Reverse: ACCCAGATTCTCCAGCAACAGTG	91	NM_001031215.2
SOD1	Forward: TTGTCTGATGGAGATCATGGCTTC Reverse: TGCTTGCCTTCAGGATTAAAGTGAG	98	NM_205064
SOD2	Forward: CAGATAGCAGCCTGTGCAAATCA Reverse: GCATGTTCCCATACATCGATTCC	86	NM_204211.1
GPX1	Forward: TTCGAGAAGTTCCTCGTGGG Reverse: CCTGCAGTTTGATGGTCTCG	79	NM_0012778553.2
GPX4	Forward: TCAACCGTGAGGGCCAAGT Reverse: CTCGGCACGCAGCTCTAC	100	NM_001346448.1
Nrf2	Forward: ACATGGACAGTTCTCCTGGG Reverse: CGGCTCCACAGAAGGAAGTA	92	NM_205117.1
β-*Actin*	Forward: GCCAACAGAGAGAAGATGACAC Reverse: GTAACACCATCACCAGAGTCCA	118	NM_205518

TABLE 4 Comparison of different treatment methods of Chinese herbal.

Item	Control group	Enzymolysis	Fermentation	SEM	*p*-value		
					ANOVA	Linear	Quadratic
Angelica (%)	7.74^{b}	8.28^{b}	17.1^{a}	0.29	<0.01	0.07	0.94
Astragalus (%)	10.6^{b}	11.9^{b}	21.1^{a}	0.14	<0.01	<0.01	0.03
Epimedium (%)	7.58	7.99	8.14	0.21	0.21	0.23	0.94
Houttuynia (%)	11.9	11.6	12.8	0.01	0.40	0.33	0.33
Leonurus (%)	1.39^{b}	2.52^{a}	1.37^{b}	0.08	<0.01	<0.01	0.09

[a–b] Means within a row with no common superscripts differ significantly (P<0.05).

days by the number of eggs. Average egg weight was calculated as total egg weight divided by the number of eggs. Feed consumption was recorded on a replicate basis at weekly intervals. The feed conversion ratio was measured as the amount of feed consumed relative to the amount of eggs produced in kilograms. Egg quality was measured on three eggs collected randomly from each replicate on the 14th, 28th, and

TABLE 5 Effect of dietary PCHM supplementation on performance.

Item	Time (week)	PCHM (%)				SEM	*p*-value		
		0	0.2	0.4	0.8		ANOVA	Linear	Quadratic
Laying rate (%)	1–2	82.4	81.4	85.3	85.5	0.95	0.32	0.13	0.76
	3–4	80.3	82.3	85.2	83.4	0.89	0.10	0.11	0.05
	5–6	79.5	84.6	85.1	83.5	0.95	0.35	0.16	0.33
	1–6	80.7	82.8	85.2	84.1	0.68	0.11	<0.05	0.24
Feed efficiency (kg/kg)	1–2	2.38	2.35	2.29	2.25	0.03	0.52	0.14	0.96
	3–4	2.49^{a}	2.29^{b}	2.36^{b}	2.28^{b}	0.03	<0.05	<0.05	0.20
	5–6	2.48	2.34	2.31	2.26	0.03	0.07	<0.05	0.46
	1–6	2.45^{a}	2.33^{a}	2.32^{a}	2.27^{b}	0.02	<0.05	<0.01	0.43
Feed consumption (g/d)	1–2	120	120	120	120	0.17	0.87	0.47	0.76
	3–4	121	121	121	121	0.26	0.91	0.71	0.91
	5–6	122	122	121	121	0.20	0.19	<0.05	0.76
	1–6	121	121	121	121	0.14	0.87	0.42	0.94
Egg weight (g/egg)	1–2	61.2	62.2	61.4	62.5	0.28	0.28	0.23	0.94
	3–4	61.3^{b}	63.6^{a}	61.7^{b}	63.2^{a}	0.25	<0.01	0.07	0.94
	5–6	61.5	63.2	61.6	63.2	0.35	<0.05	0.13	0.55
	1–6	61.3^{b}	63.0^{a}	61.5^{b}	63.0^{a}	0.23	<0.01	<0.05	0.75

$^{a-d}$ Means within a row with no common superscripts differ significantly ($p < 0.05$).

42nd days. One healthy bird was chosen at random from each replicate at the end of the feeding period (one bird per replicate, and 24 birds in total). Exsanguination of the left jugular vein with scalpels was used to collect blood samples, which were then centrifuged at 4°C at 4,000 g for 10 min to separate plasma. Following collection, plasma samples were flash-frozen at −80°C and stored in the freezer until analysis. Birds were euthanized by exsanguination and necropsied, and the liver, magnum, and uterus were separated immediately and quickly frozen at −80°C for further analysis. Approximately 2 cm medial portion sections of the uterus were removed and cleaned thoroughly with 0.9% saline, then placed in 4% formaldehyde solution for tissue fixation and histological measurement.

Egg quality and performance measurement

The rate of egg production was determined as follows: egg production rate = eggs laid per day/(birds counted per day). Feed efficiency was calculated weekly. Haugh units, yolk color, and albumen height were measured using an egg analyzer (Orka Food Technology Ltd., Ramat Hasharon, Israel). Yolk color was defined according to the Roche yolk color fan, where 1 represents bright yellow and 15 represents dark yellow. An egg force reader (Herzliya, Tel Aviv, Israel) was used to measure eggshell strength. The thickness of the eggshell (ST) was calculated as follows: [ST, mm = SW/(ES d)], where SW is the weight of the eggshell, ES is the surface area of the egg, and d is the density of the material (2.3 g/cm^3 for calcium carbonate).

Histopathologic evaluation of uterine tissue

Uterine tissues were fixed in 4% paraformaldehyde and embedded in paraffin, and the 5 μm-thick sections were stained with hematoxylin-eosin (H&E). Samples were trimmed, dehydrated, embedded, sliced, dyed, and sealed in strict accordance with the standard operating procedures for pathological experiment detection of the service-bio unit (Liu et al., 2022). Histological samples were evaluated for degree of injury, using variables such as the integrity of tissue structure and the numbers of infiltrated inflammatory cells (service-bio, Wuhan, China).

Measurement of antioxidant enzyme activity

The plasma, liver, magnum, and uterus tissue concentrations of glutathione peroxidases (GSH-Px), catalase (CAT), superoxide dismutase (SOD), and the ability to scavenge superoxide anion radicals and the hydroxyl radical malondialdehyde (MDA) were measured by enzyme-linked immunosorbent assay (ELISA) using a commercial ELISA kit for chicken (Nanjing Jiancheng Bioengineering Institute, Nanjing, China; catalog no. A003-1-2, A007-1-1, A001-1-2, A005-1-2, A052-1-1, A018-1-1) according to the manufacturer's instructions. T-SOD activity was determined by using the xanthine/xanthine oxidase method, which is based on the inhibition of nitroblue tetrazolium formazan. The activity of GSH-Px was measured using H_2O_2 as a substrate in the presence of reduced glutathione. The GSH-Px activity was reported in micromoles of oxidized NADPH per minute.

TABLE 6 Effect of dietary PCHM supplementation on egg quality.

Item	Time (week)	PCHM (%)				SEM	*p*-value		
		0	0.2	0.4	0.8		ANOVA	Linear	Quadratic
Haugh unit	1–2	66.7	68.7	66.2	69.3	0.90	0.59	0.54	0.78
	3–4	66.2^{b}	66.3^{b}	68.4^{ab}	73.5^{a}	1.02	<0.05	<0.01	0.17
	5–6	70.4	72.6	73.9	75.4	1.01	0.35	0.08	0.87
	1–6	67.8^{b}	69.2^{ab}	69.5^{ab}	72.7^{a}	0.59	<0.05	<0.01	0.37
Yolk color	1–2	5.11	6.11	5.94	5.94	0.15	0.05	0.06	0.07
	3–4	5.55	5.95	5.53	5.58	0.09	0.32	0.68	0.34
	5–6	6.17	6.44	6.59	6.83	0.13	0.24	<0.05	0.99
	1–6	5.61	6.14	6.04	6.12	0.09	0.09	0.06	0.17
Albumen height	1–2	5.08	5.13	4.92	5.25	0.09	0.68	0.71	0.48
	3–4	4.81^{b}	4.63^{b}	4.77^{b}	5.69^{a}	0.12	<0.01	<0.01	0.03
	5–6	5.71^{b}	5.84^{ab}	5.89^{ab}	6.45^{a}	0.12	<0.01	<0.05	0.34
	1–6	5.08^{b}	5.22^{b}	5.17^{b}	5.78^{a}	0.08	<0.01	<0.01	0.09
Eggshell strength (N/cm^{2})	1–2	2.49^{b}	2.96^{a}	2.88^{a}	2.82^{ab}	0.06	0.01	0.04	<0.01
	3–4	2.62	3.0	3.17	2.74	0.10	0.15	0.60	<0.05
	5–6	2.91	2.96	2.96	2.94	0.08	0.99	0.90	0.83
	1–6	2.67	3.00	3.00	2.83	0.05	0.06	0.26	<0.05
Shell thickness (μm)	1–2	0.35	0.35	0.35	0.35	0.01	0.17	0.78	<0.05
	3–4	0.35	0.35	0.35	0.35	0.01	0.17	0.78	<0.05
	5–6	0.35	0.35	0.35	0.34	0.01	0.50	0.33	0.33
	1–6	0.35	0.35	0.35	0.35	0.00	0.20	0.93	<0.05
Shape index	1–2	1.31	1.30	1.32	1.30	0.01	0.93	0.98	0.80
	3–4	1.31	1.33	1.31	1.32	0.01	0.92	0.86	0.96
	5–6	1.32	1.32	1.33	1.32	0.01	0.96	0.88	0.95
	1–6	1.32	1.31	1.31	1.32	0.04	0.99	0.96	0.87

$^{a–d}$ Means within a row with no common superscripts differ significantly ($p < 0.05$).

Quantitative PCR analysis

Total RNA was extracted from 0.1 g of liver, magnum, and uterus tissue sample using TRIzol reagent (Thermo Fisher Scientific, Shanghai, China), as per the manufacturer's instructions, and then reverse-transcribed into single-stranded cDNA using the Thermo First cDNA Synthesis Kit (Promega, Beijing, China). The expressions of nuclear factor E2 related factor 2 (*Nrf2*), Cu-Zn superoxide dismutase (*SOD*) *1*, Mn superoxide dismutase (*SOD*) *2*, catalase (*CAT*), glutathione peroxidase 1 (*GPX1*), and peroxidase 4 (*PX4*) were determined using qRT-PCR with specific primers (Table 3). An AriaMx Real-Time PCR system (Agilent Technologies, Santa Clara, California, United States) was used for quantitative PCR analysis. After initial denaturation at 95°C for 10 min, 40 cycles of amplification were carried out (95°C for 10 s and 58.2°C for 30 s), followed by the generation of melt curves that could be used to verify the specificity of amplification. The samples were tested in triplicate. The $2^{-\Delta\Delta CT}$ (Livak and Schmittgen, 2001) method was used to calculate the relative gene expression levels. Refer to the operation of (Madkour et al., 2021) for specific process.

Statistical analysis

All statistical analyses were performed using SPSS 22.0 (IBM Corp., Armonk, NY, United States). Data are presented as mean ± standard deviation of the mean (SD). Additionally, polynomial regression analysis was used to test the linear and quadratic nature of the response to the additive PCHM dosage, and Tukey's multiple comparison tests were used to analyze the differences among various treatments. $p < 0.05$ was considered significant.

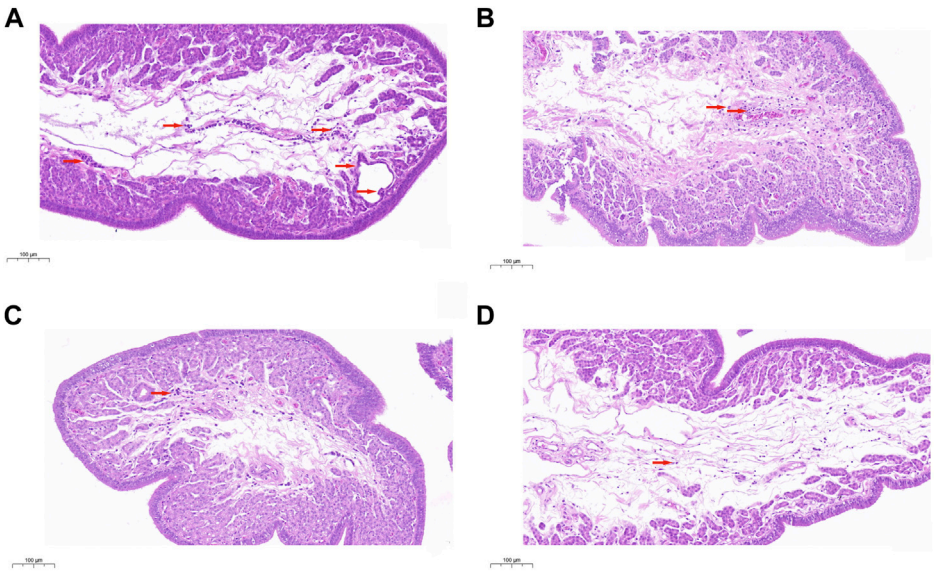

FIGURE 1
Tissues from the uterus were removed and stained with H&E. Scale bar: 100 μm. **(A)** Uterine histopathology in the control group; **(B–D)** Uterine histopathology with PCHM treatment groups at 0%, 0.2%, 0.4%, and 0.8%, respectively. The locations of tissue damage and inflammatory cell infiltration are denoted by the directional arrows in red.

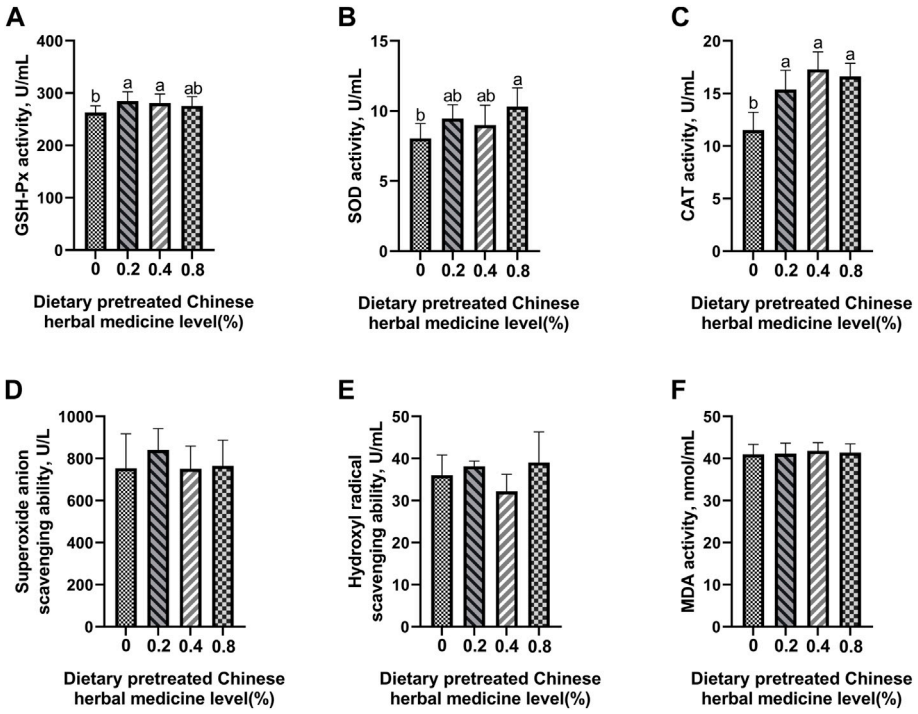

FIGURE 2
Effect of dietary PCHM supplementation on the antioxidant enzyme activity and free radicals in the plasma of late-phase laying hens. **(A–C)** Glutathione peroxidase (GSH-Px), superoxide dismutase (SOD), and catalase (CAT) activity in the plasma. **(D,E)** Scavenging free radical abilities in the plasma. **(F)** Malondialdehyde (MDA) level in the plasma. Values are expressed as means ± SEM of six birds per treatment. Means without a common letter differ ($p < 0.05$).

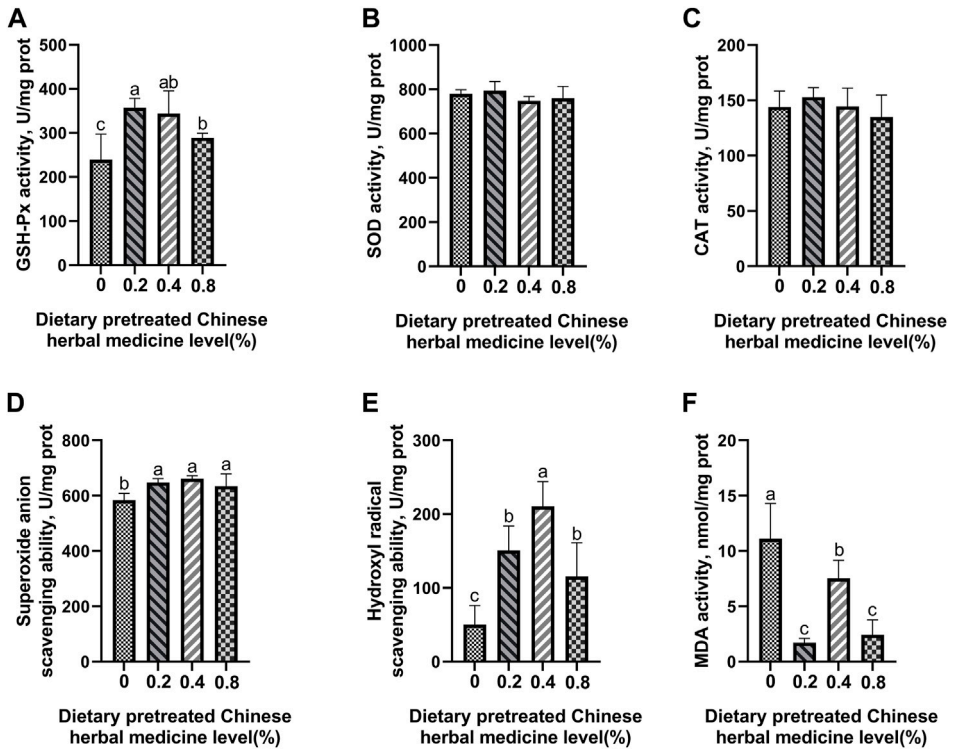

FIGURE 3
Effect of dietary PCHM supplementation on the antioxidant enzyme activity and free radicals in the liver of late-phase laying hens. **(A–C)** Glutathione peroxidase (GSH-Px), superoxide dismutase (SOD), and catalase (CAT) activity in the liver. **(D,E)** Scavenging free radical abilities in the liver. **(F)** Malondialdehyde (MDA) level in the liver. Values are expressed as means ± SEM of six birds per treatment. Means without a common letter differ ($p < 0.05$).

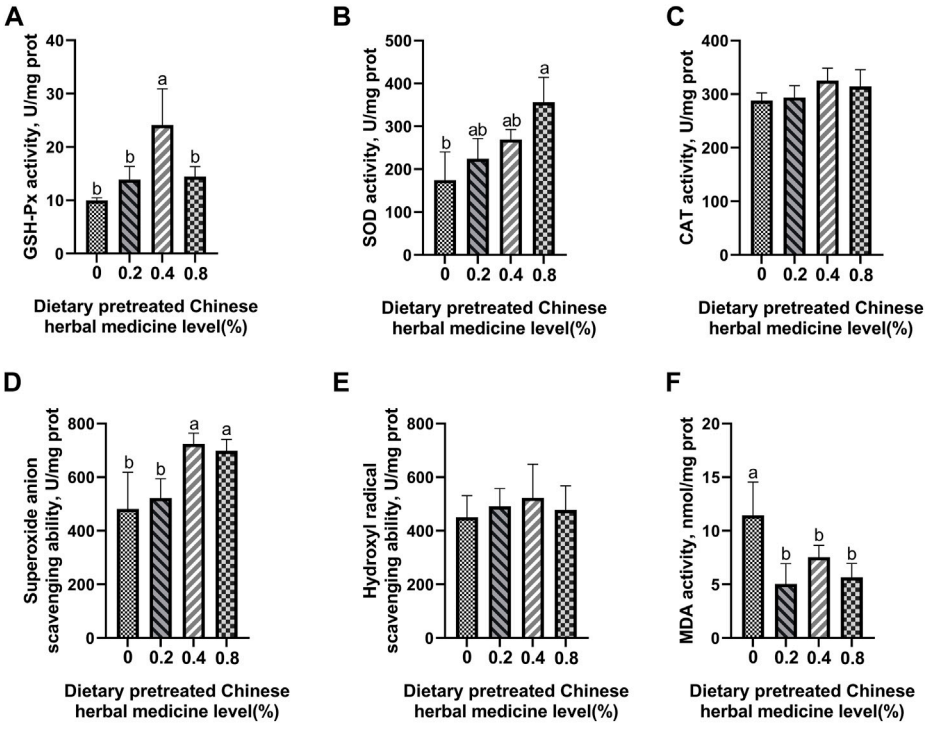

FIGURE 4
Effect of dietary PCHM supplementation on the antioxidant enzyme activity and free radicals in the magnum of late-phase laying hens. **(A–C)** Glutathione peroxidase (GSH-Px), superoxide dismutase (SOD), and catalase (CAT) activity in the magnum. **(D,E)** Scavenging free radical abilities in the magnum. **(F)** Malondialdehyde (MDA) level in the magnum. Values are expressed as means ± SEM of six birds per treatment. Means without a common letter differ ($p < 0.05$).

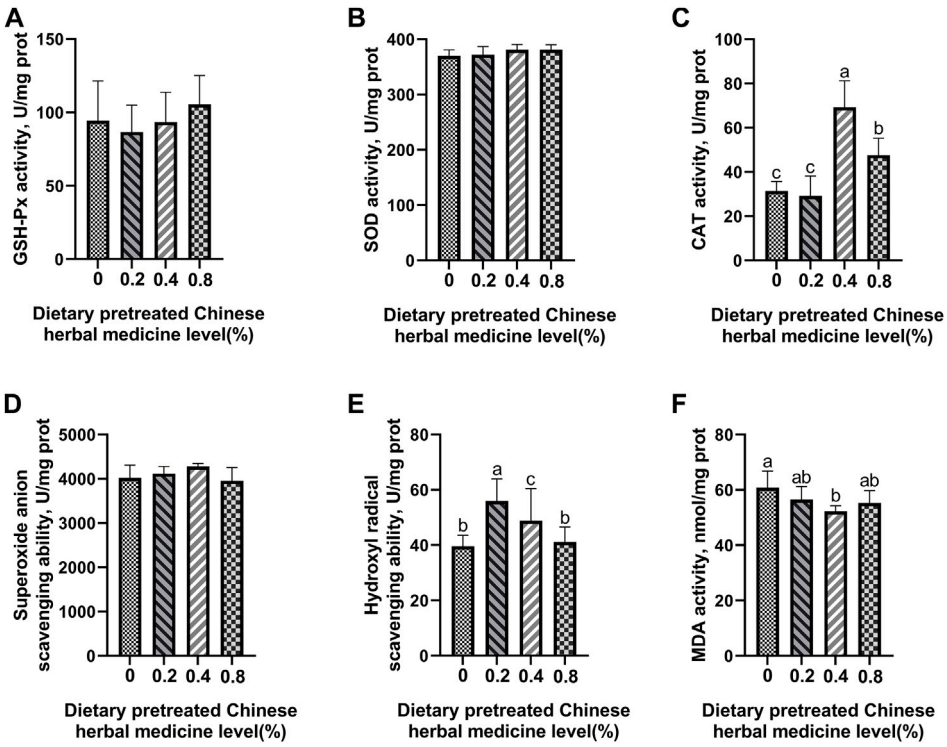

FIGURE 5
Effect of dietary PCHM supplementation on the antioxidant enzyme activity and free radicals in the uterus of late-phase laying hens. **(A–C)** Glutathione peroxidase (GSH-Px), superoxide dismutase (SOD), and catalase (CAT) activity in the uterus. **(D,E)** Scavenging free radical abilities in the uterus. **(F)** Malondialdehyde (MDA) level in the uterus. Values are expressed as means ± SEM of six birds per treatment. Means without a common letter differ ($p < 0.05$).

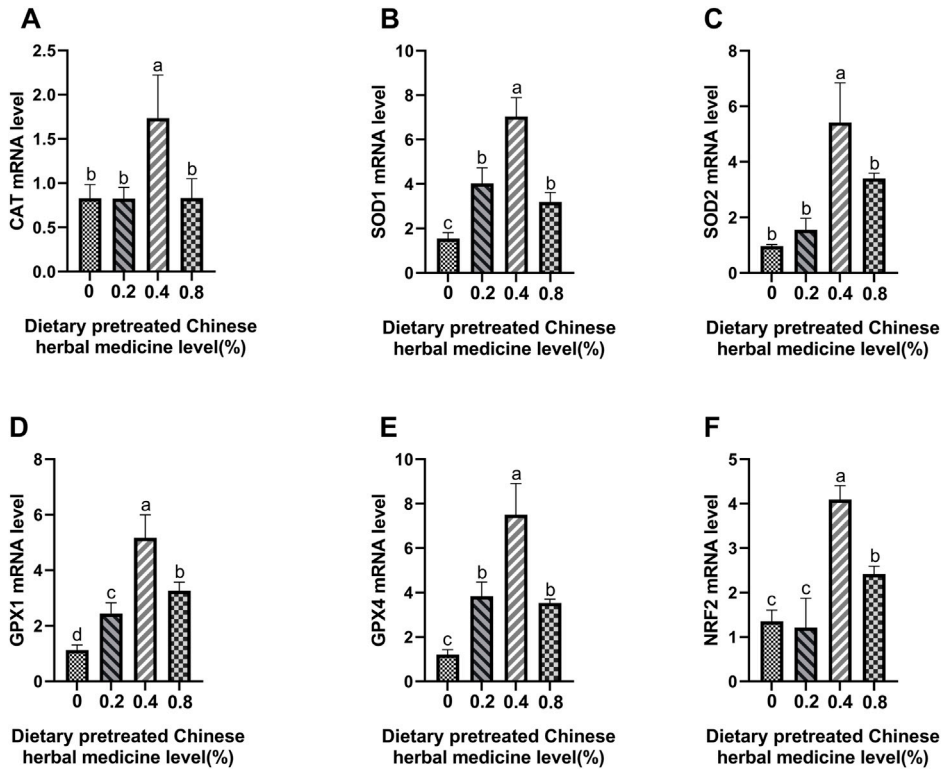

FIGURE 6
Effects of dietary PCHM supplementation on antioxidation-related mRNA expression in liver. The effect of adding PCHM (0, 0.2%, 0.4%, 0.8%) in diets in the liver, measured by quantitative PCR: **(A)** *CAT*, **(B)** *SOD1*, **(C)** *SOD2*, **(D)** *GPX1*, **(E)** *GPX4*, and **(F)** *Nrf2*. Values are expressed as means ± SEM of six birds per treatment. Means without a common letter differ ($p < 0.05$).

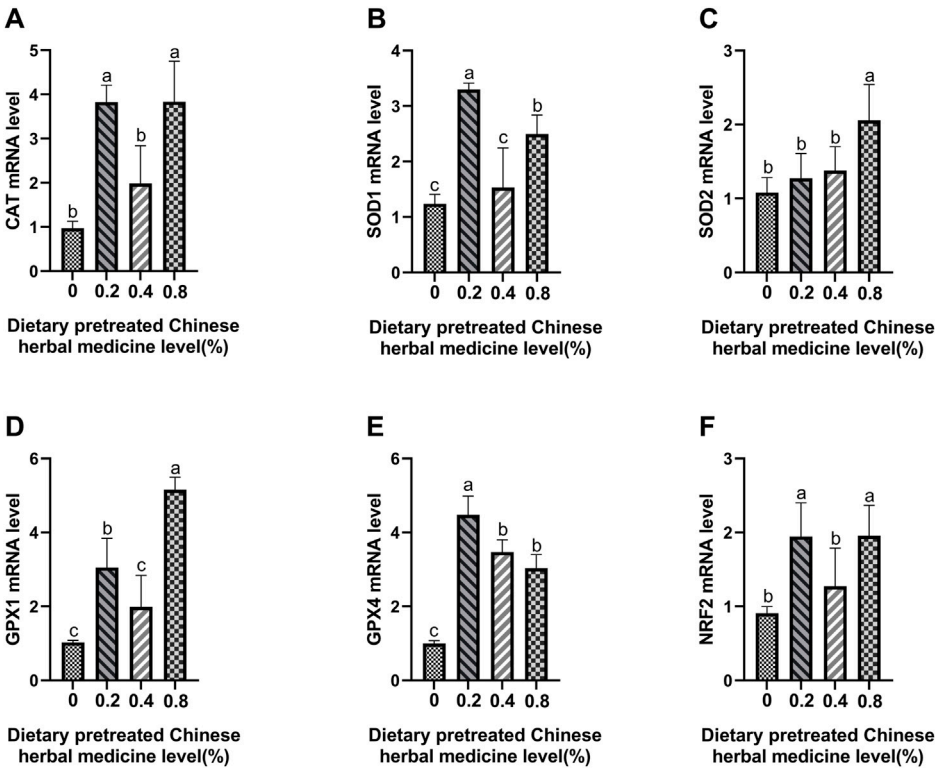

FIGURE 7
Effects of dietary PCHM supplementation on antioxidation-related mRNA expression in magnum. The effect of adding natural herbs (0, 0.2%, 0.4%, 0.8%) in diets in the magnum, measured by quantitative PCR: **(A)** *CAT*, **(B)** *SOD1*, **(C)** *SOD2*, **(D)** *GPX1*, **(E)** *GPX4*, and **(F)** *Nrf2*. Values are expressed as means ± SEM of six birds per treatment. Means without a common letter differ ($p < 0.05$).

Results

Comparison of components of Chinese herbal medicine

Comparison of the main components of five Chinese herbal medicines is shown in Table 4. *Angelica*, *Astragalus* fermentation treatment, *Leonurus* enzymolysis treatment, *Houttuynia*, and *Epimedium* did not have treatment.

Performance and egg quality

The effects of dietary supplementation of PCHM on performance and egg quality are shown in Table 5 and Table 6. PCHM supplementation had no effect on laying rate, feed consumption, yolk color, shell thickness, shape index; PCHM supplementation had a significant effect on feed efficiency, egg weight, albumen height, and eggshell strength ($p < 0.05$). With increasing dietary supplementation levels of PCHM, the Haugh unit increased linearly ($p < 0.05$).

Histopathological analysis

The histological analysis was performed to assess the pathological changes in uterine tissues. The results demonstrated that aging resulted in severe injury, including hyperemia, hemorrhage, and many inflammatory cell infiltrations (Figure 1A). However, the aging-induced pathological changes were dramatically improved by PCHM at doses of 0.2 (Figure 1B), 0.4 (Figure 1C), and 0.8% (Figure 1D). These results indicate that PCHM could effectively attenuate aging-induced pathological changes.

Antioxidant enzyme activity and free-radical scavenging ability

The effect of dietary PCHM supplementation on the antioxidant enzyme activity and free radical scavenging capabilities in plasma, liver, magnum, and uterus are depicted in Figures 2–5, respectively. The PCHM supplementation significantly influenced the antioxidant enzyme activity and free radical scavenging capabilities in plasma, liver, magnum, and uterus. In the plasma, a significant ($p < 0.05$) increased in the protein level of CAT and GSH-Px was noticed in the 0.2% and 0.4% PCHM groups, and CAT and SOD in 0.8% PCHM group supplemented were observed as compared to the other experimental groups. In liver, a significant ($p < 0.05$) increased in the protein level of GSH-Px and free radical scavenging capabilities was noticed in the PCHM supplementation as compared to the control. Further, in liver a significant ($p < 0.05$) decreased the content of MDA in the PCHM supplementation as compared to the control. In magnum, a significant ($p < 0.05$) increased of GSH-Px protein level in the 0.4% PCHM groups, SOD protein level in

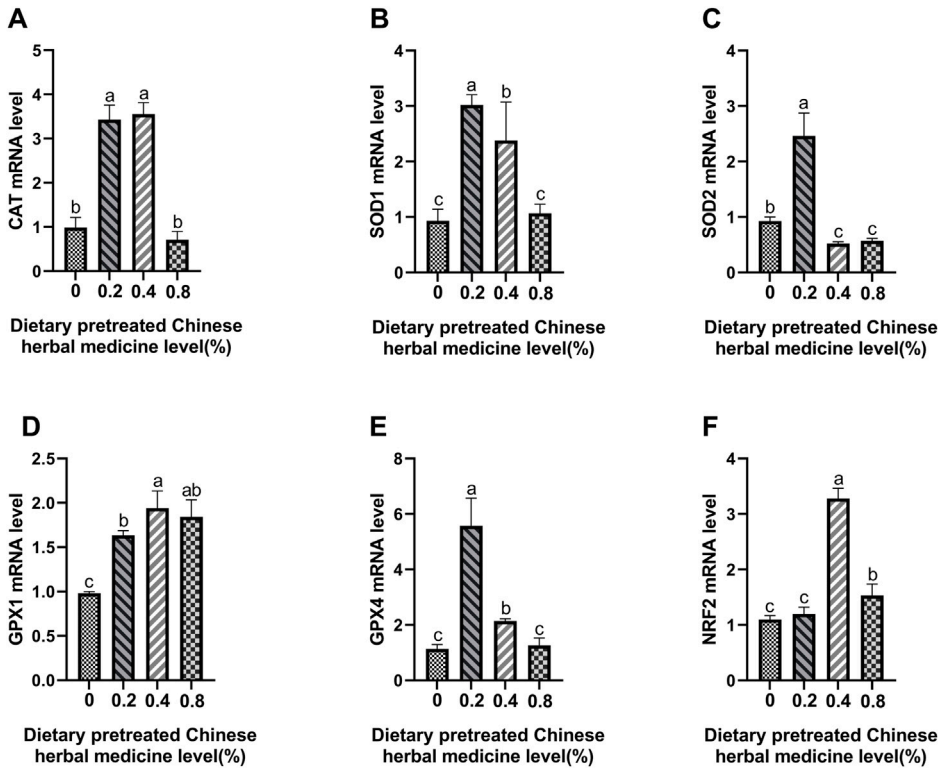

FIGURE 8
Effects of dietary PCHM supplementation on antioxidation-related mRNA expression in uterus. The effect of adding PCHM (0, 0.2%, 0.4%, 0.8%) in diets in the uterus, measured by quantitative PCR: **(A)** *CAT*, **(B)** *SOD1*, **(C)** *SOD2*, **(D)** *GPX1*, **(E)** *GPX4*, and **(F)** *Nrf2*. Values are expressed as means ± SEM of six birds per treatment. Means without a common letter differ ($p < 0.05$).

0.8% PCHM group, the ability to scavenge superoxide anion in 0.4% and 0.8% PCHM groups were observed as compared to the other experimental groups. Further, in magnum a significant ($p < 0.05$) decreased the content of MDA in the PCHM supplementation as compared to the control. Similarly, in uterus, a significant ($p < 0.05$) increased of CAT protein level in the 0.4% and 0.8% PCHM groups, the ability to scavenge hydroxyl radical in 0.2% and 0.4% PCHM group were observed as compared to the other experimental groups. Further, in uterus, significantly ($p < 0.05$) decreased the content of MDA in the 0.4% PCHM groups were evident as compared to the other experimental groups.

Antioxidant enzymes and Nrf2 mRNA expression

The effect of dietary PCHM supplementation on the expressions of *CAT*, *SOD1*, *SOD2*, *GPX1*, *GPX4*, and *Nrf2* genes in liver, magnum and uterus are depicted in Figures 6–8, respectively. The PCHM supplementation significantly influenced the expression of these genes in liver, magnum, and uterus. Expression of all the genes was significantly ($p < 0.05$) upregulated in liver with 0.4% PCHM supplementation as compared to the control. In magnum, a significant ($p < 0.05$) increase in the expression of *CAT* and *Nrf2* was noticed in the 0.2% and 0.8% PCHM supplemented as compared to the other groups. Further, in magnum, significantly ($p < 0.05$) greater expression of *SOD1* and *GPX4* in the 0.2 PCHM groups, and *SOD2* and *GPX1* in the 0.8% PCHM group were evident as compared to the other experimental groups. Similarly, in uterus, a significant ($p < 0.05$) upregulated expression of *CAT*, *SOD1* and *GPX4* in the 0.2% and 0.4% PCHM groups, *SOD2* in 0.2% PCHM group, *GPX1* in 0.2%, 0.4%, and 0.8% PCHM groups, and *Nrf2* in the 0.4% and 0.8% PCHM groups were observed as compared to the other experimental groups.

Discussion

The purpose of this study was to investigate how dietary supplementation of PCHM affected production performance, egg quality, histopathological changes in the uterus, antioxidant capacity, and antioxidant enzyme gene expression in late-phase laying hens. Egg quality is important in commercial layers (Odabasi et al., 2007). The quality of eggs, notably their shells, gradually decreases as hens age past their prime laying years (Bain et al., 2016). It has been reported that supplementation of laying hen diets with herbs can improve their egg-laying rate (Li et al., 2017), Haugh unit (Moon et al., 2021), and shell strength (Xiao et al., 2019). The present study revealed that dietary supplementation of PCHM increased average egg weight, shell thickness, eggshell strength, Haugh unit and albumen height, but did not affect the laying rate, feed consumption, yolk color, or shape index. With increasing dietary levels of PCHM, the Haugh unit increased linearly. The improvement of Haugh unit may be caused by the polysaccharide contained in PCHM (Guo et al., 2020b). Similar findings were reported by Xie et al.

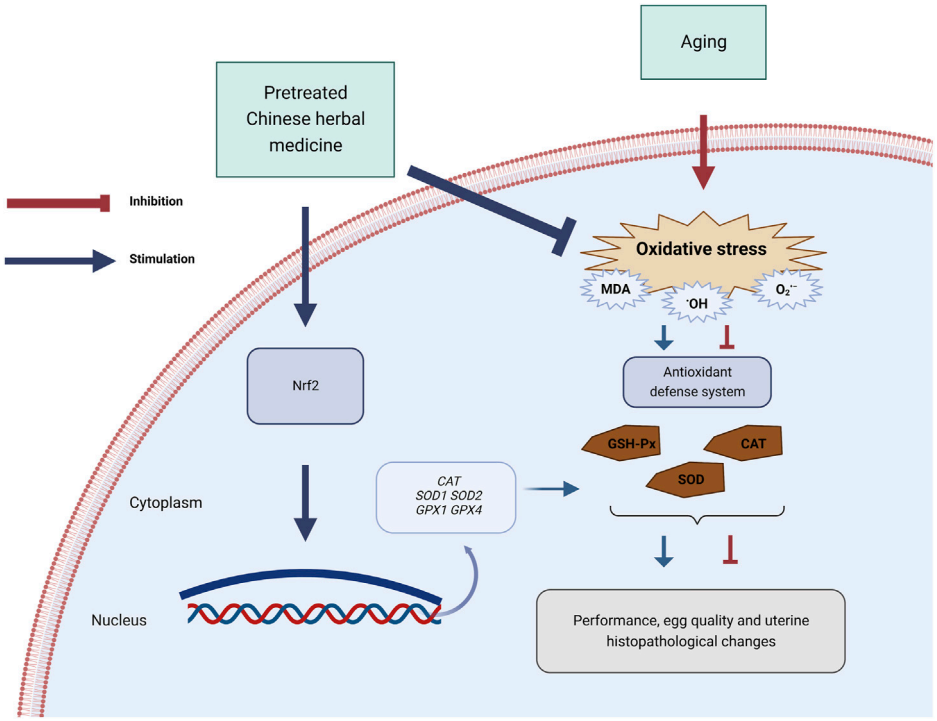

FIGURE 9
Schematic diagram summarizing the mechanisms by which pretreated Chinese herbal medicine (PCHM) promote the antioxidant defense system in late laying hens. The antioxidant defense system is downregulated in the natural aging process in laying hens. PCHM attenuated the oxidative stress in the uterus *via* the activation of the nuclear factor-erythroid 2-related factor 2 (*Nrf2*) pathway to increase the age-related up-produce in commercial laying hens. CAT, catalase; GSH-Px, glutathione peroxidase; SOD, superoxide dismutase; MDA, malondialdehyde; *SOD1*, Cu-Zn superoxide dismutase; *SOD2*, Mn superoxide dismutase; *CAT*, catalase; *GPX1*, glutathione peroxidase 1; *GPX4*, peroxidase 4. Figures generated with BioRender (https://biorender.com/).

(2019), who observed that diets including herbal medicine (*Lonicera confusa* and *Astragali Radix*) improved the albumen quality in laying hens. The antioxidant ability of PCHM's bioactive components is responsible for these changes in Haugh unit. In particular, prior investigations (Costa et al., 2019) have revealed the antioxidant capabilities of PCHM, which likely prevent protein oxidation in eggs.

To explore a possible mechanism for the improvement in egg qualities, and feed efficiency in late-phase laying hens with PCHM supplementation, histopathological changes in the uterus and antioxidant status were determined. The ability to ovulate was closely related to the uterus (Kong et al., 2012). Aging causes certain changes in the tissue morphology of uterus such as swelling of collagen fibres, elastic tissue fragments and oedema (Mulholland and Jones, 1993). Bitter ginseng bases extracted from the Chinese herb bitter ginseng were reported to significantly reduce uterine damage in a mouse model induced by *Staphylococcus aureus* lipophosphatidic acid (Jiang et al., 2019). In the current study, PCHM supplementation attenuated the uterine injury in late-phase laying hens, which the improvement of uterine injury was probably related to the ability of PCHM to inhibit lipid peroxidation (Sikiru et al., 2019). Recent research too demonstrates that ginsenoside ameliorates pathological uterine damage by boosting the anti-oxidant enzymes SOD and GSH (He et al., 2021).

Oxidative stress contributes to aging and age-related disorders (Miyazawa et al., 2009). MDA is a lipid peroxidation marker used to evaluate lipid peroxidation as a result of elevated oxidative stress (Ottolenghi et al., 2019). Oxidative stress may come from either an excess of free radical generation or a breakdown of antioxidant defense systems (Torun et al., 2009). It has been reported that some herbs possess strong radical-scavenging ability (Jarco et al., 2021). In the current study, PCHM was added to the diet to effectively improve the free-radical scavenging capacity and reduce the content of MDA in liver, magnum, and uterus. A previous report indicates that supplementation of 0.8 g/kg herbs lowers MDA content in egg yolk by increasing antioxidant enzyme activity (Chen et al., 2018). Recent studies have shown that improvement of free-radical scavenging ability may be related in part to antioxidant enzyme activity (Heng et al., 2021). It is reported that GSH-Px is an important antioxidant enzyme that can convert hydrogen peroxide into water (Zhou et al., 2021). Similarly, dietary supplementation of ginger powder increases the antioxidant enzymatic activity of laying hens (Lee et al., 2012). These results of the current study indicate that dietary supplementation with PCHM at 0.4% was most effective in improving the antioxidant capacity of late-phase laying hens.

Superoxide dismutases (SODs) are an important class of metallo-antioxidant enzymes in the metabolism of ROS in living organisms (Zhao et al., 2021). Selenium (Se) is of great importance in the treatment of diseases caused by oxidative stress (Rashid and Coombs, 2019). Previous studies have shown that selenomethionine promotes the expression of *Nrf2* transcription factor-related genes in cells during lipopolysaccharide stimulation (Adeniran et al., 2022). The change in antioxidant enzyme activity is related to gene expression (Guo et al., 2014). Previous research has demonstrated that the addition of tanshinone can boost *GPX1* mRNA expression in

macrophages (Li et al., 2008). Dietary supplementation with PCHM elevated the mRNA expression of *CAT*, *SOD1*, *SOD2*, *GPX1*, and *GPX4* in the liver and uterine in the current study. In particular, dietary supplementation with 0.4% PCHM is most effective in the liver.

Nrf2 is regarded as an important regulator of the cellular antioxidant response (Motohashi and Yamamoto, 2004). Studies have shown that with the overexpression of *Nrf2*, its downstream gene expression levels were significantly upregulated (Xiao et al., 2020; Xue et al., 2021). *Nrf2*-null affects mRNA expression of *SOD1*, *SOD2*, and *CAT* (Reisman et al., 2009). A previous study showed that theaflavin promotes resistance to oxidative stress-induced cell damage by activating *Nrf2* (Li et al., 2021). In this research, adding PCHM to the diet dramatically increased *Nrf2* mRNA levels in the liver and the uterine. The reason for improvement is related to the polysaccharide contained in PCHM (Liu et al., 2022).

In conclusion, PCHM supplementation boosted egg weight and quality in late-stage laying hens. This result may be attributable to the increase in antioxidant enzyme activity, which may be associated with increased antioxidant enzyme mRNA expression and *Nrf2* expression (Figure 9). However, the underlying mechanism through which dietary supplementation of PCHM enhances the mRNA expression of antioxidant enzymes requires additional investigation.

Data availability statement

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

Ethics statement

The animal study was reviewed and approved by the Beijing University of Agriculture's Animal Care and Use Committee approved all experimental protocols. Written informed consent was obtained from the owners for the participation of their animals in this study.

Author contributions

A-CY: Prepared and wrote the manuscript; M-AW: Designed the study and contributed to the experiment; CL: Edited the English language and wrote the manuscript; YG, H-MN, KX, and LC: Supervised and directed the experiment; X-GW, L-FX, and X-HS: Supervised and directed molecular experiments; J-TL and X-LQ: Designed the study and reviewed the manuscript.

Funding

This study was supported by Special Fund for Beijing Innovation Team Construction of Modern Agricultural Industrial Technology System (BJJQ-G11), Technology Innovation "spark action" support program of Beijing University of Agriculture (BUA-HHXD2022008) and the Project of Tianjin Jiuzhou Dadi Feed Co., Ltd. "Oviduct Health Technical Services for Laying Hens" (20210802).

Acknowledgments

Thanks to Yifan Bao, (Department of Physical Chemistry, University of Vienna) for exporting Figure 9 from Borender.com.

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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frontiers | Frontiers in Physiology

TYPE Original Research
PUBLISHED 28 March 2023
DOI 10.3389/fphys.2023.1144997

OPEN ACCESS

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SPECIALTY SECTION
This article was submitted to Avian Physiology, a section of the journal Frontiers in Physiology

RECEIVED 15 January 2023
ACCEPTED 20 March 2023
PUBLISHED 28 March 2023

CITATION
Zou Q, Meng W, Li C, Wang T and Li D (2023), Feeding broilers with wheat germ, hops and grape seed extract mixture improves growth performance. *Front. Physiol.* 14:1144997. doi: 10.3389/fphys.2023.1144997

Feeding broilers with wheat germ, hops and grape seed extract mixture improves growth performance

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In the study, Wheat germ, Hops and Grape seed extracts were made into a mixture (BX). The BX was supplemented in AA + broilers diets to investigate the effects of BX on broiler growth performance, blood indicators, microbiota, and noxious gas emissions in faeces. Four hundred and eighty 1-day-old AA + male broilers with an average initial body weight (44.82 ± 0.26) were randomly divided into four dietary treatments of six replicates each, with 20 birds per replicate. The experimental groups consisted of a group fed a basal diet and groups fed basal diet supplemented with 0.05%, 0.1%, and 0.2% BX. The trail was 42 days. The results showed that supplementing the dietary with graded levels of BX linearly increased ADG and ADFI from days 22–42 and 1–42. When dietarys supplemented with 0.2% BX significantly increased ADG and ADFI on days 22–42 and 1–42 ($p < 0.05$). The addition of BX reduced H_2S and NH_3 emissions in the faeces; the levels of *E. coli* and *Salmonella* in the faeces were significantly reduced and the levels of *Lactobacillus* were increased ($p < 0.05$). In this trial, when the diet was supplemented with 0.2% BX, faecal levels of *E. coli* and *Salmonella* were consistently at their lowest levels and *Lactobacillus* were at their highest. At the same time, NH_3 and H_2S emissions from broiler faecal also had been at their lowest levels. Conclusion: Dietary supplementation with a 0.2% BX could improve the growth performance of broilers and also reduced faecal H_2S and NH_3 emissions, as well as faecal levels of *E. coli* and *Salmonella*, and increased levels of *Lactobacillus*. Thus, BX made by Wheat germ, Hops and Grape seed extract is expected to be an alternative to antibiotics. And based on the results of this trial, the recommended dose for use in on-farm production was 0.2%.

KEYWORDS

wheat germ, hops extract, grape seed extract, broilers, growth performance, blood indicators, faecal microbiota, noxious gas

Introduction

A complete grain of wheat consists of bran, endosperm and germ, with the germ making up about 3% of the grain weight (Fardet, 2010). Wheat germ (WG) was the life source of wheat and was a major by-product of the wheat flour industry. WG contains approximately 10%–15% fat, 26%–35% protein, 17% sugar, 1.5%–4.5% fibre, and 4% minerals (Brandolini and Hidalgo, 2012). Matsui et al. (2000) obtained an angiotensin-converting enzyme inhibitory peptide with the sequence Ile-Val-Tyr by isolating a

peptide produced by the enzymatic digestion of wheat germ protein. Meanwhile, many researchers had explored the hypolipidemic (Liaqat et al., 2021), anti-aging (Zhao et al., 2021), anti-inflammatory (Sui et al., 2020), and antioxidant effects (Wang et al., 2020) of WG. Studies had shown that broilers fed wheat germ oil achieve higher body weights (Arshad et al., 2013a). When combined with *a*-lipoic acid, wheat germ oil could help improve lipid distribution in broilers (Arshad et al., 2013b). To date, no papers have been published on the effects of crushing wheat germ and feeding it directly to broilers.

Hops (Humulus lupulus) is a perennial plant of the Cannabaceae family, which has only two genera. The genus Humulus and the genus Cannabis. The hops plant plays an important role in human nutrition and culture, as its female inflorescences are used to produce beer. Extracts of the hops plant had an antibacterial effect on Gram-positive bacteria (Teuber and Schmalreck, 1973; Srinivasan et al., 2004). The addition of beta-acids from hops to broiler diets had been reported to improve the overall redox stability and nutritional properties of broilers (Zawadzki et al., 2018). It had been shown that extracts of hops (xanthohumol) could upregulate the expression of phase II enzymes while increasing the protein, activity and glutathione levels of these enzymes (Yao et al., 2015). And, the anti-inflammatory effect of xanthohumol might be related to its modulation of Nrf2-ARE signalling (Lee et al., 2011).

Grape by-products, mainly consisting of skins, seeds and stems, are rich in polyphenols. Grape Seed Extract (GSE) contains mainly proanthocyanidins (PA), catechins, quercetin and tannins. Of these, PA is the most abundant and purest, and is the main substance in the GSE that possesses a biological function. Studies had shown that GSE had antibacterial (Ahn et al., 2004), anti-inflammatory (Carini et al., 2001), antioxidant (Luther et al., 2007), and anticancer (Cheah et al., 2014) activities, with protective effects on host skin (Yamakoshi et al., 2003), cardiovascular (Nunes et al., 2016), liver (Hassan and Al-Rawi, 2013), and nervous system (Mahmoud, 2013). Farahat et al. (2017) reported that GSE increased Newcastle disease virus antibody potency in broiler serum and effectively reduced malondialdehyde levels in meat tissue. In addition, the negative effects of aflatoxin B1 could be effectively reduced by adding a certain amount of GSE to broiler diets (Ali Rajput et al., 2017).

Many studies had reported that the use of botanical preparations in animal feed as dietary supplements could effectively regulate animal metabolism and influence animal welfare and meat quality (Patra and Saxena, 2011; Zawadzki et al., 2017). Many researchers had reported on the physiological properties and beneficial effects of wheat germ, hops and grape seed extracts. However, the effect of adding a mixture of wheat germ, hops and grape seed extract to broiler dietary as a feed additive on broiler production has not been reported. Therefore, in this trial, wheat germ, hops and grape seed extract mixture (BX) was supplemented in AA + broiler diets to study its effects on broiler growth performance, blood indicators, faecal microbiota and noxious gas emissions. It is hoped that this will provide a theoretical basis for the future use of plant additives in poultry production.

Materials and methods

Ethics statement

The Animal Conservation and Utilisation Committee of the JZMU approved the animal use agreement.

Prebiotic sources

BX was supplied by Liaoning Kaiwei Biotechnology Co., Ltd. and consisted mainly of 20% wheat germ, 25% hops extract, 30% grape seed extract and 25% inert carrier (silica). Wheat germ was supplied in powder form. Hops extract contained ≥70% xanthohumol. Grape seed extract contained ≥90% proanthocyanidins.

Animals and experimental design

The trial was a completely randomised group design. Four hundred and eighty 1-day-old AA + male broilers of similar weight (44.82 ± 0.26) and health were selected and randomly divided into four groups of six replicates each, with 20 broilers in each replicate. The experimental group consisted of a base diet group fed a basic basal diet and a base diet group fed 0.05%, 0.1% and 0.2% of BX.

Animals feeding management

All birds were housed in the experimental cages (1.25 × 0.80 × 0.50 m/cage) in a test broilers room. The test broilers house was a fully enclosed house with an automatic environmental control system to ensure optimum temperature and humidity (Temperature was started at 33°C and reduced by 3°C every week up to 22°C, and 65% relative humidity). Birds were free to feed and drink. The lighting programme on days 1–7 and 36–42 was 24 h per day throughout the trial period. The lighting programme on days 8–30 provided 20 h per day and 4 h of darkness. After day 31, the darkness hours were gradually reduced. Diets were formulated to meet the nutrient requirements recommend by the National Research Council, (1994) and provided in mash form (Table 1).

Test indicator determination

Growth performance

Birds were weighed at 1, 21, and 42 days. Feed intake was recorded in replicates throughout the trial and average daily feed intake (ADG), average daily weight gain (ADFI) and meat to feed ratio (F/G) were calculated.

Blood indicators

On days 21 and 42 of the trial, 4 ml of blood was collected from the broiler's lower wing vein, left to stand for 30 min and centrifuged at 1,200 r/min for 15 min to extract the supernatant. The potency of serum antibodies to Newcastle disease and avian influenza H9 is determined by a haemagglutination inhibition test. Serum levels of

TABLE 1 Composition and nutrient levels of the basal diet.

Items	Contents	
	Days 1–21	Days 22–42
Ingredients (%)[a]		
Corn	60.4	64.05
Soybean meal	34.4	30
$CaHPO_4$	1.40	1.30
$CaCO_3$	1.21	1.12
NaCl	0.25	0.25
Soybean oil	1.00	2.00
Choline chloride	0.05	0.05
Lysine	0.08	0.10
DL-Met	0.21	0.13
Premix	1	1
Total	100.00	100.00
Nutrient levels (%)[b]		
ME (MJ/Kg)	12.14	12.51
CP	21.17	19.24
Available phosphorus	0.38	0.36
Lys	1.29	1.15
Met	0.67	0.48
Met + Cys	1.00	0.72
Ca	0.92	0.87

[a]Each kg of premix provides: VA, 5000 IU; VD, 10,000 IU; VE, 75.0 IU; VK_3, 18.8 mg; VB_1, 9.8 mg; VB_2, 28.8 mg; VB_6, 19.6 mg; VB_{12}, 0.1 mg; Biotin, 2.5 mg; Folic Acid, 4.9 mg; D-Pantothenic acid, 58.8 mg; Nicotinic acid, 196.0 mg; Zn, 37.6 mg; Fe, 40.0 mg; Cu, 4.0 mg; Mn, 50.0 mg; I, 0.2 mg; Se, 0.2 mg.
[b]The nutrient levels were calculated values.

albumin (ALB), total protein (TP), globulin (GLOB), alanine transaminase (ALT), alkaline phosphatase (ALP), and glucose (GLU) were measured using a fully automated biochemical analyser.

Faecal microbiota

On day 21 and 42 of the trial, a 1 g sample of broiler manure from each replicate was collected and transported on ice to the laboratory following the method of Dang et al. (2020). Each replicate of 1 g faecal sample was diluted and mixed with 9 ml of 1% peptone broth. The viable counts of *E. coli*, *Lactobacillus* and *Salmonella* in faecal samples were determined on McConkey agar plates, MRS agar plates and BS agar plates (in a 10 g/L peptide solution) in a biosafety cabinet. The microbial count is ultimately expressed as $\log_{10}$ colony forming units per Gram of faeces.

Noxious gas emissions

On day 21 and 42 of the trial, fresh broiler manure was collected from each replicate and ammonia and hydrogen sulphide emissions from the manure were determined using the method of Dang et al. (2020). The manure was placed in a 2 L plastic box with small holes attached to the side and fermented at room temperature (25°C) for 12, 24, and 48 h. The air sample is then collected with a gas collection pump from above the small holes on either side. NH_3 and H_2S concentrations are measured in the range of 0.00–100.00 mg/m^3.

Data analysis

The data was designed using a completely randomised grouping design. Replicate cage serves as the experimental unit. Multiple comparisons of significant differences in means were performed using the one-way ANOVA LSD method in SPSS 25.0 and visualisation was completed using Graphpad Prism 8. Orthogonal contrasts were used to examine the linear and quadratic effects in response to increasing dietary BX levels. Results are expressed as mean and standard deviation, with $p < 0.05$ indicating a significant difference.

Results

Growth performance

As shown in Table 2, Dietary supplementation with graded levels of BX linearly increased ADG and ADFI in broilers during days 22–42 days and 1–42 ($p < 0.05$), and there was a quadratic effect on ADG in broilers during days 22–42 and 1–42 ($p < 0.05$). Dietarys supplemented with 0.2% BX significantly increased ADG and ADFI at 22–42 and 1–42 days compared to controls ($p < 0.05$). There was no significant effect of dietary supplementation with BX on F/G in all periods ($p > 0.05$).

Blood indicators

As shown in Figure 1, Dietary supplementation with graded levels of BX increased the potency of Newcastle disease and avian influenza H9 antibodies in broiler serum at days 21 and 42, but the statistical results did not reach significant levels ($p > 0.05$).

As shown in Figures 2, 3, Dietary supplementation with graded levels of BX increased broiler serum levels of 21-day GLOB and 42-

TABLE 2 Effects of dietary supplementation of Wheat germ, Hops and Grape seed extract mixture (BX) on growth performance in broilers.

Items	Dietary BX levels, %				*p*-Value	
	0	0.05	0.1	0.2	Linear	Quadratic
ADG, g/d						
Days 1–21	41.96 ± 0.50	41.93 ± 0.46	42.04 ± 0.46	42.04 ± 0.46	0.670	0.913
Days 22–42	79.23 ± 2.89^{b}	80.29 ± 2.50^{ab}	82.30 ± 3.05^{ab}	83.16 ± 2.51^{a}	0.009	0.035
Days 1–42	60.60 ± 1.60^{b}	61.11 ± 1.42^{ab}	62.17 ± 1.70^{ab}	62.60 ± 1.45^{a}	0.016	0.058
ADFI, g/d						
Days 1–21	52.71 ± 0.55	52.57 ± 0.75	52.73 ± 0.43	52.72 ± 0.66	0.874	0.951
Days 22–42	134.48 ± 3.78^{b}	136.10 ± 3.81^{ab}	138.93 ± 3.70^{ab}	139.81 ± 3.84^{a}	0.010	0.036
Days 1–42	93.60 ± 2.10^{b}	94.34 ± 2.08^{ab}	95.83 ± 1.98^{ab}	96.27 ± 2.14^{a}	0.016	0.058
F/G						
Days 1–21	1.25 ± 0.01	1.26 ± 0.01	1.25 ± 0.01	1.25 ± 0.01	0.748	0.924
Days 22–42	1.70 ± 0.02	1.70 ± 0.02	1.69 ± 0.02	1.68 ± 0.01	0.076	0.214
Days 1–42	1.55 ± 0.01	1.54 ± 0.01	1.54 ± 0.02	1.54 ± 0.01	0.156	0.374

ADG, average daily gain; ADFI, average daily feed intake; F/G feed-to-weight ratio.
^{a,b}Means in the same row with different superscripts are significantly different ($p < 0.05$).

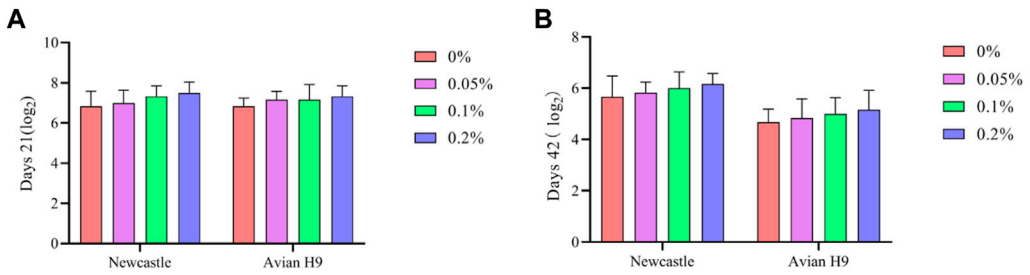

FIGURE 1
Effects of dietary supplementation of Wheat germ, Hops and Grape seed extract mixture (BX) on serum antibody potency in broilers. **(A)** Newcastle disease and Avian influenza H9 antibody potency in 21 days broiler sera; **(B)** Newcastle disease and Avian influenza H9 antibody potency in 42 days broiler sera.

day ALB, and decreased serum levels of ALT at days 21 and 42, but the statistical results did not reach significant levels ($p > 0.05$).

Faecal microbiota

As shown in Figure 4, Dietary supplementation with graded levels of BX significantly reduced *E. coli* levels in broiler faeces at 21 days ($p < 0.05$). *Salmonella* levels in 21-day broiler faeces was significantly reduced when supplemented with 0.2% BX compared to the control group ($p < 0.05$). Dietary supplementation with graded levels of BX significantly reduced *E. coli* and *Salmonella* levels in broiler faeces at 42 days ($p < 0.05$). *Lactobacillus* levels in 42-day broiler faeces was significantly increased relative to the control when supplemented at 0.1% and 0.2% ($p < 0.05$).

Noxious gas emissions

As shown in Figure 5, Dietary supplementation with graded levels of BX significantly reduced NH_3 and H_2S emissions in faeces at days 21 and 42 ($p < 0.05$). In contrast, at 0.2% BX supplementation, NH_3 and H_2S emissions were at their lowest values at each fermentation time.

Discussion

The results of this trial showed that supplementing the broiler diet with wheat germ, hops and grape seed extract mixture (BX) mainly improved the growth performance of AA + broilers in the later stages of production (22–42 days). And as the amount added increases, so does the ADG and ADFI. Although there were few reports on whether wheat germ, hops and grape seed extracts promote broiler growth, some reports suggested positive effects (Abu and Ibrahim, 2018; Goluch et al., 2023). Possible reasons for this were that natural antioxidants protect the intestinal mucosa from oxidative damage and pathogens, while limiting peristaltic activity in digestive disorders, and that some reduction in intestinal motility might lead to better nutrient absorption (Kermauner and Laurenčič, 2008). To date, there had been much evidence that wheat germ, hops and grape seed extracts had antioxidant, antibacterial

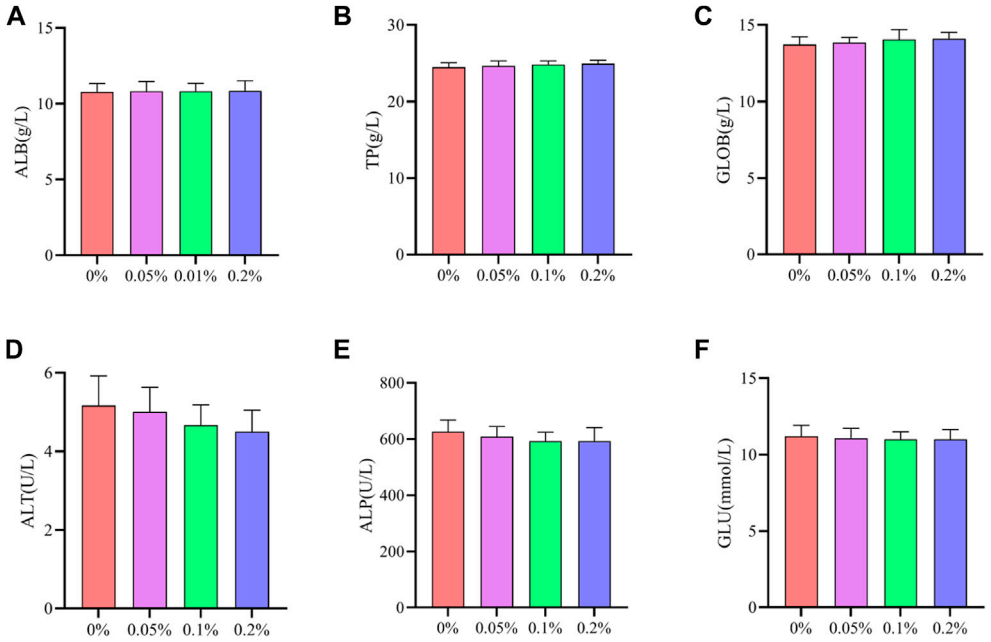

FIGURE 2
Effects of dietary supplementation of Wheat germ, Hops and Grape seed extract mixture (BX) on serum biochemical parameters in 21 days broilers. **(A)** Albumin; **(B)** Total protein; **(C)** Globulin; **(D)** Alanine transaminase; **(E)** Alkaline phosphatase; **(F)** Glucose.

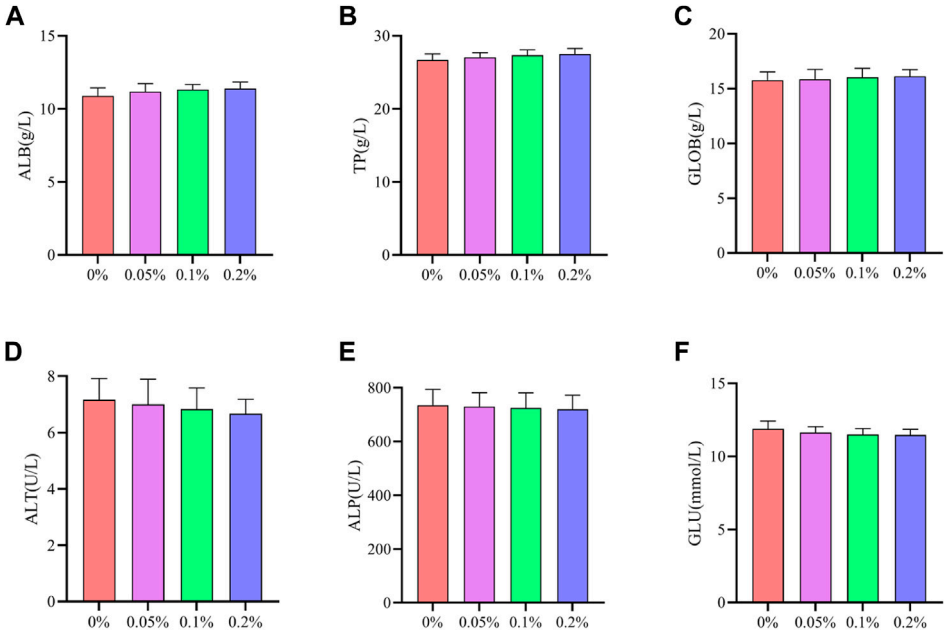

FIGURE 3
Effects of dietary supplementation of Wheat germ, Hops and Grape seed extract mixture (BX) on serum biochemical parameters in 42 days broilers. **(A)** Albumin; **(B)** Total protein; **(C)** Globulin; **(D)** Alanine transaminase; **(E)** Alkaline phosphatase; **(F)** Glucose.

and anti-inflammatory effects. However, further research is needed to determine whether they promote growth, or whether they do so in other ways.

This pilot study found that although BX increased Newcastle disease antibody potency, ALB and GLOB levels and reduced ALT levels in broiler serum, none of these reached statistically significant

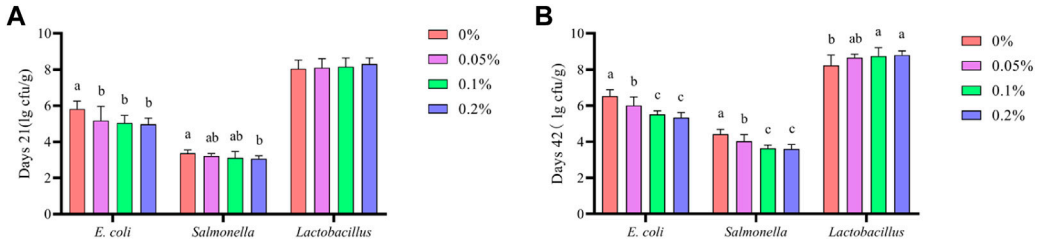

FIGURE 4
Effects of dietary supplementation of Wheat germ, Hops and Grape seed extract mixture (BX) on faecal microbiota in broilers. **(A)** Days 21; **(B)** Days 42. [a,b,c,d]Means in the different groups with different superscripts are significantly different ($p < 0.05$). The same as below.

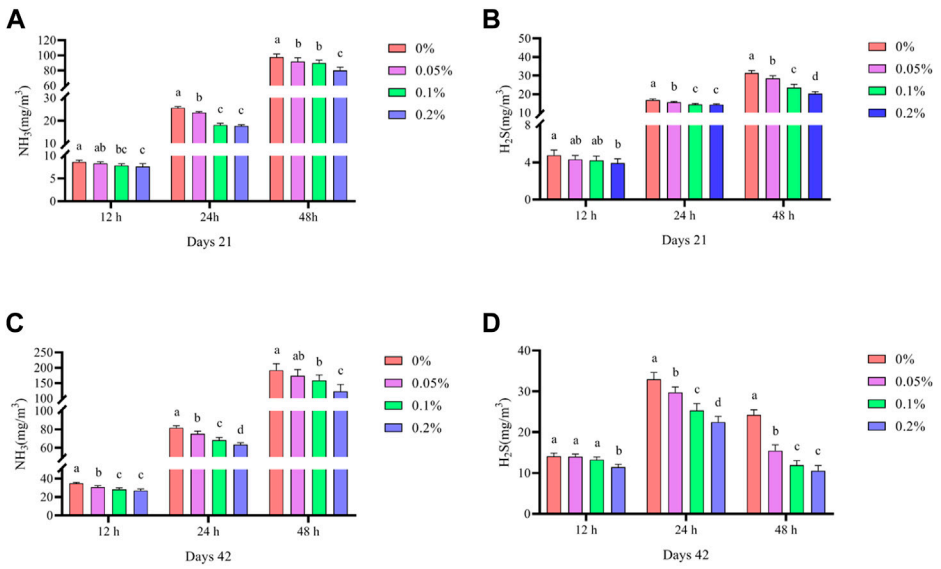

FIGURE 5
Effects of dietary supplementation of Wheat germ, Hops and Grape seed extract mixture (BX) on noxious gas emissions in broilers. **(A)** Days 21 NH_3 emissions; **(B)** Days 21 H_2S emissions; **(C)** Days 42 NH_3 emissions; **(D)** Days 21 H_2S emissions.

levels. The increased potency of Newcastle disease antibodies in serum might be due to the immunostimulatory function of grape seed extract (GSE), mainly due to its antioxidant and free radical scavenging properties, which increased the integrity and added value of B lymphocytes that differentiate into antibody-producing plasma cells (Hamilos et al., 1989). Previous studies had shown that the addition of GSE to the diet could activate the Nrf2 signalling pathway and improved resistance to oxidative stress in broilers (Long et al., 2016; Rajput et al., 2019). Ali Rajput et al. (2017) found that the addition of GSE (250 and 500 mg/kg) to the diet significantly increased serum TP, ALB, and GLOB levels. This is similar to the results of this test.

In this study, the faecal microbiota of broilers were counted at 21 and 42 days and harmful gas emissions were measured. The results showed that dietary supplementation with BX significantly reduced *E. coli* and *Salmonella* counts in broiler faeces. At the same time, the number of *Lactobacillus* increased, suggesting that the addition of BX to the diet could selectively inhibit the growth of pathogens. There was also a significant reduction in NH_3 and H_2S emissions from faeces. The antimicrobial activity of BX was attributed to lectins, tannins, flavonoids, *ß*-acids, and xanthohumol. These bioactive components exert their antimicrobial action through different mechanisms that had been reported by most researchers. Studies had confirmed that the flavonoids in GSE could promote the growth of beneficial intestinal bacteria and inhibit certain pathogenic bacteria such as *E. coli*, *Candida albicans*, and *S. aureus* (Silván et al., 2013; Brenes et al., 2016). Cao et al. (2020) reported that the polyphenols in GSE could show positive prebiotic effects by promoting the growth of *Lactobacillus* and *Bifidobacterium* to maintain intestinal health. In addition, lectins from wheat germ had been shown to be viable options for various biomedical and therapeutic applications (Ryva et al., 2019). The *ß*-acid of hops was dipentylated at the C-6 site and also has antibacterial activity (Forino et al., 2016). The results of several studies had shown that faecal odour and ammonia emissions are associated with nutrient

utilisation and the gut microbial ecosystem (Misiukiewicz et al., 2021). Along with *Pseudomonas*, *Citrobacter*, *Aeromonas* and *Salmonella*, *E. coli* was identified as the most promising H_2S producing bacteria (Maker and Washington, 1974). Fecal NH_3 and H_2S emissions were also reduced due to the addition of BX to the dietary reducing the number of *E. coli* and *Salmonella* in broiler faeces. Based on the results of this trial, we hypothesize that one of the reasons for the increased growth performance of broilers at days 22–42 due to BX supplementation in the diet is the antioxidant properties of BX. Is this also due to the antibacterial properties of BX? The reduction in harmful bacteria and the increase in beneficial bacteria maintains a healthy gut and further promotes digestion and absorption of the diet in broilers, thus improving growth performance. This conjecture has yet to be tested through subsequent studies.

Conclusion

Dietary supplementation with Wheat germ, Hops and Grape seeds mixture (BX) improved the growth performance of AA + broilers in the later stages of growth (22–42 days). Dietary supplementation with BX reduced the levels of *E. coli* and *Salmonella* in broiler faeces, as well as the emission of NH_3 and H_2S. and lead to increased levels of *Lactobacillus*.

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 Animal Ethics Committee of Jinzhou Medical University. Written informed consent was obtained from the owners for the participation of their animals in this study.

Author contributions

All authors listed have made a substantial, direct, and intellectual contribution to the work and approved it for publication.

Funding

This work was financed by the grants from the central government-guided local science and technology development fund projects (Grant No. 2021001).

Acknowledgments

We thank the College of Animal Husbandry and Veterinary Medicine of Jinzhou Medical University for its support of the experiment. We thank Liaoning Kaiwei Biological Co., Ltd. for providing the breeding base for this trial. We thank Jinzhou Zhongke Genetic Testing Service Co., Ltd. for providing the antigens for antibody detection, and for assisting us with microbiological testing.

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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frontiers | Frontiers in Physiology

TYPE Opinion
PUBLISHED 22 May 2023
DOI 10.3389/fphys.2023.1185286

OPEN ACCESS

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RECEIVED 13 March 2023
ACCEPTED 09 May 2023
PUBLISHED 22 May 2023

CITATION
Kuzmina IV (2023), The yolk sac as the main organ in the early stages of animal embryonic development.
Front. Physiol. 14:1185286.
doi: 10.3389/fphys.2023.1185286

The yolk sac as the main organ in the early stages of animal embryonic development

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KEYWORDS

yolk sac, embryo, birds, mammals, fish

1 Introduction

The yolk sac is the first of the extra-uterine organs to emerge in the course of evolution. In marsupials, the yolk sac, devoid of yolk, persists throughout pregnancy, and in placental mammals, it completes its development at the early stages of embryogenesis (Single ungulates and cloven-hoofed, and humans). In some species, the true yolk placenta exists until the end of pregnancy (rabbits and rodents). In reptiles, the yolk sac is associated with an intermediate villous membrane, the chorion (an organ that is closely related to the maternal organism), which forms a placental connection with the uterus while performing its trophic function. It is also known that during evolution, the allantois loses its trophic and respiratory functions but retains its leading role as a reservoir of metabolic products. In some cases, it disappears altogether (marsupial marten) or forms a dwarf allantois placenta (marsupial badger) (Krause and Cutts, 1984). The fate of the allantois directly depends on the competitive interaction with the yolk sac because, in the course of ontogenesis, it is the first to approach the serous membrane. In the course of these interactions, redistribution of fluid between tissues of the fetus and extra-germinal organs occurs in accordance with the requirements imposed on the forming organism by the external environment (Sklyanov et al., 2005). Nevertheless, as a manifestation of recapitulation, the yolk sac persists and then establishes new, compared to those in reptiles and birds, relations between the extra-germinal organs to implement their functions.

2 Opinion

2.1 Utilization of the yolk sac in birds

During incubation, the growth and development of the avian embryo depend on nutrients contained directly in the egg (Yadgary and Uni, 2012). For this, the avian embryo can solely rely on nutrients from the yolk, protein, and shell. The yolk sac membrane performs numerous metabolic functions and is the first line of defense against pathogens in the yolk. In terms of functions performed, this tissue acts as bone marrow for the synthesis of blood cells, the intestine for digestion and the transportation of nutrients, the liver for the synthesis of plasma carrier proteins and carbohydrate exchange, the thyroid for metabolic regulation, and the immune system for the transmission of antibodies from the hen. The rate of utilization of the contents of the residual yolk sac is related to the metabolic intensity of the embryo as well as to its size and composition at hatching (Nangsuay et al., 2013). Due to the fact that the chick receives mainly nutrients in the form of proteins and carbohydrates from feed at a day

old, it can still use the lipids stored in the remnants of the yolk sac, even when its membrane is destroyed. In addition to this, any disruption in the absorption of yolk sac contents can lead to a lack of essential nutrients and maternal antibodies, leading to mortality and poor poultry quality (Wong and Uni, 2020). It can be assumed that the utilization of the residual yolk sac during incubation and in the early post-embryonic period can affect the quality and development of chicks in the first week of rearing, as well as the development and productivity of poultry in age-related ontogeny. Podobed (2013) also states that the faster the chick gets rid of the remnants of the yolk sac, the faster it develops the ability to maximize the digestion of nutrients from the feed. Dissolution of the yolk sac in poultry in the first days of post-embryogenesis goes in parallel with the formation of their digestive system. Previously, our research results showed that the contents of the yolk sac of daily broiler chickens have high proteolytic activity. By 3 days of age, trypsin activity decreased by almost half, whereas lipolytic activity increased compared with the day-old chickens. We concluded that in the post-embryonic period of the chick, the yolk sac has no significant effect on the development of the pancreas. However, there was a stable inverse relationship: the smaller the weight of the yolk sac, the greater the weight of the duodenum (Vertiprakhov, 2022). This suggests that chickens fed immediately use more yolk than chickens fed with a delay, which may be due to higher intestinal enzymatic activity, which is probably due to peristaltic movements (Noy et al., 1996). However, the study of morphophysiological features of the yolk sac remains an interesting area of research despite the extensive information available in the literature.

2.2 Use of the yolk sac in mammals

The most primitive mammals are egg-laying mammals. Their embryogenesis is similar to that of birds. In marsupials, eggs contain a small amount of yolk, but the embryo is born underdeveloped and its further development proceeds in the maternal sac, where the mother's mammary gland nipple is connected to the baby's esophagus. For higher mammals, intrauterine development and nutrition of the embryo at the expense of the mother's body is characteristic, which is reflected in embryogenesis. Eggs are almost completely secondary to the loss of the yolk. They develop in the follicles of the ovary. After ovulation, they enter the oviduct. One of the peculiarities of mammalian development is the very early separation of the germ from the non-germ. Simultaneously with the formation of the embryonic body, one of the fetal membranes—the yolk sac—develops. The yolk sac is formed from the extra-germinal entoderm and the visceral sheet of the mesoderm. It contains a protein fluid. The wall of the yolk sac forms blood vessels. This shell performs the functions of hematopoiesis and trophic function (Bunkova, 2020). As a reservoir of yolk, the yolk sac provides its storage, cleavage, and absorption of formed monomers in a well-developed vascular network (Dubinina and Sklyanov, 2011). Placental mammalian and human oocytes are practically devoid of yolk. Nevertheless, the yolk sac in them is not only formed but also preserved until birth. This extra-natal organ (unlike that of rodents studied in laboratory experiments) belongs to the "free"-type, as it has no direct contact with tissues of the maternal organism (Freyer and Renfree, 2009). Located in the cavity of the exocelome and washed by its contents, the yolk sac appears to be able to absorb the substances it contains through its outer surface. The close connection of the exocelomic epithelium with the blood vessels also suggests the possible participation of the yolk sac tissues in providing the embryo (fetus) with nutrients through the yolk circle of vessels. Thus, the period of active functioning of the exocelomic epithelium of the yolk sac of mammals and humans is limited to the first trimester of pregnancy. This seems to be primarily due to the rapid growth of the fetus, leading to a decrease in the exocelome cavity and an increase in compression. Another possible explanation for what is happening may be a gradual change in the type of fetal nutrition. Anyway, the histogenesis and functioning of the exocelomic epithelium of the mammalian yolk sac with its subsequent involution is another striking example of the accelerated differentiation and specialization of tissues comprising the vertebrate extra-uterine organs.

2.3 Use of the yolk sac in fish

During gastrulation in fish, the first extra-germinal organ, the yolk sac, is formed, and it provides for the further development of the embryo. The epidermal layer of the yolk sac produces cells that break down the yolk into constituent elements that go toward feeding the embryo. Blood vessels appear in the mesodermal layer, through which nutrients flow to the embryo. The outer ectodermal layer of yolk sac cells performs gas exchange. The yolk sac is connected to the midgut cavity by the yolk stalk. As the nutrient material of the yolk is consumed, the sac retracts into the body of the embryo, the entoderm becomes part of the intestinal wall, the ectoderm becomes part of the skin, and the vascular system of the yolk sac is reduced. Thus, the yolk sac is a provisory (temporary) organ appearing in the evolution of vertebrates (Kokorina, 2010). The yolk sac in fish embryos should be considered, first of all, from the point of view of the necessity of this organ for food from the early stage of growth and development to the transition to mixed feeding of fish fry. The energy function of the yolk sac in fish can be considered in terms of a closed embryo–yolk system. Following the law of conservation of energy, in the yolk sac in fish embryos, there are additional irretrievable losses of nutrients for the formation of embryonic respiratory organs and mechanisms (Buslov and Sergeeva, 2013).

3 Discussion

The yolk sac is a feeding and breathing organ in the embryos of cephalopod mollusks, cartilaginous and bony fish, reptiles, birds, mammals, and humans. It appears at the early stages of embryonic development usually by fouling of the yolk with the entoderm and visceral sheet of the lateral plates and is an expanded outgrowth of the middle intestine, the cavity which,

in most animals (except higher mammals and humans), is filled with unshattered yolk. Blood cells and blood vessels are formed in the wall of the gut, providing for the transfer of nutrients to the embryo and its respiration. With the development of the embryo, the size of the yolk sac and its cavity decrease, and it gradually retracts into the body cavity and is either resorbed or rejected. In this regard, this organ can be considered one of the main organs due to its hyperfunctionality.

Author contributions

The writing, editing, translation, literature review, and funding was done by this author.

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.

Publisher's note

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frontiers | Frontiers in Physiology

TYPE Review
PUBLISHED 03 January 2024
DOI 10.3389/fphys.2023.1326809

OPEN ACCESS

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RECEIVED 23 October 2023
ACCEPTED 06 December 2023
PUBLISHED 03 January 2024

CITATION
Fathima S, Al Hakeem WG, Selvaraj RK and Shanmugasundaram R (2024), Beyond protein synthesis: the emerging role of arginine in poultry nutrition and host-microbe interactions.
Front. Physiol. 14:1326809.
doi: 10.3389/fphys.2023.1326809

Beyond protein synthesis: the emerging role of arginine in poultry nutrition and host-microbe interactions

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Arginine is a functional amino acid essential for various physiological processes in poultry. The dietary essentiality of arginine in poultry stems from the absence of the enzyme carbamoyl phosphate synthase-I. The specific requirement for arginine in poultry varies based on several factors, such as age, dietary factors, and physiological status. Additionally, arginine absorption and utilization are also influenced by the presence of antagonists. However, dietary interventions can mitigate the effect of these factors affecting arginine utilization. In poultry, arginine is utilized by four enzymes, namely, inducible nitric oxide synthase arginase, arginine decarboxylase and arginine: glycine amidinotransferase (AGAT). The intermediates and products of arginine metabolism by these enzymes mediate the different physiological functions of arginine in poultry. The most studied function of arginine in humans, as well as poultry, is its role in immune response. Arginine exerts immunomodulatory functions primarily through the metabolites nitric oxide (NO), ornithine, citrulline, and polyamines, which take part in inflammation or the resolution of inflammation. These properties of arginine and arginine metabolites potentiate its use as a nutraceutical to prevent the incidence of enteric diseases in poultry. Furthermore, arginine is utilized by the poultry gut microbiota, the metabolites of which might have important implications for gut microbial composition, immune regulation, metabolism, and overall host health. This comprehensive review provides insights into the multifaceted roles of arginine and arginine metabolites in poultry nutrition and wellbeing, with particular emphasis on the potential of arginine in immune regulation and microbial homeostasis in poultry.

KEYWORDS

arginine, poultry, gut health, nutraceuticals, immune response

1 Introduction

Amino acids are organic compounds containing both amino (—NH_2) and carboxyl (—COOH) groups. Due to the presence of asymmetric carbon, all amino acids except glycine exhibit optical activity and exist as D- and L-isoforms or enantiomers (Lehninger et al., 2005). The asymmetric α-carbon imparts chirality, a phenomenon where the molecule is not superimposable to its mirror images in space. Due to this, amino acids except glycine exist in different stereoisomeric forms (Grishin et al., 2020). The amino acids' biochemical properties and physiological functions vary widely depending on the side chains, which

impart charge to the amino acids and their isoforms (Wu, 2009; Grishin et al., 2020). There are 20 amino acids that function as building blocks of proteins in animal tissues. Based on their dietary requirements, amino acids are broadly classified as essential (indispensable) and non-essential (dispensable) for the growth and nitrogen balance of the animal. Essential amino acids are derived from the diet, as the organism cannot synthesize the carbon skeleton of those amino acids or synthesize them in amounts not adequate to meet the requirements (Watford, 2008; Wu, 2009). Conversely, non-essential amino acids can be synthesized *de novo* by the organism in sufficient amounts to meet the requirements in a species-dependent manner (Lehninger et al., 2005; D'mello, 2003a). However, some amino acids that are traditionally considered non-essential, are required in increased amounts under some pathological conditions, necessitating dietary supplementation, and are termed conditionally essential amino acids (D'mello, 2003b).

Recently, the concept of functional amino acids was introduced by Wu, (2009). Functional amino acids can be nutritionally essential, non-essential, or conditionally essential during different physiological stages of the animal. Functional amino acids play a pivotal role in gene expression (Leong et al., 2006), oxidative homeostasis, and cell signaling (Wang et al., 2008) and they regulate various physiologic and metabolic processes such as growth, development, immunity, health, reproduction, and endocrine status (Leong et al., 2006; Wang et al., 2008; Tan et al., 2009; Jimoh et al., 2021). Functional amino acids essential to maintaining whole-body homeostasis include methionine, arginine, proline, glutamine, leucine, glycine, tryptophan, and cysteine (Wu, 2010). These amino acids exert their functional roles directly or through their metabolites, exerting antioxidant, immunomodulatory, and growth-promoting effects (Fagundes et al., 2020; Liu et al., 2023). The cellular mechanisms by which these amino acids, notably arginine (Rubin et al., 2007; Tan et al., 2009; Tan et al., 2010; Al-Daraji and Salih, 2012; Tan et al., 2014; Zhang et al., 2017; Dao et al., 2022a), exert their beneficial effects, their functional roles, and their potential use as nutraceuticals in poultry feed have been investigated lately.

Arginine is a functional amino acid essential for growth, energy metabolism, immune response, wound healing, and protein synthesis (Wu et al., 2009). Additionally, arginine is the precursor for various bioactive molecules such as NO, polyamines, agmatine, creatine, glutamine, glutamate, and proline (Almquist et al., 1941; Montanez et al., 2008). Supplementation of arginine and its metabolites such as guanidinoacetic acid (GAA) and citrulline in poultry feed improves growth performance, carcass yield, lean meat yield, bone development, immunity, and antioxidant capacity (Al-Daraji and Salih, 2012; Tan et al., 2014; Chowdhury et al., 2017; Zhang et al., 2018; Dao et al., 2021a; Dao et al., 2022a). This review article delves into the intricate facets of arginine, shedding light on its absorption, metabolism, and physiologic functions in poultry. This review also briefly explores the commercially available arginine metabolites, GAA, and citrulline, shedding light on their roles within the broader context of poultry physiology and health. A particular emphasis is given to the interaction of arginine with the gut microbial community during health and disease, with a specific focus on necrotic enteritis as the disease model. Therefore, this review aims to offer an encompassing perspective on the present understanding of arginine's functional role in enhancing poultry health and production.

2 Arginine in poultry production

Arginine is a dibasic amino acid (Rubin et al., 2007) consisting of a linear chain of four carbon molecules and a distal complex guanidinium group, displaying resonance hybrid properties that impart the chemical properties of arginine (Khajali and Wideman, 2010). Arginine is an essential amino acid in poultry due to the absence of a functional urea cycle (as illustrated in Figure 1) (Application of Nutritional Immunology, 2022). This dietary indispensability of arginine in chickens arises from the lack of the enzyme carbamoyl phosphate synthase-I, which is necessary for the synthesis of L-arginine from ornithine, ammonia, and amino-nitrogen of aspartate. Additionally, poultry exhibits lower activities of ornithine transcarbamoylase and hepatic arginase (Khajali and Wideman, 2010), reinforcing their dependency on dietary arginine. Nevertheless, in the presence of dietary citrulline, arginine synthesis can occur in chicken macrophages and kidneys (Allen and Fetterer, 2000). Citrulline can replace arginine in the diet because of argininosuccinate and argininosuccinate synthetase enzymes in poultry. However, due to the lack of the enzyme carbamoyl phosphate synthetase, chicks cannot utilize ornithine (Tamir and Ratner, 1963a) as a source of arginine. In addition, hepatic arginine synthesis does not occur in chickens as the arginase activity is relatively higher in the liver.

Arginine is the fifth-limiting amino acid in a corn-soybean meal-based poultry diet. The National Research Council (NRC) requirement of arginine for broilers is 1.25%, 1.10%, and 1.00% of the diet for up to 3 weeks, 3–6 weeks, and 6–8 weeks of age, respectively (National Research Council, 1994b). However, the last updated version of the NRC recommendations for poultry was published in 1994. Commercial broilers were genetically selected in the last few decades to improve body weight gain, feed efficiency, and breast muscle yield (Applegate and Angel, 2014). The requirements for this increased growth and production performance, changes in management practices, and feed-related changes have not been accounted for in the NRC (1994) recommendations (Applegate and Angel, 2014). According to recent research findings, the NRC recommendations for arginine might not be adequate to support increased growth, prevent pulmonary hypertension due to stressful environmental conditions, and support arginine-dependent immune responses (Khajali and Wideman, 2010). However, other studies suggest the arginine requirements are close to the NRC (1994) recommendations (Jahanian, 2009). Nonetheless, the arginine requirement for optimum cellular and humoral immune responses in poultry is thought to be higher than that required for maximum growth rate in poultry (Jahanian, 2009).

Arginine is not a limiting amino acid in a corn-soybean meal-based poultry diet with an arginine: lysine ratio ranging from 100 to 107 (2022-Cobb500-Broiler-Performance-Nutrition-Supplement, 2022; Ross-BroilerNutritionSpecifications2022-EN, 2022). However, recent studies indicate that a higher arginine: lysine ratio is recommended for improved

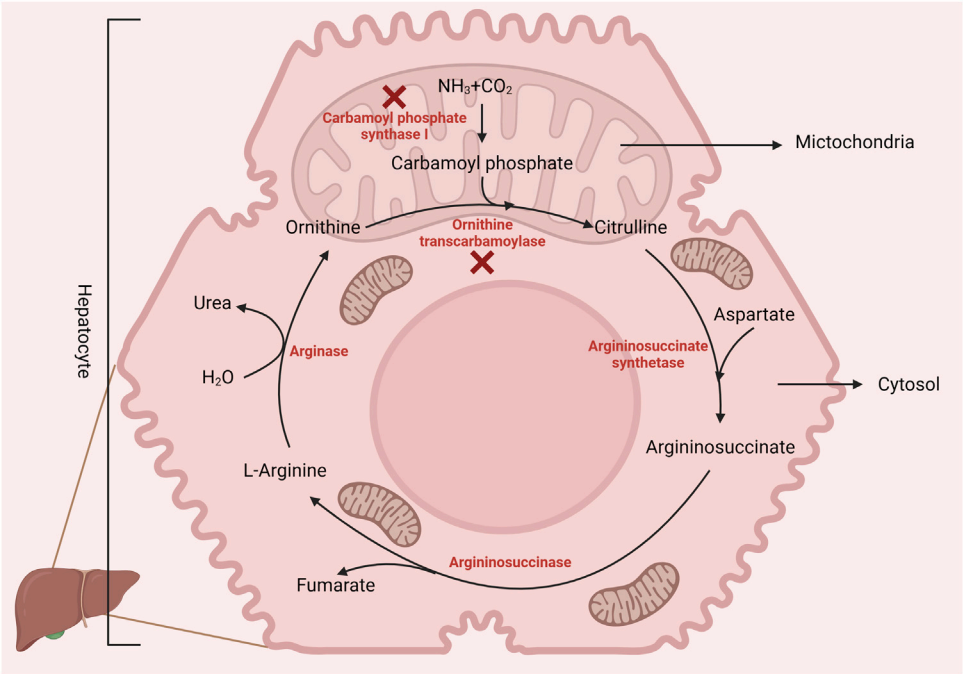

FIGURE 1
Birds lack the enzyme carbamoyl phosphate synthase-1 (incomplete urea cycle), making L-arginine dietary essential. However, poultry can synthesize arginine from citrulline via arginosuccinate synthase and lyase. Created with biorender.com (21 May 2022).

BWG and FCR (Zampiga et al., 2018; Sirathonpong et al., 2019; Corzo et al., 2021). Arginine supplementation is also recommended when birds are raised at high altitudes, during heat and cold stress, and when increasing the stocking density (Brake et al., 1998; SRINONGKOTE et al., 2004; Kodambashi Emami et al., 2017). The increased use of low crude protein feed formulations, replacement of soybean meal with by-products such as corn distillers' dried grains, and reduced use of animal sources of protein in poultry diet necessitates arginine supplementation (DeGroot et al., 2018). The requirement for arginine can vary depending on several other factors, such as dietary protein level, source of protein, digestibility of feed ingredients, stage of growth, and physiological status of the bird (McNab, 1994).

The proteins' nutritional value and amino acid composition vary with the dietary ingredients used in poultry feed formulation. Amino acid availability is a valuable measure and indicator of protein quality (McNab, 1994; Ravindran et al., 1999) Estimating the amino acid availability of feed ingredients enables the efficient formulation of poultry feed, accounting for endogenous losses. Amino acid availability is defined as "the proportion of dietary amino acids that is in a form suitable for digestion, absorption, and utilization by the animal" (McNab, 1994). Amino acid digestibility is a sensitive indicator of dietary amino acid availability for poultry. Excreta analysis is the most common method used to assess the amino acid digestibility in poultry. Nonetheless, since the urine and feces are excreted together in poultry, excreta analysis measures amino acid metabolizability rather than digestibility (Ravindran et al., 1999). Analysis of ileal contents is a more reliable method for assessing amino acid digestibility in poultry as it takes into account the hindgut fermentation, preventing underestimation of the amino acid requirement (Investigation of protein quality, 1968). In addition, there are differences in the amino acid digestibility among different feed ingredients. Amino acid digestibility is highest in oilseed meals, particularly soybean and sunflower meals. Arginine digestibility was highest in oilseed meals (except for cottonseed meal), grain legumes, wheat middling, and rice polishings. Among animal protein sources, blood meal had the highest amino acid digestibility coefficient, followed by fish meal, meat meal, meat, bone meal, and feather meal, respectively (Ravindran et al., 2005). These factors affecting the digestibility and estimation of the digestibility of arginine influence the arginine requirement of the birds as well.

In growing chicks, the requirement for arginine will be greatly increased (when expressed as g/day) with high demand for the amino acid for muscle protein accretion (Ball et al., 2007). Similarly, during infections, the immune system is activated, which significantly affects the amino acid availability for muscle protein accretion, thus compromising growth. Arginine requirement will be increased during an active inflammatory response (Rochell et al., 2017; Nogueira et al., 2021) indicated by a decreased plasma arginine concentration in infected birds. The increased demand for arginine during enteric infections such as coccidiosis might be due to its role in polyamine synthesis which is required for mucosal tissue repair (Rochell et al., 2017). Thus, the body prioritizes the immune response over protein deposition during stress conditions (Nogueira et al., 2021) reducing the metabolic availability of arginine and negatively impacting growth (Allen and Fetterer, 2000). Similarly, during stress conditions, especially during heat stress, the sodium-dependent and sodium-independent uptake of arginine is depressed, increasing the arginine requirement in birds (Khajali and Wideman, 2010). The requirement for arginine

can also vary based on its relationship other amino acids such as lysine and methionine (Chamruspollert et al., 2002), methodology used to estimate the requirement, and type of birds used (Lima et al., 2020). However, determining the actual requirement for arginine hinges on its bioavailability, contingent upon both digestibility and post-digestion utilization of the amino acid. Thus, comprehending the intricate processes of absorption, transport, and metabolism of arginine in poultry is crucial, and these aspects will be explored further in the subsequent sections.

3 Arginine absorption and transport mechanisms

Arginine, being a cationic amino acid, shares transport proteins with other cationic amino acids such as ornithine and lysine (Closs and Mann, 2000). The carrier proteins for cationic amino acids belong to the solute carrier family 7 (SLC7) and include members 1,7 and 9. The sodium-independent transporter SLC7A1 preferably transports arginine, followed by lysine and histidine. However, the sodium-dependent transporters SLC7A7 and SLC7A9 prefer lysine (Bröer and Fairweather, 2018). Arginine, in most cells, is taken up by a Na^+-independent transport system, termed system y^+. The system y^+ is constituted by the cationic amino acid transporter (CAT) proteins CAT-1, CAT-2B, and CAT-3 (Closs et al., 2004). The CAT transporters cater to the cationic amino acid requirements for protein synthesis and the synthesis of bioactive substances such as NO, creatine, proline, polyamines, agmatine, glutamine, and urea (San Martín and Sobrevia, 2006). The glycoprotein-associated heterodimeric $b^{0,+}$AT/rBAT transporter is a Na^+-independent transporter located in the luminal side of the epithelium and facilitates the inward transport of dibasic amino acids such as arginine and lysine in exchange for neutral amino acids (Torras-Llort et al., 2001). The neutral amino acids necessary for exchange with arginine are transported by PepT1, PepT2, or B^0AT and y^+LAT1 at the apical and basolateral membranes, respectively (Closs et al., 2004). In addition, amino acid transporters are present on the basolateral membrane of the enterocytes that facilitate the exchange of amino acids between the vascular system and the epithelial cells. The transporters y^+ LAT1 and y^+ LAT2 transport neutral and cationic amino acids, whereas CAT1 and CAT2 transport cationic amino acids across the basolateral membrane of the enterocytes (Miska and Fetterer, 2017).

The expression of these amino acid transporters is significantly decreased during infections, leading to malabsorption, weight loss, and immune dysfunction (Miska and Fetterer, 2017). Further, intestinal immunopathology is significantly increased during infection-associated arginine deficiency. This infection-induced damage can be reversed by administering supplemental arginine (Zhang et al., 2019). During such conditions, arginine is mobilized from body protein to satisfy the increased demand or to compensate for the decreased availability (Faure et al., 2007). However, the absorption and utilization of arginine is also influenced by the amino acid balance, acid-base balance, and the presence of antagonists in the diet (Jones et al., 1967; Khajali and Wideman, 2010). These interrelationships of arginine with other dietary components affecting its absorption and utilization are discussed below.

4 Nutritional antagonism: interaction of other amino acids with arginine

A dietary balance of micronutrients, such as essential amino acids, is important for optimum growth and development (Zampiga et al., 2018). The amino acids interact with each other to maximize the growth and production performance in poultry (Kidd et al., 1997). A change in the dietary inclusion level of one amino acid can cause a marginal deficiency of other amino acids if the balance is not maintained. Antagonism occurs due to the competition among amino acids for absorption and transport systems and common enzymes used in their catabolism due to similarities in their chemical structures. Moreover, antagonists might inhibit the uptake or utilization of the amino acid, affecting its availability for physiological functions. Factors such as dietary imbalances in amino acid composition, competition for transporters, or metabolic interactions can contribute to amino acid antagonism (Bell, 2003; Maynard and Kidd, 2022).

The balance between arginine and lysine is important in poultry feed. The nutritional antagonism of arginine and lysine was first identified in 1952 (Anderson and Combs, 1952). The antagonism is explained by the fact that arginine and lysine are basic amino acids competing for renal tubular reabsorption. The antagonism is more pronounced with excess lysine than with excess arginine. The antagonistic effects are observable when the lysine content in the poultry diet is approximately 2%–3.5% or when the lysine-to-arginine ratio is 2.2–2.6: 1 (Ball et al., 2007). A high lysine: arginine ratio enhances renal arginase activity, leading to increased degradation and urinary excretion of arginine (Khajali and Wideman, 2010). Excess lysine affects the muscle amino acid concentration and growth in poultry. The suppression of weight gain by diets high in lysine was first reported by Anderson and Combs (Anderson and Combs, 1952) whereas, the growth-depressing effect of a high arginine diet was first reported by D'mello and Lewis (D'mello and Lewis, 1970). The optimum dietary arginine: lysine ratio recommended by the NRC is 1.14, 1.10, and 1.18 for 0–3 weeks, 3–6 weeks, and 6–8 weeks respectively (National Research Council, 1994a). The effect of high lysine on arginine can be attenuated by supplementing sodium, potassium, calcium, or magnesium salts of organic acids, such as sodium and potassium acetate (Khajali and Wideman, 2010).

In contrast to the above-discussed findings, in a study conducted by Kadirvel and Kratzer (1974), it was observed that leucine significantly inhibited the uptake of arginine more than lysine. This antagonism can be due to the faster absorption of leucine and lysine. Arginine deficiency produced by lysine can be due to the metabolic effect rather than competitive inhibition of intestinal absorption as well (Jones et al., 1967). Kidney arginase activity and urea excretion have a significant impact on arginine requirements and homeostasis. Several amino acids such as lysine, isoleucine, phenylalanine, histidine, and tyrosine significantly increase kidney arginase activity (Austic and Nesheim, 1970) while glycine and threonine suppress kidney arginase activity (Austic and Nesheim, 1970).

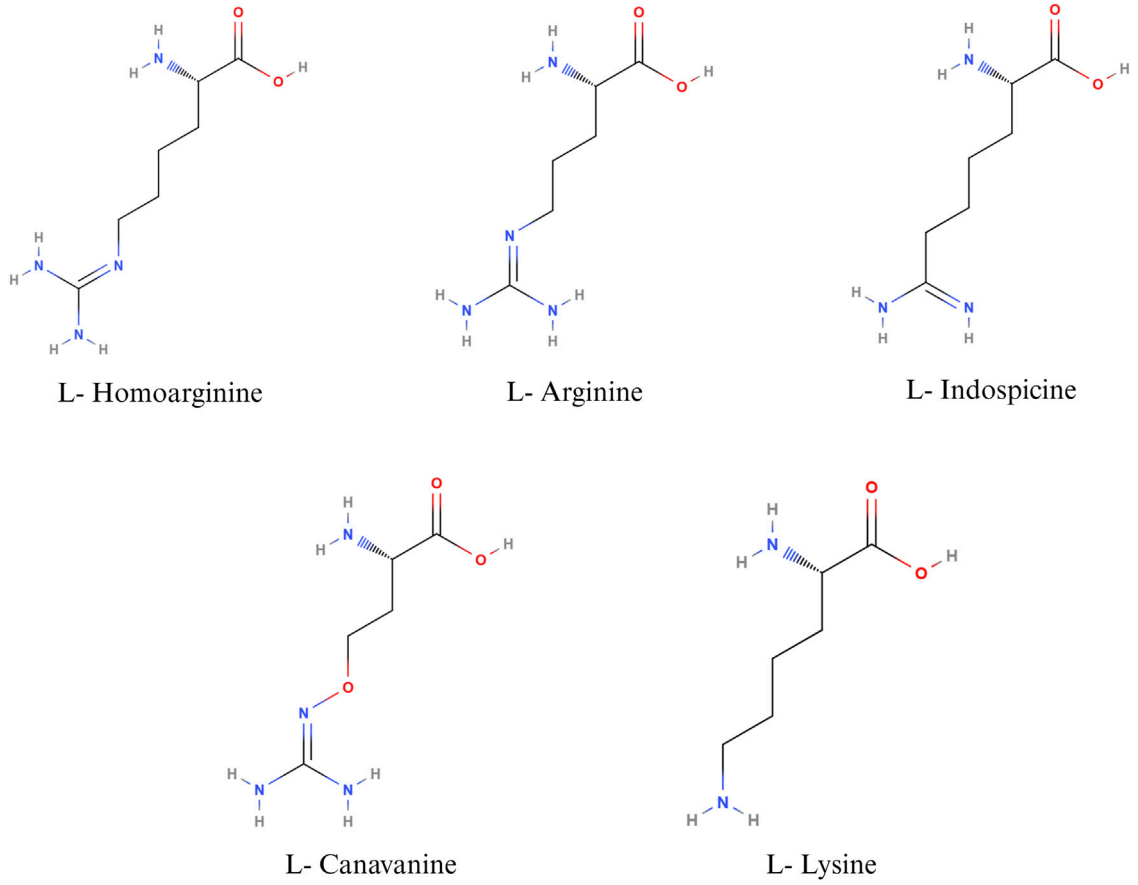

FIGURE 2
Nutritional antagonists of arginine. Created with BioRender.com (26 November 2023).

Non-protein amino acids such as canavanine, homoarginine, and indospicine are structural analogues of arginine (Figure 2), implicated in antagonistic activity against arginine. Canavanine is a non-protein structural analogue of arginine and is found predominantly in legumes and crops such as alfalfa, clover, bitter vetch, and trefoils. The seed of bitter vetch contains 28.5% crude protein and can be used as an alternative source of protein in poultry feeds. However, the presence of canavanine limits its use as an alternative feedstuff in monogastric animals (Sadeghi et al., 2004; Sadeghi et al., 2009a; Sadeghi et al., 2009b). Canavanine is stored in leguminous plants as a chemical barrier against diseases causing pathogens and predation. In animals, canavanine can replace arginine during protein synthesis, leading to the synthesis of non-functional proteins (Sadeghi et al., 2009b). In addition, canavanine replaces ornithine in the urea cycle, leading to the formation of canavaninosuccinate. Canavaninosuccinate inhibits the ornithine decarboxylate enzyme, hindering the biosynthesis of polyamines such as spermine, spermidine, and putrescine (D'mello, 2003a). Canavanine also inhibits Na^+- dependent transport of arginine across the intestinal epithelium (Khajali and Wideman, 2010). Canavanine in poultry feed can adversely affect growth performance and cause pancreatic hypertrophy (Sadeghi et al., 2004). However, canavanine in the feedstuffs can be inactivated by different treatment methods, primarily soaking, acid treatment, alkali treatment, or heat treatment (Sadeghi et al., 2004).

L-homoarginine, a non-protein amino acid, is synthesized from the catabolism of lysine or transamination of arginine in the small intestine, liver, and kidneys (Adams et al., 2019). Homoarginine can affect NO production by acting as a substrate for the enzyme NOS. As L-Homoarginine uses the same intestinal amino acid transporter as lysine, feeding homoarginine was found to decrease feed consumption in birds and cause lysine deficiency in rats (Adams et al., 2019). Homoarginine acts as a competing substrate for the enzymes that use arginine as a substrate (Haghikia et al., 2017). The effect of homoarginine on nitric oxide production can be positive or negative, depending on several factors, such as the cell type, intracellular and extracellular concentrations of arginine, and the activity of other arginine metabolizing enzymes. Feeding homoarginine in poultry inhibits the secretion of alkaline phosphatase, which is important for the maintenance of gut health and intestinal homeostasis (Adams et al., 2019). Alkaline phosphatase plays an important role in bone formation as well; hence, homoarginine levels are inversely proportional to the parameters of bone formation (Linder, 2016; Adams et al., 2019). Hence, homoarginine plays an important role in different metabolic processes in poultry. However, the normal level of serum homoarginine and its implications for poultry health and wellbeing have not been well established.

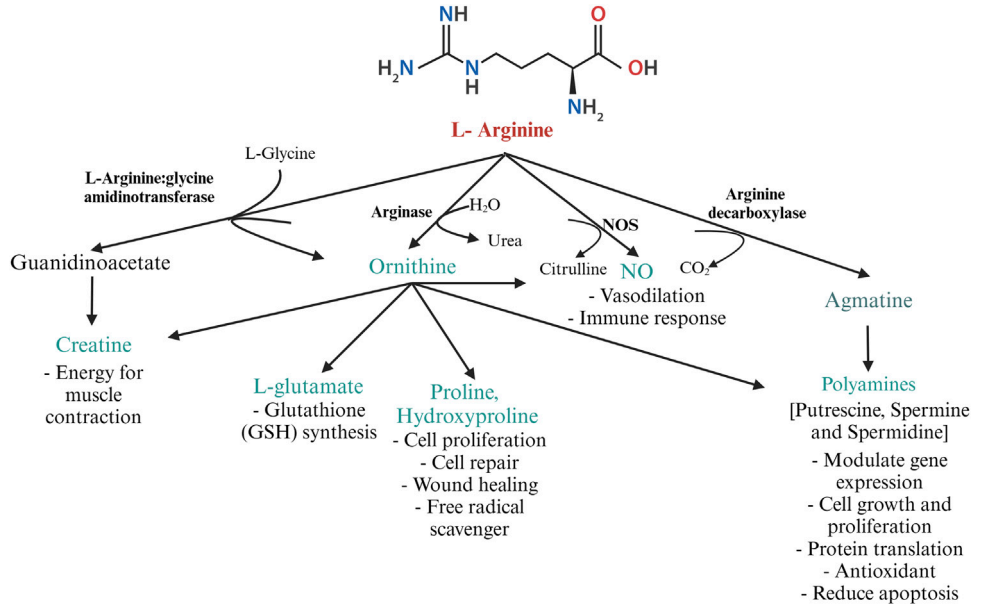

FIGURE 3
Metabolism of arginine by the major arginine metabolizing enzymes L-arginine: glycine amidinotransferase, arginase, NOS, and arginine decarboxylase in poultry. Created with Biorender.com (8 October 2022).

Indospicine, a non-proteinogenic amino acid, is a competitive inhibitor of arginine. Indospicine is found in *Indigofera* plant species. The compound causing *Indigofera* toxicity was identified in 1970 as indospicine by Hegarty and Pound (Hegarty and Pound, 1970). Indospicine acts as a cation on ion exchange resins, is not metabolized by arginase, and interferes with the incorporation of arginine in proteins (Hegarty and Pound, 1970; Bell, 2003). Livestock, grazing on pasture, accumulates toxins in their meat, which leads to the secondary poisoning of animals consuming the meat of grazers. Indospicine interferes with the arginine metabolic pathways in mammals and is highly hepatotoxic and teratogenic (Fletcher et al., 2015). In poultry, feeding 5% *I. spicata* meal caused decreased growth rate and paralysis of the neck, wings, and legs, followed by death (Rosenberg and Zoebisch, 1952). It has been suggested that poultry, being a uricotelic species, is less susceptible to the adverse effects of indospicine than ureotelic animals (Bell, 2003). Nevertheless, the current literature on the effect of indospicine on poultry health is sparse.

5 Arginine metabolism and physiological effects of metabolites in poultry

In poultry, the fate of arginine is determined by the activities of CATs, arginosuccinate synthase, and the arginine-degrading enzymes- NOS and arginases (Wu and Morris, 1998). The key enzymes involved in arginine catabolism are 1. NOS, 2. arginase 3. arginine decarboxylase (ADC), and 4. arginine: glycine amidinotransferase (AGAT), summarized in Figure 3. The expression of these enzymes is cell-specific. The three isozymes of NOS, namely, neuronal NOS (nNOS or NOS 1), endothelial NOS (eNOS or NOS 3), and iNOS or NOS2 differ in their structure, distribution, and synthetic capacity, but catalyze the same reaction (Stuehr et al., 2004). The enzyme NOS incorporates molecular oxygen at the terminal guanidino nitrogen group of arginine, yielding NO and citrulline. The gene expressions of nNOS and eNOS are constitutive, whereas the expression of iNOS is inducible. While nNOS and eNOS-mediated production of NO is "low-output" and is important for normal physiological functions, the production of NO by iNOS is classified as "high-output" and is involved in infection and inflammation (MacMicking et al., 1997).

Under physiological conditions, NO (produced by the expression of eNOS and nNOS) is necessary for vasodilation, parasympathetic neuronal action, smooth muscle relaxation, spermatogenesis, gene expression, and embryogenesis in poultry. For instance, *in ovo*, inoculation of arginine in chicks improves egg weight, hatchability, chick weight, production performance, lymphoid organ weight, and liver and pectoral muscle energy storage (Nabi et al., 2022), that might contribute to the increased survivability of chicks. In addition, arginine supplementation in poultry raised at high altitudes helps to regulate vasodilation and prevent heart disease and subsequent ascites syndrome in poultry (Miri et al., 2022) due to the production of NO. eNOS expression is upregulated during hypertension, hypoxia, and hypoxemia. This will promote calcium entry into the endothelial cells transiently, which forms the calcium-calmodulin complex and stimulates NO production. NO acts as a vasodilator, relieving hypertension and increasing the blood supply to the tissues (Bowen et al., 2007). However, during inflammation, pro-inflammatory cytokines such as IFN-γ, IL-1β, IL-12, tumor necrosis factor- α (TNF-α), and bacterial lipopolysaccharides (LPS) induce the expression of iNOS (Qureshi, 2003; Bowen et al., 2007). The activity of the different isoforms of NOS has been reviewed previously (MacMicking et al., 1997).

Arginine is the only known substrate for all three isoforms of NOS and the precursor of NO in the body (Wu and Morris, 1998) and hence, NOS competes with other arginine-degrading enzymes, such as arginase.

Arginase exists predominantly in two isoforms-liver-type arginase I and non-hepatic-type arginase II. Arginase I is present in the cytosol of hepatocytes and erythrocytes, whereas arginase II is present in the mitochondrial matrix of enterocytes and the cells of the kidney. In poultry, arginase activity is highest in the kidney, liver, and macrophages (Tamir and Ratner, 1963b). Expression of arginase I in macrophages is induced by the cytokines IL-4 and IL-13. Arginase downregulates NO production by competing with NOS for arginine (MacMicking et al., 1997). Prolonged production of NO is toxic to macrophages and other cells in the vicinity. Arginase, induced during the later stages of inflammation, depletes intracellular arginine, thus preventing the overproduction of NO. Arginase acts on L-arginine, yielding ornithine, which is decarboxylated by ornithine decarboxylase to form putrescine (MacMicking et al., 1997). In the presence of decarboxylated S-adenosylmethionine, spermine and spermidine can be formed from putrescine by ornithine decarboxylase and S-adenosylmethionine decarboxylase (Seiler, 1987). The polyamines spermine, spermidine, and putrescine are associated with cell repair, cell proliferation, and wound healing (Wu and Morris, 1998). Ornithine can be converted to pyrroline-5-carboxylate further converted to proline and hydroxyproline. Proline and its metabolites regulate gene expression, mTOR pathway (van Meijl et al., 2010), protein synthesis, cell survival, and scavenge free radicals (Kaul et al., 2008). Besides, hydroxyproline is required for the synthesize of glycine, glucose, and pyruvate and is known to scavenge free radicals (Phang et al., 2008).

Decarboxylation of arginine by the mitochondrial enzyme ADC yields the cationic amine agmatine. Agmatine is a precursor for synthesizing polyamines and is important for maintaining mitochondrial membrane permeability (Akasaka and Fujiwara, 2020). Agmatine is a pleiotropic molecule involved in various physiological functions such as NO synthesis, polyamine metabolism, glucose metabolism, carnitine synthesis, and neurotransmission (Molderings and Haenisch, 2012). Agmatine has been discovered to have therapeutic applications and is considered a nutraceutical in mammals (Molderings and Haenisch, 2012). The role of agmatine in poultry is largely unexplored. ADC activity is highest in the kidney and liver. Studies on agmatine revealed the antagonistic activity of agmatine aldehyde on NOS (Satriano, 2004). Agmatine inhibits polyamine biosynthesis by binding to the enzyme ornithine decarboxylase and promoting its degradation. Additionally, agmatine induces the antizyme- I, an enzyme that converts higher-order polyamines to lower-order polyamines (spermine → spermidine → putrescine) (Satriano, 2004). Thus, in general, agmatine possesses antiproliferative and anti-inflammatory activity.

Creatine cannot be synthesized in birds *de novo*. The creatine balance in poultry is dependent on dietary arginine. The enzyme transamidinase (AGAT) catalyzes the transfer of an amidino group from arginine to the N-terminal amine of glycine to yield ornithine and guanidinoacetate (GAA). Guanidinoacetate methyltransferase catalyzes the methyl group transfer from S-adenosylmethionine to GAA, yielding creatine (Portocarero and Braun, 2021). Creatine plays a pivotal role in energy metabolism by acting as a phosphate reservoir for adenosine triphosphate (ATP) formation (Portocarero and Braun, 2021). Though creatine can be endogenously synthesized in mammals, birds fully rely on dietary sources. Creatine is highly unstable and is not approved as a feed additive for poultry. However, GAA acts as a precursor of creatine and is approved as a feed additive in broilers (Majdeddin et al., 2020).

6 Arginine sparing effects of arginine metabolites

The lack of commercially available, economical sources of L-Arginine prompted the use of arginine metabolites that fuel arginine's non-protein functions, sparing more arginine for muscle protein accretion (DeGroot et al., 2018). Citrulline and GAA are metabolites of arginine that are commercially available and exhibit arginine-sparing effects. GAA, also known as glycocyamine, is formed from arginine and glycine by the activity of the enzyme arginine: glycine amidinotransaminase in the kidneys. GAA is methylated in the liver by the action of the enzyme guanidinoacetate-N-methyltransferase to form creatine (Khajali et al., 2020). Creatine is transported to the tissues with high energy demands such as the skeletal muscles, spermatozoa, brain, heart, and retina. Creatine and phosphocreatine play a significant role in cellular energy metabolism through the formation of high-energy phosphate bonds (Lemme et al., 2007). However, the tissues have a limited storage capacity for creatine and hence, high circulating creatine levels induce a negative feedback mechanism that inhibits the formation of GAA (Khajali et al., 2020). Though creatine synthesis represents a major proportion of arginine utilization, the thermal instability of creatine limits its use as a feed additive (Vraneš et al., 2017; Khajali et al., 2020). Synthetic GAA is highly thermostable and has a high recovery rate from feed, making it a suitable feed additive in pelleted and extruded feed (Vraneš et al., 2017). GAA supplementation also bypasses the negative feedback inhibition by creatine (DeGroot et al., 2018). Hence, GAA can be considered as a readily available source of creatine and can reduce or spare arginine requirement in broilers (Arginine sparing potential of guanidinoacetic acid in broiler nutrition, 2018). DeGroot, A. A., Braun, U., & Dilger, R. N. (2018) demonstrated that supplementation of 0.12% GAA in an arginine-deficient diet fed to broiler chickens reversed the arginine deficiency-induced reduction in growth performance, muscle glycogen concentration, and muscle phosphagen concentration (DeGroot et al., 2018). GAA supplementation in a low-protein diet during heat stress in chickens improves growth performance and feed conversion ratio (Amiri et al., 2019). GAA supplementation also improves sperm concentration and motility and decreases sperm abnormality in broiler breeder roosters, contributing to improved semen quality and fertility. Creatine phosphate is important for the energy homeostasis of sperm and is required for sperm motility (Tapeh et al., 2017). Creatine also has anti-apoptotic and anti-oxidative effects on cells, which aids in maintaining the plasma membrane integrity of spermatozoa and preventing abnormalities (Meyer et al., 2006). Creatine supplementation also plays a significant role in muscle

development, indicated by an improved feed: gain ratio in broilers supplemented with creatine monohydrate. GAA supplementation in energy energy-deficient diet partially reverses the adverse effects of dietary energy reduction on the growth performance of poultry (Fosoul et al., 2018). Supplementation of GAA, even in arginine-sufficient diets, is found to have an arginine-sparing effect, diverting arginine from creatine formation to protein accretion in broilers (Portocarero and Braun, 2021). Supplementation of GAA in an adequate protein diet for broilers improves BWG and FCR, which might be due to increased energy efficiency. Moreover, GAA also promotes polyamine synthesis required for the synthesis of RNA, DNA, and proteins, and promotes the production of growth hormones (Ahmadipour et al., 2018).

Citrulline is a non-protein amino acid formed from arginine by the action of the enzyme nitric oxide synthase. Citrulline can be converted to arginine by the sequential action of the enzymes argininosuccinate synthase and argininosuccinate lyase (Chowdhury et al., 2017). Dietary arginine is metabolized by the hepatic arginase during first-pass metabolism or is degraded by the intestinal mucosal arginase, limiting its presence in plasma (Zheng et al., 2017). Citrulline bypasses the hepatic metabolism and can be converted to arginine in the kidneys and released into the bloodstream (El-Hattab et al., 2012). Hence, citrulline can be used for the *de novo* synthesis of arginine in poultry (Uyanga et al., 2023). Several studies in human subjects highlight the therapeutic applications of citrulline in different conditions such as skeletal muscle atrophy (Ham et al., 2015), metabolic syndrome (Sailer et al., 2013), and urea cycle disorders (Johnson, 2017). However, the potential role of citrulline in poultry health and disease is not understood completely. Citrulline can be used to partially replace arginine in broiler diets without causing a detrimental effect on the growth performance and intestinal health of the birds (Uyanga et al., 2023). Citrulline supplementation in poultry increases the activity of the NOS enzyme, improves antioxidant synthesis, reduces lipid peroxidation, and modulates the availability of the free amino acids arginine, ornithine, and citrulline (Uyanga et al., 2020). Citrulline supplementation during heat stress in chicks was found to be beneficial in reducing the rectal temperature down to the level of non-heat-stressed birds (Chowdhury et al., 2017). The regulation of core body temperature by citrulline is mediated through its effects on the secretion of inflammatory cytokines, initiating a neuroendocrine immunoregulatory cascade (Uyanga et al., 2022). Citrulline supplementation also promotes muscle protein synthesis by activating the mTORC1 pathway (Osowska et al., 2006; Le Plenier et al., 2011).

Though citrulline and GAA were able to replace arginine in low-protein poultry diets and demonstrate arginine-sparing effects (Esser et al., 2017), GAA was found to be less effective in replacing arginine (Dao et al., 2021b). However, the inclusion levels of the GAA and citrulline supplementation should also be considered; GAA at doses higher than 0.15% in poultry diets is demonstrated to have toxic effects on day 35 in Ross 308 male cockerels fed a low protein diet (Dao et al., 2021b), whereas doses ranging from 0.06%–0.12% promote growth and production in Ross 308 cockerels fed a basal diet on day 35 (Tossenberger et al., 2016). However, as indicated by several studies, the physiological effects of GAA and citrulline depend on factors such as the dose supplemented and the physiological status of the birds ((Ahmadipour et al., 2018; Uyanga et al., 2022)). Further studies are warranted to elucidate the biological events that underlie the response of poultry to citrulline and GAA supplementation.

7 Arginine and the macrophage dichotomy

Arginine and its metabolites serve as important mediators of several physiological processes affecting the health and production of poultry, extensively elaborated elsewhere (Ghamari Monavvar et al., 2020). This review focuses on the role of arginine and its metabolites in immune responses of poultry. Arginine is demonstrated to play a pivotal role in humoral and cell mediated immune responses in poultry (Ruan et al., 2020). Macrophages are professional cells of the innate immune system, which performs diverse functions. Macrophages are involved in the induction and resolution of an inflammatory reaction, tissue repair, and the activation of lymphocyte-mediated adaptive immune response (Miyashita et al., 2022). Macrophages adapt to the respective microenvironment and tissue niches in which they function. This adaptability enables macrophage polarization, which is the process by which macrophages mount a specific phenotypic and functional response to the microenvironmental stimuli encountered in a specific tissue (Sica and Mantovani, 2012). The polarization of macrophages is not fixed due to their multifaceted functions. Polarization of macrophages occurs in response to cell-to-cell interactions and cell-to-molecule interactions during an inflammatory response to maintain homeostasis. Macrophage polarization is regulated by arginine availability in the microenvironment and its metabolism by macrophages (Gharavi et al., 2022). Macrophage polarization can be categorized into M1 (classically activated macrophages or pro-inflammatory) and M2 (alternatively activated macrophages or anti-inflammatory) macrophages based on the arginine metabolism (Lumeng et al., 2007). M1 macrophages are induced by inflammatory mediators such as bacterial lipopolysaccharides and are characterized by the production of proinflammatory cytokines such as IFN-γ, IL-1β, IL-12, iNOS, TNF-α, and reactive oxygen species. M2 macrophages are induced by IL-4 and IL-13, which are Th2 cytokines important for the resolution of inflammation, tissue repair, and wound healing (Benoit et al., 2008). M1 macrophages are microbicidal and inflammatory, whereas M2 macrophages are anti-inflammatory and poor microbicides (Benoit et al., 2008).

Polarization of macrophages along the M1 and M2 axes occurs based on the activities of the arginine metabolizing enzymes NOS and arginase, respectively (Wentzel). Activation of macrophages by microbial products such as LPS, Th1 cytokines such as IL-1β, IFN-γ, or TNF-α, or stress such as hypoxia recruits macrophages to the M1 pathway and induces the expression of the iNOS gene, also known as macNOS because it was first discovered in activated macrophages (Molecular and epigenetic basis of, 2015). Furthermore, the M1 macrophages mediate their anti-microbial activity through the activation of the nicotinamide adenine dinucleotide phosphate (NADPH) oxidase system and the subsequent production of reactive oxygen species (Shapouri-Moghaddam et al., 2018). The availability of arginine and

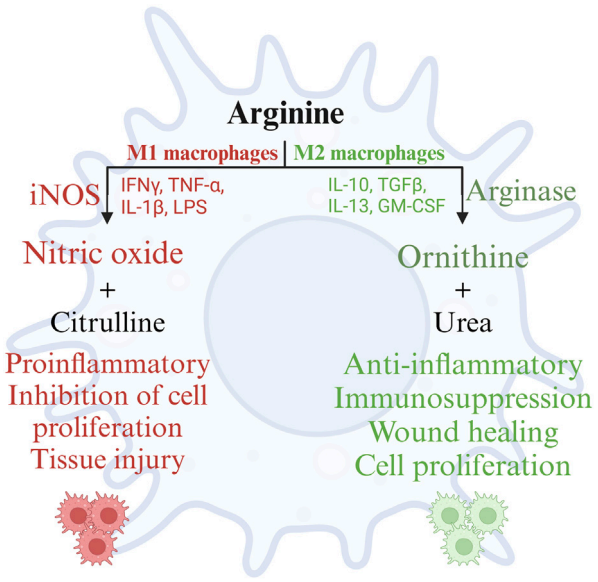

FIGURE 4
Metabolism of arginine in M1 and M2 macrophages. Created with BioRender.com (18 Nov 2022).

endogenous or pharmacological analogs of arginine can constrain NO synthesis. However, the effects of NO can be detrimental to the host tissues as well. The local concentrations of NO play an important role in cytotoxicity; under normal physiological conditions, NO is produced in picomolar quantities, whereas during inflammation, NO is produced in micromolar quantities (Abramson et al., 2001). Sustained increased production of NO damages the surrounding cells and tissues of the host as well, in addition to the pathogens (Abramson et al., 2001). Furthermore, NO causes lipid peroxidation and decreases the activity of serum antioxidants such as glutathione, causing oxidative stress (Qiu et al., 2019).

Unlike M1 macrophages, M2 macrophages metabolize arginine using the arginase pathway, which is stimulated by cytokines such as IL-4, IL-6, IL-10, IL-13, TGF-β, and other factors such as GM-CSF, PGE-2, cAMP, and catecholamines (Martí i Líndez and Reith, 2021). As the cytokines indicate, the arginase pathway in macrophages primarily promotes wound repair, matrix deposition, and healing (Martí i Líndez and Reith, 2021). These functions are mediated by the metabolism of L-arginine by arginase, yielding ornithine and urea. Ornithine is further metabolized by ornithine aminotransferase and ornithine decarboxylase to proline and polyamines, respectively. Proline is essential for collagen synthesis, while polyamines mediate diverse functions such as gene expression, translation, cell proliferation, cell growth, cell signaling, membrane stability, and cell death (Kusano et al., 2008; Li et al., 2022). Arginine supplementation thus reduces inflammation, intestinal injury, and oxidative stress, restoring intestinal homeostasis (Qiu et al., 2019). Arginase competes with NOSs for the common endogenous substrate L-arginine, preventing the overproduction of NO and associated tissue damage during prolonged inflammation. In short, during inflammation, the metabolism of arginine follows a biphasic pattern; initially, there will be a burst of microbicidal NO synthesis followed by an increase in the synthesis of ornithine, proline, and polyamines to promote the resolution of inflammation and wound healing (Martí i Líndez and Reith, 2021). However, iNOS can control arginase activity by generating hydroxy-L-arginine, an intermediate in NO synthesis, to inhibit arginase activity. Arginase, in turn, can deplete arginine in the extracellular milieu, thus regulating NO production (Choi et al., 2009). This chasm between the metabolic pathways of arginine in M1 and M2 macrophages is summarized in Figure 4.

Thus, arginine plays an important role in macrophage activation and function by serving as the sole endogenous substrate for the macrophage enzymes iNOS and arginase, mediating inflammation and resolution of inflammation, respectively.

8 Arginine and the gut microbiota

L-arginine is a metabolically versatile amino acid that serves as a source of carbon, nitrogen, and energy through different catabolic pathways in bacteria. Even though there is a vast diversity of gut microbiota, their metabolic redundancy and interaction with other microbiota species make their survival easier. The interaction between different species of gut microbiota and their metabolic products can have important implications for gut microbial composition, immune regulation, metabolism, and host health as well. It is thought that microbial amino acid utilization in the small intestine is for the synthesis of bacterial proteins. In contrast, amino acid catabolism dominates in the large intestine due to the lower availability of carbohydrates (Dai et al., 2011).

Arginine biosynthesis in bacteria occurs through the linear pathway, present in *E. coli*, or the recycling pathway, present in *Bacillus*. In the linear pathway, acetyl CoA condenses with glutamate to yield arginine through a series of eight steps, while in the recycling pathway, the acetyl group from acetylornithine is transferred to glutamate by the enzyme ornithine acetyltransferase. Both pathways

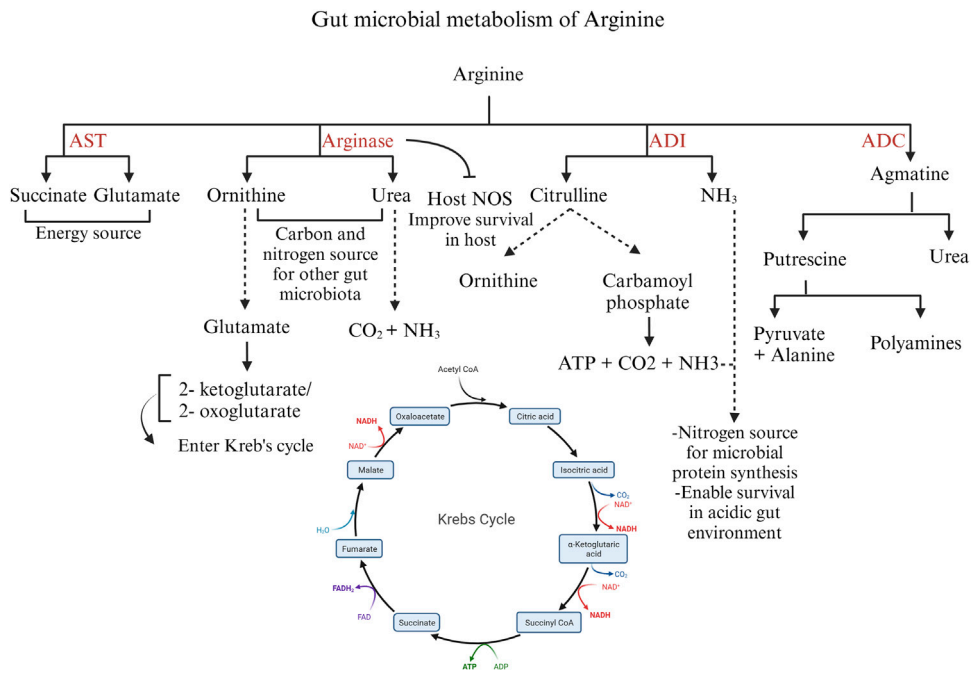

FIGURE 5
Different pathways of arginine catabolism in gut microbiota. Created with BioRender.com (16 September 2023).

are regulated by a negative feedback mechanism based on the concentration of arginine (Lu, 2006). The expression of these enzymes can be affected by other gut microbes and the intestinal compartment as well. In the case of *Lactobacillus plantarum*, it was observed that the expression of argininosuccinate synthase, an enzyme involved in arginine biosynthesis, increased significantly in mice's gastrointestinal tract compared to its *in vitro* expression (Bron et al., 2004). Furthermore, the expression of argininosuccinate synthase is specifically induced in the small intestine of mice, compared to other sections of the gastrointestinal tract (Marco et al., 2007).

The bacteria catabolize arginine via the arginase pathway, arginine deaminase pathway, arginine dehydrogenase/ transaminase/oxidase pathway, and arginine succinyl transferase pathway (Lu, 2006). The expression of these enzymes can be affected by other gut microbes and the intestinal compartment. An outline of the bacterial catabolism by the four major enzymes is schematically represented in Figure 5.

The arginase pathway in bacteria is important to modulate the intracellular levels of arginine and ornithine in response to environmental conditions and physiological needs. Arginase expression or activity increases in the presence of exogenous arginine (Ide et al., 2020) In the arginase pathway, ornithine and urea are formed from arginine. Ornithine is transformed into glutamate by the enzymes ornithine aminotransferase and Δ-pyrroline-5-carboxylate dehydrogenase. Glutamate is converted into 2-ketoglutarate or 2-oxoglutarate, entering the Krebs cycle. Ornithine and urea generated by the arginase pathway can serve as carbon and nitrogen sources for other gut microbial species (Lu, 2006). In microorganisms producing the enzyme urease, the urea formed as a by-product is further hydrolyzed to carbon dioxide and ammonia, serving as a source of nitrogen (Hernández et al., 2021) Moreover, the arginase pathway serves as a survival mechanism for pathogenic bacteria such as *H. pylori*. *H. pylori* arginase inhibits the host NO synthesis by depleting the substrate arginine, thus promoting bacterial survival (Gobert et al., 2001).

The enzyme arginine decarboxylase (ADC) decarboxylates arginine to yield agmatine, which is further hydrolyzed to urea and putrescine by agmatinase. Putrescine, in turn, can be metabolized to pyruvate and alanine. Though putrescine can be metabolized to pyruvate, the ADC pathway is aimed at polyamine synthesis rather than an energy source (Schriek et al., 2007).

The arginine deiminase (ADI) pathway is induced in bacteria under microaerobic and anaerobic conditions. The ADI gene is expressed by several microbes such as *Bacillus licheniformis*, *Clostridium perfringens*, and *Enterococcus faecalis* (Lu, 2006). ADI deiminates arginine to citrulline and ammonia. Citrulline is further converted into ornithine and carbamoyl phosphate by the enzyme ornithine transcarbamoylase. Carbamate kinase mediates ATP production from carbamoyl phosphate with carbon dioxide and ammonia as by-products. Thus, the ADI pathway produces ATP for energy and ammonia as a nitrogen source for the bacteria (Lu, 2006). In addition, ammonia aids in the survival of pathogenic bacteria, such as *C. perfringens,* under acidic conditions (Myers et al., 2006) In bacteria expressing arginase and ADI enzymes, the arginase pathway is predominant under aerobic conditions, whereas the ADI pathway predominates during anaerobic conditions (Hernández et al., 2021).

The arginine succinyl transferase (AST) enzyme mediates the transfer of the succinyl group from succinyl CoA to arginine and further the production of succinate and glutamate through a series of chemical reactions. The AST pathway is the preferred pathway for arginine catabolism in *Pseudomonas* (Hernández et al., 2021);

however, in *E. coli*, the AST pathway is stimulated during carbon starvation, when the priority is cell survival using arginine as a nitrogen source. In some *Pseudomonas* species, such as *P. aeruginosa*, arginine transaminase supplements succinate production under aerobic conditions. Arginine transaminase uses ketoarginine as a substrate, produced by L-arginine: pyruvate transaminase, arginine oxidase, and arginine dehydrogenase (Li and Lu, 2009). Thus, arginine functions as a microbial energy source, governs the expression of bacterial virulence genes, and actively modulates the host's immune response to the gut microbiota (Choi et al., 2012). Despite these known roles, understanding the specific impact of arginine on poultry gut microbiota and enteric pathogens, and its precise involvement in shaping the pathogenesis of enteric diseases requires further elucidation.

9 Arginine and necrotic enteritis

The use of arginine in low-protein diets to improve gut health is recently being investigated in poultry, especially in relation to the control of necrotic enteritis (Zhang et al., 2019; Dao et al., 2022a; Dao et al., 2022b; Dao et al., 2022c). Arginine can modulate the birds' innate and adaptive immune responses to the *C. perfringens* challenge (Zhang et al., 2019). Arginine exerts its effect primarily through the metabolites NO and ornithine, which further take part in downstream reactions or are metabolized to bioactive molecules that take part in inflammation or the resolution of inflammation (Zhang et al., 2019). Arginine modulates macrophage polarization towards the M1 or M2 pathway, significantly affecting the innate immune response to pathogens, including *C. perfringens* (Kim et al., 2022). Apart from its role in shaping the innate immune response, arginine is important in regulating the adaptative immune response to necrotic enteritis and alleviating inflammatory damage caused by necrotic enteritis (Zhang et al., 2019).

During infection by *C. perfringens*, the Toll-like Receptor (TLR)-2 recognizes lipoteichoic acid in the cell wall of *C. perfringens* and initiates downstream signaling, leading to the activation of the transcription factor NFκB, which translocates to the nucleus and induces the expression of iNOS (Korhonen et al., 2002). Inducible NOS converts arginine to NO. Nitric oxide (NO) reacts with superoxide anions to form peroxynitrite (ONOO-), which causes DNA damage, membrane lipid peroxidation, protein dysfunction by nitration of tyrosine residues, and the disruption of tight junction proteins (Potoka et al., 2002), thus increasing intestinal permeability (Korhonen et al., 2002). nNOS and eNOS are expressed and produce picomolar quantities of NO. In contrast, iNOS, expressed in response to inflammation, produces micromolar quantities of NO (Nathan and Xie, 1994). The apoptotic effect of sustained high concentrations of NO and peroxynitrite is due to the inhibition of mitochondrial respiration by S-nitrosylation of complex-I. Additionally, the inhibition of mitochondrial respiration can decrease the transmembrane potential, leading to the release of cytochrome-c, which interacts with the cytoplasmic apoptosis activating factor-1 (Apaf-1) and procaspase-9 initiating the apoptotic cascade (Potoka et al., 2002). Previous research findings indicate an upregulation of NOS gene expression, increased gut permeability, and decreased expression of tight junction proteins and nutrient transporters during necrotic enteritis (Dao et al., 2022d). However, NO can play a significant role in the resolution of enteritis as well. NO can cause nitrosation of the p50 subunit of NFκB or activate IκB, the inhibitor protein for NFκB, and thus regulate the production of proinflammatory cytokines (McCafferty et al., 1997). Sustained overproduction of NO reduces the circulating levels of IL-6 and TNF, downregulating the expression of adhesion molecules. This, in turn, will reduce neutrophil adhesion in inflammatory sites and host tissue damage (Muñoz-Fernández and Fresno, 1998). A schematic representation of the pathway through which arginine mediates the proinflammatory and anti-inflammatory roles during necrotic enteritis is shown in Figure 6.

At the cellular level, the mechanistic target of rapamycin complex I (mTORC1) regulates eukaryotic cell metabolism, growth, proliferation, and survival in response to environmental signals such as nutrients and growth factors (Cummings and Lamming, 2017). Under adequate arginine conditions, the cytosolic arginine sensor cellular arginine sensor for mTORC1 (CASTOR) interacts with the GAP activity toward Rags (GATOR), a negative regulator of mTORC1. GATOR2 lies upstream of GATOR1 and suppresses the RagA/B GTPase-Activating Protein (GAP) activity of GATOR1 under sufficient arginine conditions. Activated RagA/B binds GTP, and RagC/D binds GDP and is anchored to the lysosome by the Ragulator protein (Wolfson et al., 2016; Jung et al., 2019). Rag proteins mediate lysosomal recruitment of mTORC1, which is subsequently activated by Ras homologs enriched in the brain (Rheb) present on the lysosomal membrane. Activation of mTORC1 leads to the phosphorylation of S6 kinase-1 (S6K1) and eukaryotic translation initiation factor 4E-binding protein-1 (4EBP1), which stimulates protein translation and cell growth (Wolfson and Sabatini, 2017). A diagrammatic representation of the regulatory pathway of mTORC1 in cells is presented in Figure 7.

L-arginine is one of the three amino acids (arginine, glutamine, and leucine) that can directly regulate mTORC1 activation and, thus, cell proliferation and apoptosis. Arginine interacts with the transcriptional regulators in the mTOR pathway, enhancing T-cell survival and memory T-cell formation (Geiger et al., 2016). Intracellular arginine availability is thus an important determinant of T-cell function. Arginine metabolism by arginase depletes arginine in the microenvironment, causing T-cell hypo responsiveness. Arginine depletion inhibits proliferation, downregulates the expression of activation markers, and decreases cytokine production in T-cells. Arginase-mediated arginine starvation arrests the cell cycle at the G_0-G_1 phase (Rodriguez et al., 2007). Arginine availability in cells regulates T-cell survival and activity by producing NO. NO exerts proapoptotic effects on T-cells by regulating the intracellular signaling protein expression (Choi et al., 2009).

Arginine supplementation during necrotic enteritis depletes the arginine degradation pathways in gut microbiota, including *C. perfringens*, sparing arginine for T-cell proliferation and function and thus inhibiting disease progression (Dao et al., 2022c) T-lymphocytes, particularly Th1 cells, play an important role in pathogen clearance and adaptive immunity during necrotic enteritis (Fathima et al., 2022). Dietary arginine supplementation increases the T-cell population and promotes T-cell activation and survival (Kishton et al., 2016), thus helping faster recovery. Naïve or quiescent T-cells use oxidative phosphorylation for their energy supply and require little nutrients, whereas activated T-cells rely on glycolytic and glutaminolytic pathways for their energy needs and

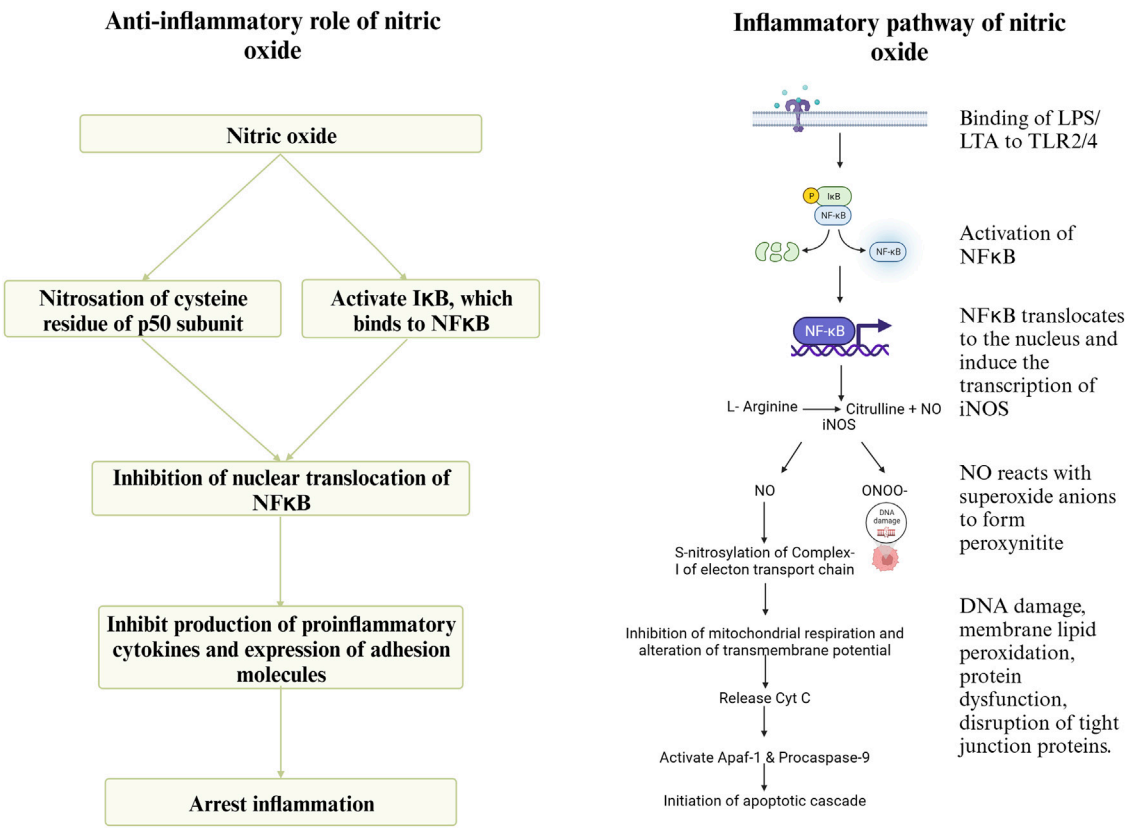

FIGURE 6
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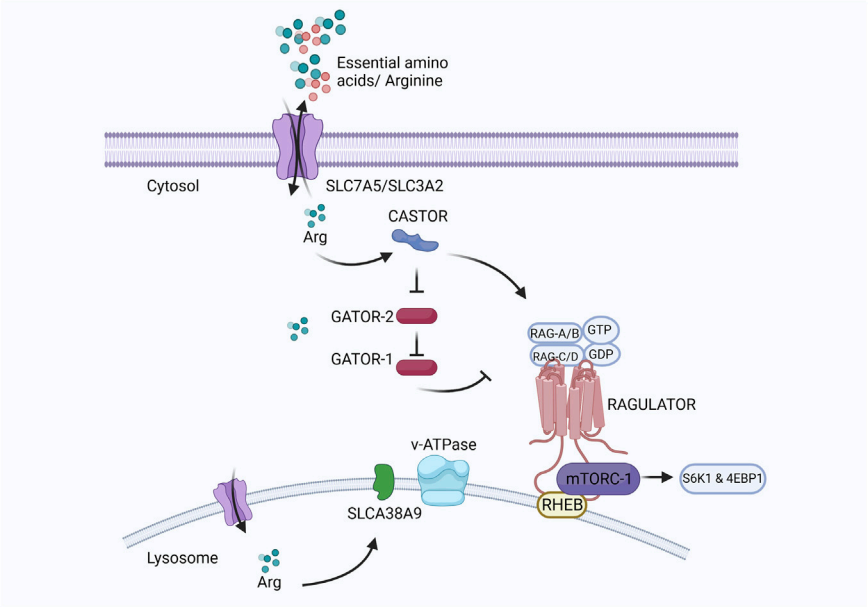

FIGURE 7
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consume large amounts of amino acids, glucose, and fatty acids in the process (Geiger et al., 2016). Activated T-cells heavily consume arginine, causing a marked drop in serum arginine levels. This drop in serum arginine is observed during poultry coccidiosis (Allen and Fetterer, 2000), an important predisposing factor for necrotic enteritis. This can be due to the increased requirement for nutrients to enhance the survival of T-cells during infection and the development of memory T-cells during recovery. Further, this can be correlated with the increased proliferation of intestinal epithelial cells, protein synthesis, and reduced intestinal epithelial cell damage during arginine supplementation *in vitro* (Tan et al., 2010). L-arginine supplementation upregulates the mRNA expression of the tight junction proteins ZO-1, claudin-1, and occludin, resulting in reduced intestinal injury, improved intestinal permeability, and increased villus height: crypt ratio in poultry. Arginine supplementation also inhibits *C. perfringens* colonization, reduces the gross pathology associated with necrotic enteritis and hepatic translocation of *C. perfringens*, improves intestinal absorption and barrier function, and attenuates intestinal inflammatory responses (Zhang et al., 2017; Zhang et al., 2019).

10 Emerging trends and future prospects of arginine in poultry production

Arginine is a functional amino acid of paramount importance in ensuring the health and wellbeing of poultry. It assumes a multitude of critical roles within avian physiology, encompassing functions such as growth, metabolism, immune response, and gut microbial homeostasis. Together, these interconnected aspects highlight the pivotal role of arginine in shaping the nutritional status, immune response, and overall wellbeing of poultry. Arginine offers a promising avenue for improving poultry health and the sustainability of the poultry industry. Though existing research acknowledges the importance of arginine in poultry nutrition beyond protein synthesis, further research is warranted to investigate the optimum levels of arginine and arginine metabolites in poultry diets under different production systems, stages of production, breeds, and physiological states. The potential role of arginine in preventing enteric diseases such as coccidiosis and necrotic enteritis in poultry has been explored to some extent. Still, it offers wider arenas for further understanding arginine's specific mode of action during these disease processes. The possible modulation of gut microbiota by arginine and its association with disease incidence, severity, and gut health during enteric diseases is poorly investigated in poultry. An understanding of the impact of arginine on gut barrier function, immune response, and gut microbial homeostasis can give insights into the potential use of arginine for improving the health and production in poultry.

Author contributions

SF: Conceptualization, Writing–original draft, Writing–review and editing. WA: Writing–review and editing. ReS: Writing–review and editing. ReS: Funding acquisition, Writing–review and editing.

Funding

The author(s) declare financial support was received for the research, authorship, and/or publication of this article. Open access publication fees were supported by the United States Department of Agriculture – Agricultural Research Service (USDA- ARS) grant 6040-42000-046-000D awarded to ReS.

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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frontiers | Frontiers in Physiology

TYPE Original Research
PUBLISHED 10 January 2024
DOI 10.3389/fphys.2023.1229152

OPEN ACCESS

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RECEIVED 26 May 2023
ACCEPTED 26 December 2023
PUBLISHED 10 January 2024

CITATION
Murray BA and Machin KL (2024), Utilizing NMR fecal metabolomics as a novel technique for detecting the physiological effects of food shortages in waterfowl.
Front. Physiol. 14:1229152.
doi: 10.3389/fphys.2023.1229152

Utilizing NMR fecal metabolomics as a novel technique for detecting the physiological effects of food shortages in waterfowl

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Metabolomics is the study of small, endogenous metabolites that participate in metabolic reactions, including responses to stressors. Anthropogenic and environmental changes that alter habitat and food supply can act as stressors in wild waterfowl. These alterations invoke a series of physiological processes to provide energy to restore homeostasis and increase survival. In this study, we utilized fecal metabolomics to measure metabolites and identify pathways related to a 6-day feed restriction in captive mallard ducks (*Anas platyrhynchos*, n = 9). Fecal samples were collected before (baseline) and during feed restriction (treatment). H^1 Nuclear Magnetic Resonance (NMR) spectroscopy was performed to identify metabolites. We found that fecal metabolite profiles could be used to distinguish between the feed-restricted and baseline samples. We identified metabolites related to pathways for energy production and metabolism endpoints, and metabolites indicative of gut microbiota changes. We also demonstrated that mallard ducks could utilize endogenous reserves in times of limited caloric intake. Fecal metabolomics shows promise as a non-invasive novel tool in identifying and characterizing physiological responses associated with stressors in a captive wild bird species.

KEYWORDS

stress physiology, waterfowl, metabolomics, fecal metabolomics, feed restriction, mallard duck, metabolism

Introduction

The Prairie Pothole Region (PPR, a habitat composed of shallow ephemeral wetlands in 3 prairie provinces (Alberta, Saskatchewan and Manitoba) and five states) is one of North America's most important habitats for nesting and migratory waterfowl (Mann, 1974). These wetlands are vulnerable and are experiencing drying due to climate change and are undergoing drainage for agricultural and urban development (Niemuth et al., 2014). Changes in this vital habitat are expected to cause waterfowl population declines through decreased nesting habitat and food availability (Niemuth et al., 2014).

Acquiring appropriate and sufficient nutritional resources is essential for wild populations during every life cycle stage (Fokidis et al., 2012). Shortages or unpredictable food availability is an environmental stressor for many wild species. Exposure to stressors causes the hypothalamic-pituitary-adrenal (HPA) axis to activate and release glucocorticoids (corticosterone (CORT) in birds) (Rich and Romero, 2005; Strochlic and Romero, 2008). When glucocorticoids are increased, they incite metabolic changes and biochemical responses, increasing available energy (accelerating the rate of

gluconeogenesis) and improving the chance of survival (Teague et al., 2007). These metabolic alterations increase circulating glucose to help supply the increased energy demand and to overcome the stressor while temporarily suspending non-essential activities (e.g., digestion, growth) in favour of survival (MacDougall-Shackleton et al., 2019). Metabolic processes like gluconeogenesis, protein degradation, lipolysis, and metabolites such as glycerol, and ketone bodies are promoted to produce energy sources when circulating glucose levels are depleted (Teague et al., 2007).

While the short-lived physiological response to a stressor enables survival, chronic exposure to unpredictable or uncontrollable environmental challenges can result in prolonged CORT elevations that may have broad implications affecting survival (Sapolsky et al., 2000). With sustained chronic elevations of CORT, costly, non-essential processes (such as growth and reproduction) remain persistently inhibited in order to initiate pathways that produce energy to recover homeostasis (Wingfield et al., 1995; Blas, 2015). Consequently, the continued release of glucocorticoids reduces glycogen production and protein synthesis rates which can interfere with energy conservation through the persistence of catabolic actions, stimulating the degradation of fatty acids and proteins (Fokidis et al., 2012). Domestic chickens experiencing heat stress had a decrease in muscle protein synthesis and amino acid uptake, indicating muscle tissue catabolism and inhibition of growth (Lu et al., 2018). Another study in chickens exposed to heat stress found increased liver lipid metabolism, suggesting break down of fat stores for energy (Jastrebski et al., 2017). Similar studies in wild birds are lacking.

To better understand the metabolic consequences of the stress response, a process known as metabolomics can be utilized. Non-targeted metabolomics, a quantitative analysis of metabolites in biofluids (e.g., blood plasma or serum, urine, etc.), tissues or feces, can detect changes in an organism's metabolome. The metabolome is a collection of metabolites that participate in metabolic reactions required for growth, maintenance, and responses to disease and stressors (Samuelsson and Larsson, 2008). Changes in the metabolome may reflect differences in organ function, thus giving vital clues about the physiological status and health of the individual (Viant, 2008). Over the past decade, there has been a rapid increase in environmental metabolomics studies to discover biomarkers of toxicant exposure and disease that can be applied to future conservation efforts (Viant et al., 2003). Nuclear magnetic resonance (NMR) spectrometry-based metabolomics is one of the dominant analytical methods used for environmental metabolomics studies The broad range of metabolites detected and ease of sample preparation makes NMR an easily reproducible tool making it suitable for repeated sampling for monitoring wildlife (Valerio et al., 2019).

Fecal metabolomics can not only provide quantification of the endpoints of metabolism but can also identify metabolites that result from the host-microbiome interaction and give an integrated picture of the metabolic consequences of the stress response. The gut microbiome, comprised of trillions of species of bacteria, can affect the host metabolism and energy homeostasis (Kogut, 2013; Schröder, 2022). Noguera et al. (2018), demonstrated that the composition of microbial community can be altered by glucocorticoids in a wild yellow-legged gulls (*Larus michahellis*) (Noguera et al., 2018). The metagenomic metabolite link between the gut and brain may be bidirectional through the gut-brain axis. In fact, gut microbiota are crucial for maintaining the homeostasis of the local, systemic, and brain systems (Bistoletti et al., 2020). The complex network between the HPA axis and autonomic nervous system facilitate communication between the gut and brain which regulates the host physiological homeostasis. Commensal gut bacteria-host interactions release endocrine messengers, neurotransmitters such as serotonin and gamma-aminobutyric acid (GABA), and metabolites such as short-chain fatty acids (SCFA) acetate, propionate, and butyrate which, in turn, influence brain function (Cryan et al., 2019).

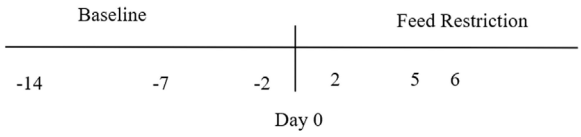

FIGURE 1
Sampling timeline for fecal collection from nine mallard ducks before (baseline) and during feed restriction. Baseline (19 samples), Day 2 (7 samples), Day 5 (7 samples), Day 6 (4 samples).

To enhance our understanding of the physiological consequences of an ecologically relevant stressor, we subjected mallard ducks (*Anas platyrhynchos*) to 6 days of food restriction. This study aimed to quantify and identify fecal metabolites during chronic food shortages. We predicted that NMR spectrometry could be used to assess the fecal metabolome and distinguish fecal profiles of mallards before and during feed restriction. We hypothesize that ducks would utilize pathways to increase energy by metabolizing alternative sources such as fat and muscles during nutritional stress. Fecal metabolomics has rarely been applied in birds, although it has been used in mammals to non-invasively assess the consequences of stressors (Valerio et al., 2019). This is the first study to utilize fecal metabolomics in a wild waterfowl species and one of the first to be applied in a non-domestic species of birds.

Materials and methods

To examine the metabolic response of waterfowl to a chronic stressor, a captive flock of adult (1 year old) mallard ducks (n = 9, 7 female, 2 male) were subjected to feed restriction for 6 days. At the start of the study (day 0), ducks had a mean weight of 1080 g ± 140 std dev (range 900–1350 g). Ducks were considered healthy based on a physical exam. No illnesses were noted during winter housing (October 2018-May 2019) or during the study. The ducks were housed in two outdoor fenced pens to mimic natural conditions 10 km south of St. Denis, Saskatchewan (52°06′32.5″N 106°04′25.7″W) in May 2019. The outdoor enclosure included upland grass (26.8 m^2) and a natural pond (13.4 m^2). These enclosures allowed ducks to exhibit natural nesting and dabbling behaviours. The pond area was lined, and pond water was pumped as needed to maintain adequate water levels and provide fresh water to the ducks. Access to pond water permitted supplemental feeding of natural aquatic invertebrates. Ducks were provided with a commercial nutritionally balanced diet providing 2850 kcal/kg,

(crude protein (actual): 19%, crude fat (actual): 3.8%, crude fiber (actual): 5%) *ad libitum* except during food restriction. We followed the guidelines of the Canadian Council on Animal Care as defined by the Guide to the Care and Use of Experimental Animals. This project was approved by the University of Saskatchewan Animal Research Ethics Board—protocol no. 20030021.

This study was divided into two activities: baseline (1) and food restriction (2) (Figure 1).

1) Fresh fecal samples were collected to characterize the baseline values of fecal metabolites 1 hour after the food was introduced in the morning on days −14, −7, −2. Baseline samples were taken over 2 weeks to ensure that repetitive handling did not impact the results and normal metabolome of the ducks was represented.
2) Ducks underwent a restricted food trial for 6 days to mimic a chronic stressful event where an uncontrolled environmental challenge leads to decreased food availability. Mallards were fed a diet that met 75% of their basal metabolic rate (BMR, minimum number of calories needed for the body to perform necessary functions) for 6 days. All ducks were weighed before (baseline days −14, −7, −2) and when the birds were sampled. The weight at the end of feed restriction was subtracted from the baseline weight (day 0) to calculate weight loss during the experiment.

BMR was calculated based on the weight of the birds during the baseline period using an equation developed for mallards (Prince, 1979). The BMR and metabolic energy of the feed were used to calculate the feed amount for all the birds daily. The mass of food (g) estimated to meet the BMR/bird/day was calculated.

$$\text{BMR (kcal/bird/day)} = 87.9\,\text{W}^{0.734}, \text{where W} = \text{mass (kg)}$$

The constant 87.9 (SE = 2.3) (Prince, 1979)

$$g = \text{BMR}_{\text{bird}}/\text{ME}_{\text{Food}}$$

Feed was divided into individual bowls placed within a divided feeding structure to ensure that all ducks had equal access to food. Ducks were monitored for signs of aggression defined as chasing, pecking, feather pulling or fighting for a minimum of 20 min after introducing the feed. Fresh fecal samples were collected 1 h after the food was introduced in the morning during baseline and on days 2, 5 and 6 of the feed restriction period. We expected that fecal metabolite changes would not be immediate, so began sampling on day 2. As we were interested in chronic changes, we sampled again on days 5 and 6. We avoided daily sampling because we suspected frequent capture and handling would be stressful and potentially influence the results. Feces were collected by placing ducks into separate dry rectangular plastic storage boxes with plastic netting lids for airflow and ventilation on sampling days. Ducks were kept in fecal collection containers until they defecated but released from containers if they failed to defecate within 45 min of capture. Fecal samples were collected from the bottom of the fecal collection containers into cryovials and flash-frozen in liquid nitrogen to stop metabolite degradation. Samples were stored at −80 C until sample analysis. During feed restriction, ducks produced small and less frequent defecation which decreased the number of samples available for analysis.

Fecal samples were collected at baseline and on days 2, 5, and 6. Samples were collected at each time point with one or two birds a day exceeding the maximal allowable confinement time, preventing sufficient sample collection (Table 1). Each sample was lyophilized in a Labconco Freezone (Labcono Corporation, Kansas City, MO, United States) to remove water and standardize sample weight. However, day 6 samples were not analyzed as there was insufficient freeze-dried sample (minimum of 10 mg required) from the majority of the ducks (4/9 ducks). Freeze-dried fecal powder (10 mg) was extracted in random order to remove potential processing bias according to previously published protocols in chickens (Le Roy et al., 2016) using 500 µL of 0.2M pH7.4 + NaN_3 3 mM + trimethylsilylpropanoic acid - TMSP ($C_6H_{14}O_2Si$ Sigma Aldrich 269913) in 0.1% phosphate buffer in D2O. TMSP was used as a chemical shift reference as an internal standard. Then 200 µL of supernatant was pipetted into a 5 mm NMR stem tube for non-targeted ^{1}H NMR spectroscopy (Bruker Advance III HD 600 MHz NMR spectrometer). Tuning and shimming was completed for each sample. A standard 1- dimensional noesypr1d pulse sequence (noesypr1d, 90° pulse length 15 µs, total acquisition time of 4 s) with water suppression applied during relaxation delay (1 s) and mixing time (100 m) at 298 k. For each sample, 128 scans were recorded.

To investigate variation in metabolite profiles among baseline and during feed restriction, raw NMR spectra were processed in Topspin 3.6 (Bruker Corporation, MA, United States) and then imported into Chenomx NMR Suite 7.0 (Chenomx, Edmonton, Canada) for manual baseline and shimming correction and TMSP was calibrated as 0.0 ppm. Peaks were assigned to individual metabolites by comparing chemical shifts of 1D 1H-NMR spectra of the samples to metabolites available at the Chenomx Compound Library. Metabolite profiling for samples was performed by a single person (B.A.M.) with the metabolite fitting algorithm available within the Chenomx software. Concentrations of compounds were exported into Excel and used for statistical analysis in MetaboAnalyst 5.0 (https://www.metaboanalyst.ca) (Pang et al., 2021).

Concentration data were normalized via constant sum; data were transformed with log normalization and mean-centered data scaling prior to statistical analysis. To ensure that repetitive handling did not impact the results we used a multiple-baseline design utilizing concentration data from every baseline day (Christ, 2007). There was no detectable difference in metabolome between days (ANOVA utilizing MetaboAnalyst identified 0 significant features) (baseline days −14, −7, −2) and will be referred to as baseline hereafter. Chemometric analyses of principal component analysis (PCA) and partial least squares discriminate analysis (PLS-DA) methods were applied to investigate differences in metabolite profiles between the baseline and food restriction periods. The PCA was used as an unsupervised approach to investigate the separation between samples, followed by a supervised PLS-DA to identify natural groupings and evaluate the difference between treatments. The PLS-DA model performance was validated using i) 10-fold CV, which used accuracy and R2 and Q2 values, and (ii) permutation tests, which used prediction accuracy after

TABLE 1 Sample collection and body mass changes for nince mallard ducks prior to (baseline) and after feed restriction.

Duck	Sex	Body mass day 0 (g)	Body mass post-feed restriction (g)	Body mass loss (g)	Percent body mass lost (%)	Number of samples collected during baseline	Sample collected on day 5 of feed restriction?
1	F	1200	756	444	37	3	Yes
2	F	1100	1000	100	9.1	3	No
3	F	950	900	50	5.3	1	Yes
4	M	1350	1200	150	11.1	3	Yes
5	F	1125	1000	125	11.1	3	Yes
6	M	1100	950	150	13.5	1	Yes
7	F	900	725	175	19.4	2	No
8	F	1050	850	200	19.0	1	Yes
9	F	950	850	100	10.5	2	Yes

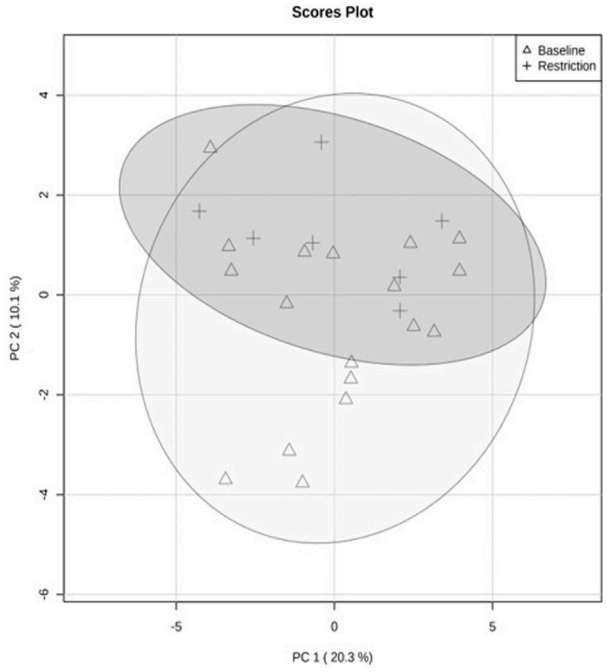

FIGURE 2
Quantitative analysis of fecal metabolite profiles from mallard ducks (n = 9) collected prior to (baseline days –14,-7,-2, 19 samples) and during feed restriction (day 5, 7 samples) using Principal Component Analysis (PCA). The separation of 95% confidence regions in clusters indicates a notable difference between metabolite profiles.

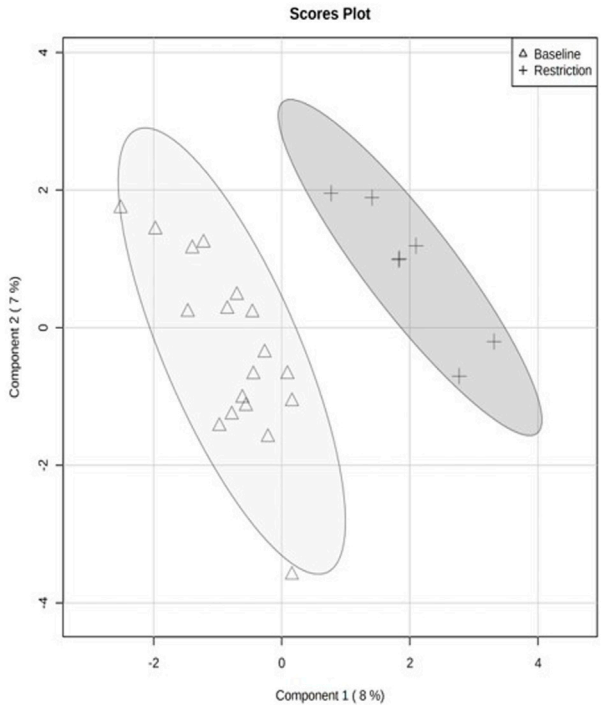

FIGURE 3
Quantitative analysis of fecal metabolite profiles from mallard ducks (n = 9) collected prior to (baseline days –14,-7,-2, 19 samples) and during feed restriction (day 5, 7 samples) using Partial Least Square Discriminant Analysis (PLS-DA). Separation of 95% confidence regions in clusters indicates a notable difference between metabolite profiles.

100 iterations. When 10-fold CV values >0.6 and permutation test $p < 0.05$, PLS-DA models are considered accurate and predictive. The PLS-DA variable importance in projection plot (VIP) identified metabolites responsible for the variations between treatments. All metabolites with VIP score values greater than one were considered essential in separation between treatments. To determine if ducks with greater mass loss foraged more for alternative sources of food (i.e., insects (Becker et al., 1996)), we examined the relationship between trehalose and body mass loss using a Pearson's product correlation.

The Pathway Analysis tool in MetaboAnlayst utilizing significant VIP metabolites (hereafter, VIP metabolites) was used to identify metabolic pathways. The human metabolome database (HMDB Version 4.0) was used to investigate the pathways

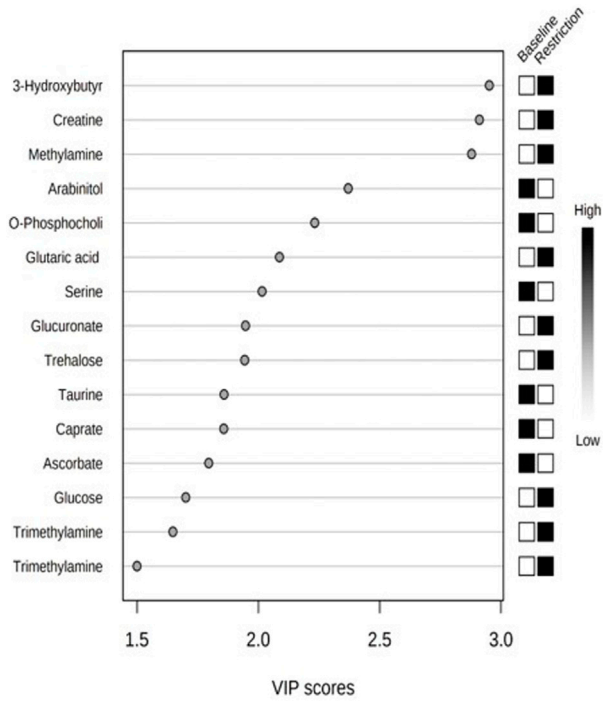

FIGURE 4
Top 14 metabolites based on variable importance in projection (VIP) scores from PLS-DA from mallard duck feces collected prior to (baseline days −14,-7,-2, 19 samples) and during feed restriction (day 5, 7 samples).

associated with the identified metabolites. Pathway analysis was performed with MetaboAnalyst 5.0 using the chicken (*Gallus gallus*) database. Enrichment analysis was performed with over-representation analysis (ORA) was used to analyze the subset of metabolites identified with the VIP plot to identify the pathways associated with each sampling period (baseline vs feed restriction). The pathway library of the Kyoto Encyclopedia of genes and genomes (KEGG) database was used as the reference (Ogata et al., 1999).

Results

Ducks did not exhibit any signs of aggression during feeding, before or during the food restriction period. During the 6-day feed restriction, ducks lost an average of 15% ± 9 of body mass (mean = 154.9 , SD = 63.9 range = 75 (6.9%) - 294 g (28%) Table 1). The Canadian Council on Animal Care (CCAC) considers 20% body mass reduction significant and in need of intervention. No duck reached this point prior to the last day of the study when they were provided with *ad-lib* food.

The PCA revealed separation between baseline and day 5 feed restriction metabolite profiles (Figure 2). The PCA fecal profiles started separating during the feed restriction day 2 (results not shown), and maximum separation was achieved on day 5. The two principal components made up 30.3% of the total variation (PC1 20.3% and PC2 10.1%). The PLS-DA showed separation between the baseline and feed restriction samples (Figure 3). The two principal components made up 15% of the total variation (PC1 8% and PC2 7%). The cross-validation of the PLS-DA model via leave-one-out cross-validation (LOOCV) showed that the first two components showed very high scores of accuracy (0.84). The multiple correlation coefficients (R^2, 0.91) and cross-validated R^2 (Q^2, 0.17) indicated the PLS-DA model's good fit and performance.

We putatively annotated 134 metabolites in the NMR spectra of the mallard feces. Variable importance in projection (VIP) scores from PLS-DA found 14 metabolites that explain the variation in metabolic profiles between treatments using the chicken (*Gallus gallus*) pathway library (Figure 4; Table 2). Compared to the baseline, mallard fecal sample during feed restriction contained higher concentrations of 3-hydroxybutyrate, creatine, methylamine, glucose, glutaric acid, trehalose, glucuronate, and trimethylamine and lower levels of arabinitol, O-Phosphocholine, ascorbate, caprate and amino acids (taurine, and serine) compared to baseline (Table 2). There was no relationship between trehalose and body mass loss ($r = -0.331$, $p = 0.211$).

We performed a metabolite site enrichment analysis (MSEA) with an over-representation analysis (ORA) to test if there were biologically relevant groups of metabolites. The MSEA is a method that helps to determine if metabolites identified in the VIP analysis are biologically relevant. This determination was made by identifying pathways or diseases associated with identified metabolites. The enrichment analysis uses prior knowledge of metabolites obtained through the Human Metabolome Database and identifies related pathways in the Kyoto Encyclopedia of Genes and Genomes (Xia et al., 2009). Metabolites with a higher concentration during the baseline period (arabinitol, O-phosphocholine, ascorbate, caprate, taurine, and serine) produced a metabolite set enrichment analysis that revealed the metabolites were associated with pathways related to taurine and hypotaurine metabolism, phosphatidylcholine biosynthesis, beta-oxidation of very-long-chain fatty acids, phospholipid biosynthesis, fatty acid biosynthesis, sphingolipid metabolism and bile acid biosynthesis. Metabolites with a higher concentration during the feed restriction period (3-hydroxybutyrate, creatine, methylamine, glutaric acid, glucose, trehalose, glucuronate, trimethylamine) produced a metabolite set enrichment analysis that revealed the metabolites were associated with pathways related to lactose degradation, trehalose degradation, glucose-alanine cycles, lactose synthesis, transfer of acetyl groups into mitochondria, glycolysis, starch and sucrose metabolism, inositol metabolism, gluconeogenesis, fatty acid biosynthesis, galactose metabolism, sphingolipid metabolism, arginine and proline metabolism, Warburg effect, glycine and serine metabolism and tyrosine metabolism.

Discussion

In this study, we used NMR metabolomics to assess changes in mallard duck fecal metabolome exposed to an ecologically relevant stressor of chronic feed restriction. Using fecal metabolite profiles, we could differentiate between baseline and feed restriction, and 14 metabolites explained the variation between samples.

In order to maintain homeostasis, energy metabolism is required to provide continuous fuel for cellular processes (Wilson and Matschinsky, 2021). Carbohydrate metabolism is vital for energy production, the most essential being glucose (Scanes, 2022). Glucose

TABLE 2 Top metabolites based on variable importance in projection (VIP) scores from PLS-DA from mallard duck feces collected prior to (baseline days −14, −7, −2, 19 samples) and during stress (food restriction day 5, 7 samples).

Metabolites decreased during feed restriction	Metabolites increased during feed restriction
•O-phosphocholine	•3-hydroxybutryrate
•Serine	•Creatine
•Taurine	•Methylamine
•Caparate	•Arabinitol
•Ascorbate	•Glutaric acid
	•Glucuronate
	•Trehalose
	•Glucose
	•Trimethylamine

is required for neuronal function (von Eugen et al., 2022). The brain is the most energy-intensive tissue, but avian neurons require approximately three times less glucose than their mammalian counterparts (von Eugen et al., 2022). Studies in birds have shown higher plasma glucose concentrations when compared to mammals that have similar body mass (Sweazea, 2022). Birds utilize glucose for various physiological functions such as cellular oxidation, glycogen (polysaccharide) synthesis (to store energy) in the liver and glycolytic flight muscles (pectoral and supracoracoideus), synthesis of fatty acids (lipogenesis) and other non-essential amino acids and vitamins (vitamin C) (Scanes, 2022).

Glucocorticoids promote hyperglycemia by activating multiple pathways to create energy. Glycerol is mobilized from tri- and di-glyceride lipids via lipolysis from adipose tissue, which can create glucose in the liver via gluconeogenesis. Glycogenolysis of glycogen in the skeletal muscles creates glucose-1-phosphate, which can be metabolized through glycolysis to maintain blood glucose levels (Goldstein et al., 1993; Landys et al., 2004). All mallards in our study exhibited a body mass reduction during feed restriction because energy expenditure was higher than metabolizable energy intake (75% of BMR). Although the mass loss between birds was variable, some individuals lost more than others despite receiving a similar feed allocation throughout the restriction period. This may have been due to individual genetics and invertebrate forging success among birds.

Baseline

During the baseline period, metabolites identified were associated with phospholipid, bile acid, and fatty acid biosynthesis pathways and cell production. Amino acids, serine and taurine were higher during the baseline than during feed restriction. These metabolites form proteins and are essential for growth through the production of muscle and fat reserves (serine) and homeostatic maintenance through regulating digestion (taurine) (Kalhan and Hanson, 2012; Uyanga et al., 2022). Serine can be obtained from nutritional sources but can also be readily produced through the serine synthesis branch of glycolysis (Klaassen and Biebach, 1994). Ducks during feed restriction may have undergone serine deprivation, which has been shown to alter mitochondrial glucose and glutamine metabolism, reducing adenosine triphosphate (ATP) production (Gao et al., 2018). Declines in ATP can lead to a diminished growth rate, which may impede homeostasis. It is impossible to determine if depletion in serine occurred because of the inability to produce serine or if serine was utilized by the microbiota as an energy source when nutrients were limited (Velayudhan et al., 2004). More studies should be completed to investigate the turnover of serine in tissues during feed restriction. Taurine is an amino acid utilized in bile acid biosynthesis in the liver, facilitating the digestion of fats and oils. In chickens, taurine is typically regarded as a non-essential amino acid obtained through the diet and internal synthesis (Martin, 1972). However, when exposed to stressors, taurine may become semi-essential, meaning that the synthesis capacity may be insufficient to meet the increased demand. In these situations, taurine must be obtained through the diet. (Surai et al., 2020). Taurine increases glycogen synthesis, glycolysis and glucose uptake and inhibits gluconeogenic enzymes in the liver from releasing hepatic glucose (Batista et al., 2012).

Hepatic energy turnover is a dynamic process where the liver takes up, stores, and releases energy to maintain blood glucose concentrations to ensure energy supply to the tissues (Halperin and Cheema-Dhadli, 1989). As ducks were fed *ad lib* during baseline, substantial metabolism of hepatic energy reserves would not have occurred as there was sufficient glucose to maintain homeostasis. Interestingly, a human study found that plasma taurine increased after 3 days of fasting but decreased to baseline levels after 5 days of fasting (Tang et al., 2021). Those authors suggested that systemic metabolic reprogramming may occur after a period of continuous starvation (Tang et al., 2021). While the blood and fecal metabolomes are not the same, changes in the blood metabolome can be indicative of shifts or alterations in gut microbiota activity and various metabolic processes that may alter the fecal metabolome (Behr et al., 2019). Future work is needed in birds to determine if metabolic reprogramming occurs after a long fasting period. This flexibility may be a necessary physiological adaptation for migratory birds.

Multiple factors such as genetics, life-history stage, and environmental conditions can influence metabolic processes

(Wingfield et al., 1998; Viant et al., 2003). Birds may rely on endogenous energy stores (e.g., glycogen, fatty acids, protein) during extended energetic effort and fasting (LaGrange and Dinsmore, 1988; Nord and Williams, 2015). Fat and muscle are essential energy reserves in long-distance migratory birds like mallards that experience prolonged periods of exertion where energetic costs are high. These life history stages are often accompanied by prolonged reductions in energy intake, limiting protein synthesis and leading to muscle catabolism and proteolysis (Moyano et al., 1998). Powered flight during migration causes the activation of flight muscles, which account for 10%–25% of the bird's body mass, requiring large amounts of energy (DuBay et al., 2020). A study in garden warblers (*Sylvia borin*) found a 22% decrease in leg muscle mass after migration, likely through muscle catabolism needed to provide energy (Bauchinger and Biebach, 2005).

Higher concentrations of antioxidants associated with preventing cell damage, like ascorbate (ascorbic acid, vitamin C) and gut protectants, like O-phosphocholine, were observed during the baseline. Ascorbate is required for amino acid and mineral metabolism, which may be disrupted during periods of physiological stress (McDowell, 1989). A study in chickens exposed to heat stress demonstrated decreased ascorbate concentrations (Attia et al., 2016). Unlike mammals, birds can produce ascorbate in their liver, which is required to meet physiological needs through the enzymatic conversion of sugars (Khan et al., 2012). Under stressful conditions such as prolonged feed restriction, the ascorbate requirement may exceed endogenous production capacity, resulting in a net deficiency. During nutritional deprivation, birds may conserve endogenous sugars for neuronal function. O-phosphocholine involved in phospholipid biosynthesis, is found in cell membranes and is associated with a healthy gut (Wishart et al., 2018). Phosphatidylcholines are thought to protect the large intestine wall by producing a mucosal layer (Parlesak et al., 2007). This layer is a critical aspect of gut health and protects the intestines by creating a barrier against bacteria (Chen et al., 2015). Heat stress has been shown to cause gut barrier failure (Pearce et al., 2013). During feed restriction, possible gut mucosal barrier atrophy may have decreased gut health, although this was not assessed. A study in chickens found that gut segments had increased intestinal fragility (loss of tensile strength) with increasing duration of feed withdrawal, especially when longer than 14 h (Bilgili and Hess, 1997). Adequate tensile strength is crucial for gut health as increased fragility can lead to the leakage of digestive juices, bacteria, or undigested food into the abdominal cavity, potentially causing infection or inflammation (Egorov et al., 2002). Similarly a study in rats found short-term fasting-induced intestinal mucosal barrier atrophy, which may have occurred in feed-restricted ducks (Dock et al., 2004).

Medium-chain fatty acids have become increasingly popular in poultry feeds because of their antimicrobial effects (Çenesiz and Çiftci, 2020). Caprate (also called capric acid or decanoic acid) is a medium-chain fatty acid found in milk lipids and oils of several plants (Zentek et al., 2011). We observed a higher concentration of caprate during the baseline period. As caprate is a metabolite not synthesized by ducks, the presence of caprate in the feces is unknown but may have been present in the commercial diet. The caprate concentration in the feces was lower during feed restriction, likely due to reduced feed consumption.

Feed restriction

Several extracellular and cellular mechanisms work together to form the mucosal barrier function to absorb nutrients. Many animals regulate glucose during feed restriction by mobilizing internal energy sources.

Mobilization of energy (glucose) can be achieved through the metabolizing of stored glycogen and alternative energy sources like amino acids (methylamine, 3-hydroxybutyrate, glucuronate, glutaric acid), through protein degradation and tissue metabolism (creatine), and bacterial utilization of alternative energy sources such as trehalose and trimethylamine (TMA). During fasting, metabolism will shift from homeostatic maintenance to increased utilization of energy reserves to increase glucose through glucocorticoid stimulation of gluconeogenesis in the liver (Sapolsky et al., 2000). During feed restriction; there was a significant increase in glucose in the feces. It is possible that a damaged mucosal barrier prevented the absorption of glucose. Jastrebski et al. (2017) found elevated glucose levels in the liver of birds exposed to chronic heat stress but did not measure fecal glucose (Jastrebski et al., 2017). In contrast, a study by Lu et al. (2018) found that birds exposed to chronic heat stress for 14 days exhibited lower serum glucose concentrations, possibly indicating exhaustion of the bird's endogenous energy reserve (Lu et al., 2018). While ducks in this study were not heat stressed, environmental stressors are known to affect fecal metabolites in cattle (Valerio et al., 2019). This mobilization of energy reserves is beneficial in the short term, but long-term catabolism can lead to deleterious effects such as muscle wasting and death. In this experiment, methylamine was increased during feed restriction. Methylamine is an endogenous short aliphatic amine involved in the central regulation of food intake (Pirisino et al., 2004; Zendehdel et al., 2020).

With food restriction and body mass loss, endogenous energy shortage requires mobilization of alternate energy sources to help maintain ATP synthesis like ketone bodies such as 3-hydroxybutyrate (β-Hydroxybutyric acid). Ketone bodies are produced in the liver when glucose from the glycerol reserve starts to decline, causing fatty acids and phospholipid metabolism (Mierziak et al., 2021). Thus, ketone increases may be beneficial in extended times of exertion, such as migration (Costantini, 2008). Numerous studies in migrant birds have reported increases in 3-hydroxybutyrate during flight (Landys et al., 2005; Bairlein et al., 2015; Gutiérrez et al., 2019). The amount of fuel stored before migration can limit migration distance (Gutiérrez et al., 2019). Mallards are capital breeders, meaning they arrive at the breeding grounds with the endogenous reserves needed for reproduction (Meijer and Drent, 1999). Thus, mallards will stop along migration flyways to replenish energy reserves depleted during flight (Beatty et al., 2017).

Incubation is another life history stage where female mallards rely on alternative energy sources, such as ketone bodies, as they lose an average of 25% of their body mass (Krapu, 1981). A study in nesting King eider (*Somateria spectabilis*) hens demonstrated reliance on endogenous and exogenous energy sources. Females had high concentrations of free fatty acids, β-hydroxybutyrate, and glycerol throughout incubation, indicating that fat reserves were a primary energy source (Bentzen et al., 2008). A study in king penguins (*Aptenodytes patagonicus*) demonstrated elevated 3-

hydroxybutyrate concentrations after a 150-day natural fasting period, suggesting that ketone bodies are necessary to resist starvation (Cherel and Le Maho, 1985). While mallards are not as resistant to starvation, they appear to be able to utilize fat reserves to create energy during periods of food shortage.

In addition to ketone bodies, we observed higher concentrations of metabolites associated with pathways for alternative energy production, such as glucuronate (glucuronic acid), glutaric acid, and creatine, during feed restriction. This may demonstrate the mallard ducks' ability to utilize endogenous reserves in times of limited caloric intake. Glucuronate is derived from glucose and is converted from less common sugars to ones that can be more readily metabolized to provide energy when ATP is limited (Bhagavan, 2002). Glucuronate is also a precursor of ascorbate (vitamin C). Glucuronate concentrations were higher during the feed restriction, while ascorbate was higher during the baseline period, indicating that ducks were utilizing glucuronate and could not generate ascorbate during caloric deficiency (Linster and Van Schaftingen, 2006). Glutaric acid was also found in higher concentrations during feed restriction. Glutaric acid is produced by amino acid metabolism and is correlated with metabolic acidosis during starvation (Wishart et al., 2018). Higher concentrations of glutaric acid in ducks experiencing a caloric deficit may be due to the utilization of alternative energy pathways.

During feed restriction, there was an increase in creatine, an amino acid that supplies energy to the cells, primarily the muscles (Hedemann and Damgaard, 2012). Muscle catabolism generally seen during severe malnutrition and occurs when there are no other sources of energy (Carbone et al., 2012). A study in rats found that 16-h fasting produced a 3-fold increase in serum creatine but did not measure muscle catabolism (Robertson et al., 2011). Ducks may have catabolized muscle tissue during feed restriction for energy, given the 8%–23% reduction in body mass.

Metabolites associated with microbiome

While gut microbiota was not measured, metabolite changes suggest the possibility that microbes were altered during feed restriction. Starvation causes alterations in the mucosal structure and transport function of the small intestine, leading to changes in fluid and ion secretion (Ferraris and Carey, 2000). For example, a study in chickens found that a 70% reduction in feed caused changes in the gut microbiota composition (Yan et al., 2021). Additionally, a study in Asian seabass (*Lates calcarifer*) found that the intestinal microbial community composition changes in response to starvation in less than 3 days (Xia et al., 2014). The microbiome can achieve its previous microbial diversity after periods of starvation (Trinh et al., 2023). Metabolites associated with a healthy gut (arabinitol) were found in higher concentrations during the baseline. Arabinitol is a sugar alcohol produced specifically by *Candida spp*., a yeast-like fungus that is a ubiquitous inhabitant in healthy bird crops and gastrointestinal tracts (Talazadeh et al., 2022). Arabinitol is not absorbed efficiently by the intestine and is converted to pentose sugars by bacteria such as *Lachnospiraceae* and *Ruminococcaceae* (Ramirez et al., 2020). Decreases in arabinitol may indicate a change in the microbiome during food restriction.

During feed restriction, trehalose and trimethylamine (TMA) were higher; this suggests bacterial utilization of alternative energy sources and possible alteration of the gut microbiota. Trehalose is a sugar synthesized by numerous bacteria species, insects and plants. It is produced by bacteria such as *Escherichia coli, Corynebacterium sp*. and *Propionibacterium freudenreichii* (Strom and Kaasen, 1993; Cardoso et al., 2007; Ruhal et al., 2013). Trehalose has been found to accumulate in bacterial cells in response to stress. Trehalose can be metabolized from choline in the colon through host-microbial interaction (Cardoso et al., 2007; Rath et al., 2017). Alternatively, increases in trehalose may be associated with increased invertebrate consumption as trehalose is the primary blood sugar in insects (Becker et al., 1996). Although we found no relationship between the degree of mass loss and trehalose concentration, the ducks may have opportunistically consumed insects. Trimethylamine (TMA) has been shown to be produced by the gut microbiota from dietary quaternary amines such as choline and carnitine (Rath et al., 2017). Synthesis of TMA can be influenced by differences in gut microbiota (Delgado et al., 2006) and has been associated with severe cardiovascular disease in humans (Rath et al., 2017). It is possible that increases in trehalose and TMA may be associated with alterations in the gut microbiota in response to feed restriction in this study.

Conclusion

This study demonstrated that mallards utilize endogenous reserves in times of limited caloric intake. We suggest that similar metabolism shifts would occur during migration and egg incubation in free-ranging mallards when energy use exceeds energy acquisition. As the ducks in this study were fed a commercial diet, extrapolating these results to wild mallards must be interpreted cautiously. Thus, more research is needed to investigate the response of the fecal metabolome to feed restriction in different life history stages of the annual cycle.

Data availability statement

The data presented in this study are openly available at Murray, Breanne (2022), "Utilizing NMR fecal metabolomics as a novel technique for detecting the physiological effects of food shortages, chronic stressors, in waterfowl", Mendeley Data, V1, doi: 10.17632/yphnb98s2m.1.

Ethics statement

The animal study was approved by we followed the guidelines of the Canadian Council on Animal Care as defined by the Guide to the Care and Use of Experimental Animals. This project was approved by the University of Saskatchewan Animal Research Ethics Board—protocol no. 20030021. The study was conducted in accordance with the local legislation and institutional requirements.

Author contributions

Conceptualization, Methodology, Formal Analysis, Investigation, Resources, Data Curation, Writing by BM and KM. All authors contributed to the article and approved the submitted version.

Funding

The Western College of Veterinary Medicine Wildlife Health Research Fund, University of Saskatchewan, Devolved Scholarship, University of Saskatchewan, Fish and Wildlife Development Fund, Government of Saskatchewan, and Environment and Climate Change Canada Grants and Contribution.

Acknowledgments

We would like to thank Max Machin-Brua with sample collection and Dr. Robert Brua, Dr. Jianfeng Zhu, and the Saskatchewan Structural Sciences Center for their assistance with data collection and analysis.

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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frontiers | Frontiers in Physiology

TYPE Opinion
PUBLISHED 21 February 2024
DOI 10.3389/fphys.2024.1358524

OPEN ACCESS

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RECEIVED 19 December 2023
ACCEPTED 05 February 2024
PUBLISHED 21 February 2024

CITATION
Kuzmina IV, Tolpygo SM, Kotov AV, Shoibonov BB, Zamolodchikova TS and Ovchinnikova NV (2024), Intestinal digestion in poultry compared to other animal species with a diverse diet.
Front. Physiol. 15:1358524.
doi: 10.3389/fphys.2024.1358524

Intestinal digestion in poultry compared to other animal species with a diverse diet

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KEYWORDS

poultry, intestine, digestive enzyme activity, mammals, trypsin

Introduction

The intestines of farm animals and poultry play an important role not only in the assimilation of nutrients from feed but also in maintaining the immune defense of the organism (Grozina, 2014; Fisinin et al., 2016). The barrier function of intestinal villi (microvillus cylindrical epithelium) cannot completely protect the organism from the introduction of pathogenic bacteria and viruses without populating with a variety of beneficial microflora. It is proved that the intestinal microbiota of birds performs numerous functions to maintain homeostasis and resistance. It is known that it takes part in the normal functioning of cardiovascular, endocrine, hematopoietic, nervous, and other systems (Kochish et al., 2020). At the same time, in order to obtain the healthiest birds possible, it is necessary to know their genetic potential and improve nutrition based on physiological features. It is proved that the growth and development of poultry is determined by the formation of the digestive system. At the same time, for the last few decades (30–40 years), serious shifts in the world indicators of productivity of farm animals have been noted (Vertiprakhov et al., 2016a). In order to move forward, it is necessary to clearly represent the physiological digestive processes occurring in the body of any animal, including birds. In this regard, a clear understanding of the digestive processes in the intestine of different animal species, their development in the process of evolution, and the peculiarities of their differences is required (Batoev, 2001). Therefore, it can be considered that the enzymatic activity in the intestine and blood of birds and mammalian animals can be used to "predict" the further growth and development of the organism as a whole. For a deep understanding of the fundamental regularities of food assimilation, it is necessary to know the peculiarities of functioning of the digestive system in animals with different specialization in nutrition.

Opinion

Activity of digestive enzymes in the intestine and blood in poultry

The results of studies on fistulated poultry showed that the chyme of the duodenum of meat chickens is characterized by high activity of digestive enzymes, which is consistent with the data from Fisinin et al. (2019). The results obtained on the activity of digestive enzymes in parental lines of broilers (Fisinin et al., 2017a) indicate that hybrids (chickens)

have a higher amylase activity in duodenal chyme than hens of maternal and paternal lines. This is most likely due to the diet of poultry (Fisinin et al., 2017b) since the diet of broilers is lower in fiber content and higher in digestible carbohydrates compared to that of the original lines. Since the diet of broiler chickens has a higher crude fat content compared to parental individuals, the lipase activity in the duodenal chyme of hybrids is correspondingly higher as well. A different pattern was observed for the activity of proteolytic enzymes in the intestine. At high crude protein content in broiler diets, protease activity is lower than that in hens of the original maternal line and higher than that in chickens of the paternal line. This may be due to the uneven hydrolysis of different feed components along the digestive tract, where fats are primarily broken down (mainly in the 12-intestine), while amylase and proteases are predominant in the jejunum (Wiseman, 2006). Consequently, the digestive system in meat chickens develops adequately to the nutritional content of incoming feed depending on genetic features that determine the intensity of metabolic processes. The results of studies of amylolytic enzyme activity in the blood plasma in meat chickens have an opposite tendency compared to that in the intestine. In the blood plasma of broiler chickens, the activity of amylase is reduced relative to the indicators in young chickens of maternal and paternal lines. Lipase and protease activities in the plasma had non-significant differences. These results are consistent with the hypothesis of the enteropancreatic circulation of digestive enzymes. The observation of the presence of pancreatic enzymes in the blood confirms the finding that they can be absorbed into the bloodstream, enter the pancreas through the blood, and be secreted into the intestine repeatedly without being broken down to amino acids in the intestine (Esmaeilipour et al., 2012; Vertiprakhov et al., 2016b).

Activity of digestive enzymes in the intestine and blood in animals with different types of nutrition

In experiments on rabbits, noticeable changes in the activity of blood enzymes at the level of the gastrointestinal tract were revealed. It was determined that blood passing through the capillary network of the digestive tract in rabbits is additionally saturated with protein, but the activity of amylase decreases. The biochemical analysis of the blood of minipigs showed that when blood passes through the gastrointestinal tract, the activity of blood enzymes changes, but the pattern is somewhat different, where the activity of amylase in the proximal small intestine increases. Analyzing these results, we observe that these changes in the activity of enzymes occur not only due to the absorption of nutrients but also as a result of plasma protein biosynthesis, activation, or deactivation of enzymes. The distinctive features of the dynamics of enzyme activity in the outflow blood in rabbits and minipigs are probably related to species-specific morphophysiological features of the digestive tract and differences in animal diets (Ksenofontov and Ksenofontova, 2022). The complex mechanisms of digestion in cattle can be well demonstrated by the dynamics of the activity of digestive enzymes in chyme. Experimental data indicate that the highest activity of protease and lipase is established in the first hours of intestinal chyme sampling, followed by its decrease after 3 h. Amylase activity, on the contrary, decreased in the first 3 h of sampling and increased further 24 h after chyme sampling in the experimental animal (Lebedev et al., 2018).

In numerous experiments on mono- and polygastric animals with duodenal anastomoses implanted behind the ducts of bile and pancreatic juice, it was shown that the size of fluctuations in the content of substances in duodenal chyme is limited. Thus, for 1 kg of dry matter of the consumed feed by cattle, sheep, pigs, and horses, 14 + 0.5 kg of digestive juices is secreted, the amount of which directly depends on the body weight of both mono- and polygastric animals, which leads to the formation of chyme in the duodenum in the amount directly proportional to the body weight of the animal. At the same time, the withdrawal of duodenal chyme leads to digestive disorders; changes in chyme evacuation; disturbance in the cardiovascular system, nervous system, and other pathological phenomena; and later even to lethal outcomes. If the intestine of such animals is being injected with their chyme or chyme taken from another animal, the physiological state normalizes. In addition, it was shown that the nervous system plays a huge role in the regulation of digestive and metabolic functions of the gastrointestinal canal and also revealed the ability of digestive glands to adapt to the food substrate, subtly responding to changes in the chemistry of the diet (Aliev, 2007).

For comparison, an experiment was conducted to keep rats for 10 days on a meat and fish paste diet with 20% higher protein levels used in growing animals. The rats showed a significant increase in pepsin activity in the gastric mucosa and pancreatic homogenate and a decrease in the amylase activity in the small intestinal mucosa. At the same time, studies on mink, when feeding predators a diet with different levels of protein, fat, and carbohydrates, showed that the animals do not show a definite regularity in the changes in the activity of digestive enzymes depending on the ratio of the above components of the diet (Oleinik, 1997). It can be concluded that adaptation of the digestive enzyme spectrum during diet change in mammals requires a long time compared to that in birds. In farm animals (cows, horses, pigs, etc.), adaptation of digestive enzymes occurs much faster, almost as in hens or broilers, with one difference in the level of enzyme activity.

Trypsin activity in the blood in different types of animals by the type of nutrition

Trypsin is a PAR-activating protease that plays the role of a major digestive enzyme in the duodenum. The results of studies have shown that serum trypsin activity varies between animals. Porcine trypsin contains 4 histidine residues; bovine, sheep, human, and turkey trypsin contain 3 residues; human and turkey trypsin contain 8 polycystin residues; and bovine, pig, and sheep each contain 12 residues (Sukhanova et al., 2018). The results showed that in terms of trypsin activity, the maximum activity in the serum was observed in broiler chickens, which exceeds the level of laying hens by 385.4% in cows, 89.4% in goats, 22.6 in laying hens, and 70% in rats. Biochemical studies of the rat blood serum revealed multidirectional changes in trypsin activity under conditions of thirst and starvation. Thus, the highest activity of this enzyme was observed in animals under water deprivation; on account of food deprivation, on the contrary, a significant decrease in the activity of this enzyme was found in rats. One hour after providing deprived animals with water and feed, a

unidirectional significant decrease in enzyme activity was detected in both experimental groups (Kuzmina and Ovchinnikova, 2023). The dominant position of birds in the activity of this enzyme indicates a higher level of metabolism in relation to mammalian animals. It has been established that the indicator of trypsin activity in the blood can be used to judge the state of intestinal health in birds and the processes of digestive adaptation to the composition of the diet (Trukhachev et al., 1999; Vertiprakhov et al., 2023).

Conclusion

The biological law that determines the development of the organism depending on the genotype and environmental factors provides birds with more intensive digestive processes in the metabolic system compared to other animal species. Since digestion of feed nutrients depends on the intensity of hydrolysis in the intestine, digestive processes in birds are 10 times faster compared to horses and ruminants and 2–3 times more active than digestion in pigs and rats.

Author contributions

IK: writing–original draft and writing–review and editing. ST: data curation and writing–review and editing. AK: writing–original draft and writing–review and editing. BS: writing–original draft. TZ: writing–original draft. NO: data curation and writing–original draft.

Funding

The author(s) declare that no financial support was received for the research, authorship, and/or publication of this article.

Conflict of interest

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frontiers | Frontiers in Physiology

TYPE Original Research
PUBLISHED 15 May 2024
DOI 10.3389/fphys.2024.1381548

OPEN ACCESS

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RECEIVED 04 February 2024
ACCEPTED 24 April 2024
PUBLISHED 15 May 2024

CITATION
Sharma MK, Liu G, Choppa VSR, Rafieian-Naeini HR, Mahdavi FS, Marshall B, Gogal RM Jr and Kim WK (2024), Effects of *Artemisia annua* supplementation on the performance and gut health of laying hens challenged with mixed *Eimeria* species. *Front. Physiol.* 15:1381548. doi: 10.3389/fphys.2024.1381548

Effects of *Artemisia annua* supplementation on the performance and gut health of laying hens challenged with mixed *Eimeria* species

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Background: Coccidiosis outbreaks in susceptible laying hens can significantly decrease egg production and cause substantial economic loss to the egg industry. The supplementation of poultry diets with chemotherapeutic agents is limited due to antimicrobial resistance and residue in poultry meat or processed products. Therefore, alternative strategies to control coccidiosis are needed, and *Artemisia annua* (**AA**) might have the potential to be a phytogenic feed additive, an alternative to anticoccidial agents. This study aimed to investigate the effect of the dietary supplementation of powdered AA on the performance and gut health of laying hens infected with coccidiosis by *Eimeria* spp.

Methods: A total of 225 Hy-Line W-36 laying hens at 23 weeks of age were allocated into 5 treatment groups: 1) control (**NC**), 2) pair-fed (**PF**) control, 3) challenged control (**CC**), 4) CC with dietary inclusion of 0.5% AA (**0.5AA**), and v) CC with dietary inclusion of 1% AA (**1AA**). The hens in the CC, 0.5AA, and 1AA groups were orally inoculated with sporulated oocysts of *Eimeria maxima* (12,500), *Eimeria tenella* (12,500), and *Eimeria acervulina* (62,500) at week 25. The PF hens received the same amount of feed consumed by the CC hens from 0–14 days post-inoculation (**dpi**) of *Eimeria* spp. The performance of the laying hens, including body weight (**BW**), hen–day egg production (**HDEP**), feed intake (**FI**), and feed conversion ratio (**FCR**), was measured weekly. Additionally, markers of intestinal health, including gut permeability, lesion score, intestinal morphometry, and immune responses, were evaluated at 6, 14, and 21 dpi.

Results: At 6 and 14 dpi, laying hens challenged with *Eimeria* spp. had a lower BW than PF and NC hens ($p < 0.0001$). Supplementation of 1% AA improved the HDEP by 8.1% compared to CC hens; however, it was still 15.4% lower than that of PF hens ($p < 0.0001$). The inclusion of 1% AA did not have any beneficial effect on FI; however, the FCR was improved by 0.61 (2.46) than that of CC hens (3.07; $p < 0.0001$). The inclusion of 1% AA reduced the severity of the intestinal lesions and increased the recovery of intestinal villi ($p < 0.05$). Additionally, gut permeability was significantly different between the challenged and non-challenged hens;

however, among the challenged hens, the inclusion of AA reduced the gut permeability by 29% compared to CC hens ($p < 0.0001$). Furthermore, the inclusion of 0.5% AA reduced the inflammatory responses in the infected hens.

Conclusion: Dietary inclusion of AA partially restored the performance and gut health of the laying hens and modulated their inflammatory immune response following *Eimeria* infection; however, further studies are needed to better understand the mode of action and effective dosages to improve the gut health without negative impacts on the performance.

KEYWORDS

***Artemisia annua*, phytogenic feed additive, laying hens, coccidiosis, gut health, egg production**

Introduction

The poultry industry worldwide is under tremendous pressure to control and minimize the risk associated with avian coccidiosis (Abbas et al., 2011). Despite several strategies (biosecurity, vaccination, or the use of coccidiostat or coccidiocidal drugs) being employed to control coccidiosis in commercial poultry production, coccidiosis is still the leading cause of economic loss in the poultry industry (Peek and Landman, 2011; Liu et al., 2023). Unfortunately, the development of resistance against currently available coccidiostat or coccidiocidal drugs, potential drug residues in the final products, and consumer interests have challenged the industry for alternatives to control coccidiosis. Recently, the use of phytogenic feed additives has been increasing as it has been shown to improve the performance, immune response, and intestinal barrier functions against coccidiosis (Choi and Kim, 2020; Yadav et al., 2020; de Andrade et al., 2022; El-Shall et al., 2022; Paneru et al., 2022; 2023).

Artemisia annua (AA), also known as sweet wormwood, is an herb used in traditional Chinese medicine to treat malaria caused by multidrug-resistant *Plasmodium* spp (Klayman, 1985). Artemisinin, a bioactive flavonoid found in AA leaves, inhibits the growth of several stages of *Plasmodium* spp. (Meshnick, 2002; Golenser et al., 2006). Like *Plasmodium* spp., *Eimeria* spp. is an intracellular parasite belonging to the same family, Apicomplexa. The use of AA and its extract, artemisinin, exhibit anticoccidial effects against *Eimeria tenella* (Allen et al., 1997; Del Cacho et al., 2010; Hady and Zaki, 2012; Drăgan et al., 2014; Jiao et al., 2018) when infected by it alone. However, in the field, infection from only one species is rare. The use of either dried AA leaves or its extract, artemisinin, has shown potential in improving lesion scores, reducing oocyst shedding and sporulation, and modulating the humoral and immune response in chickens (Allen et al., 1997; Arab et al., 2012; Gholamrezaie et al., 2013; Kaboutari et al., 2014; Wan et al., 2016; Song et al., 2017; Yang et al., 2021). It has been observed that apicomplexan parasites have developed an anti-apoptotic mechanism to survive intracellularly in the host (Jiao et al., 2018). During coccidia infection, *Eimeria* alters the host cellular pathways, inhibiting the apoptosis of infected cells by upregulating the transcriptional factor (**NF-κB**) and the anti-apoptotic factor in chickens as a defense mechanism. However, artemisinin suppresses the expression of NF-κB and promotes the apoptotic pathways, limiting the *Eimeria* infection and inflammation (Jiao et al., 2018). Furthermore, artemisinin from AA alters the cell wall formation of the oocysts by disrupting the Ca^{2+} homeostasis of the oocysts, leading to the death of the developing oocysts and a reduction in the sporulation rate (Del Cacho et al., 2010). Artemisinin disrupts Ca^{2+} homeostasis in developing oocysts, affecting Ca-dependent pathways involved in protein secretion and differentiation. This disruption inhibits cell wall formation and leads to the degeneration of micro/macrogametes, ultimately reducing sporulation rates and oocyst shedding (Del Cacho et al., 2010). Therefore, this study aimed to evaluate the effect of dried AA leaves on the performance, gut health, and immune response of laying hens infected with mixed *Eimeria* species.

Materials and methods

Laying hen husbandry and experimental design

The Institutional Animal Care and Use Committee of the University of Georgia approved the animal study protocol (A2021 12–012). A total of 225 23-week-old Hy-Line W-36 laying hens were randomly divided into 5 treatment groups (n = 45), each comprising 5 replicates. The laying hens were adapted to the diets for 2 weeks. At 25 weeks of age, the hens were challenged with mixed *Eimeria* species (12,500 *Eimeria maxima*, 12,500 *E. tenella*, and 62,500 *Eimeria acervulina* oocysts) according to the treatment groups. The five different treatments were 1) non-challenged and fed a basal diet (**NC**), 2) non-challenged and pair-fed (**PF**), 3) basal diet with mixed *Eimeria* spp. challenge (**CC**), 4) dietary inclusion of 0.5% AA leaf powder and challenged with mixed *Eimeria* spp. (**0.5AA**), and 5) dietary inclusion of 1% AA leaf powder and challenged with mixed *Eimeria* spp. (**1AA**). The powdered AA leaves were purchased from Artennua® (Barcelona, Spain). The experimental mash diets were isocaloric and isonitrogenous and formulated to meet the nutrient requirements of laying hens (Hy-Line International, Mansfield, GA). The nutritional composition of AA powder and diets are shown in Tables 1, 2, respectively. Feed and water were provided a*d libitum* throughout the experimental period except for the PF group. The PF group was fed the exact amount of the basal diet consumed by the CC group from 0–14 days post-inoculation (dpi) of *Eimeria* spp. (**dpi**). The feed consumption of the CC group was measured daily at the same time following the *Eimeria*

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TABLE 1 Nutritional composition and artemisinin content of dried *Artemisia annua* leaves.

Item[a]	Value	Unit
Gross energy	3,080	kcal/kg
Crude protein	9.80	%
Crude fiber	7.30	%
Ether extract	4.30	%
Total lysine	0.47	%
Total methionine	0.13	%
Total arginine	0.37	%
Total leucine	0.62	%
Total isoleucine	0.39	%
Total valine	0.46	%
Total threonine	0.34	%
Total cysteine	0.11	%
Total phenylalanine	0.41	%
Total tryptophan	<0.02	%
Total artemisinin	0.93	%

[a]The samples were sent to the University of Missouri agricultural experiment station chemical laboratories for the above-mentioned analysis.

challenge, and the average daily feed consumption was calculated. The exact amount of feed was then provided to the PF group on the next day. To ensure that the PF group had the same amount of feed as the CC group, feed addition and consumption were carefully monitored to prevent any wastage. The laying hens were housed in environmentally controlled battery cages (475 cm^2/hen) equipped with trough feeders and nipple drinkers. The lighting regime was adjusted based on the breeder management guidelines (Hy-Line International, Mansfield, GA).

Laying hen performance

The laying hens were weighed at 23 and 25 weeks of age and 6, 14, and 21 dpi for growth performance. The number of eggs laid per replicate was recorded daily, and hen–day egg production (**HDEP**) was calculated. The daily feed consumption (**ADFI**) and feed conversion ratio (**FCR**) were measured. The FCR was expressed as the kg of feed consumed per dozen eggs produced (Sharma et al., 2022b).

Gut permeability, lesion score, and oocyst shedding

At 5 dpi, 2.2 mg/mL of FITC-d (MW 4000; Sigma-Aldrich, Canada) was orally inoculated to one hen per replicate, and blood was collected 2 hours post-inoculation from the wing vein. The concentration of FITC-d that leaked into the bloodstream was measured using a plate reader at an excitation/emission wavelength of 485/525 nm (SpectraMax ABS Plus, Molecular Devices, San Jose, CA). At 6 dpi, 2 hens per replicate were euthanized, and intestinal lesion scoring was carried out for *E. acervulina* (in the duodenum), *E. maxima* (in the jejunum and ileum), and *E. tenella* (in the ceca) on a scale of 0–4 following the methods described by Johnson and Reid, (1970). In brief, the lesion scores were ranked based on the severity of lesions from 0 to 4, with 0 indicating no lesions and 4 indicating extremely severe lesions. The severity of intestinal lesions was determined based on the density of the characteristic lesion of each *Eimeria* species, inflammation and thickening of the intestinal wall, and intestinal content at their predilection sites (Johnson and Reid, 1970). On 5, 6, 7, and 8 dpi, feces were allowed to accumulate on a manure belt. On day 8, the feces on the belt were thoroughly mixed within each replicate, and feces samples were collected for oocyst shedding. The oocyst counts for *E. acervulina*, *E. maxima*, and *E. tenella* were counted following the procedure proposed by Goo et al. (2023). In brief, 5 g of the feces was mixed in 35 mL of tap water and left to soften for 48 h at room temperature. Afterward, the sample was vortexed and diluted in a saturated saltwater solution (1:11 ratio). Then, 650 µL of the diluted solution was added to a McMaster counting chamber (Vetlab Supply, Palmetto Bay, FL) for oocyst enumeration. The different species were identified under a microscope based on the size and morphology of the oocysts.

Sample collection and analysis

At 6, 14, and 21 dpi, 1 hen per replicate was randomly selected for sampling and was euthanized by cervical dislocation. For intestinal morphometric analysis, 2-cm-long sections of the duodenum, jejunum, and ileum were collected and fixed in 10% neutral-buffered formalin. The villus height (**VH**), crypt depth (**CD**), and VH-to-CD (**VHCD**) ratio were measured following

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TABLE 2 Diet formulation and nutritional composition of experimental diets.

Ingredients (%)	Control	0.5AA	1AA
Corn	58.55	58.03	57.55
Soybean meal	22.00	22.00	22.00
Limestone[a]	9.34	9.34	9.34
Soybean oil	3.00	3.00	3.00
Distiller's dried grains with soluble	2.50	2.50	2.50
Artemisia Annua	0.00	0.50	1.00
Glycine	1.74	1.68	1.68
Dicalcium phosphate	1.70	1.70	1.71
Salt	0.39	0.39	0.39
DL-methionine	0.33	0.40	0.39
Vitamin premix[b]	0.10	0.10	0.10
L-lysine	0.10	0.10	0.11
L-valine	0.09	0.09	0.10
L-threonine	0.08	0.09	0.10
Mineral mix[c]	0.075	0.075	0.075
Calculated nutritional composition			
ME (kcal/kg)	2910	2910	2910
Crude protein (%)	16.70	16.69	16.69
Total calcium (%)	4.00	4.00	4.00
Available P (%)	0.45	0.45	0.45
dLys (%)	0.80	0.80	0.80
dMet (%)	0.54	0.54	0.57
dTSAA (%)	0.73	0.73	0.76
dThr (%)	0.56	0.56	0.56
dTrp	0.16	0.16	0.16
dArg	0.89	0.89	0.89
dVal	0.71	0.70	0.70
dILeu	0.59	0.59	0.59

[a]The level of the fine and coarse limestone ratio was maintained at 50:50.

[b]Vitamin mix provided the following in mg/100 g diet: folic acid, 7.5; riboflavin, 1.5; nicotinic acid amide, 15; vitamin B-12 (source concentration, 0.1%) pyridoxine-HCl, 1.2; d-biotin, 3; thiamine-HCl, 1.5; 2; d-calcium pantothenate 4; menadione sodium bisulfite, 1.98; cholecalciferol (5,000,000 IU/g), 0.09; α-tocopherol acetate (500,000 IU/g), 22.8; retinyl palmitate (500,000 IU/g), 2.8; ethoxyquin, 13.34; dextrose, 762.2; and I-inositol, 2.5.

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

the procedure outlined by Sharma et al. (2024b). Furthermore, samples of the jejunum and cecal tonsils were collected and snap-frozen in liquid nitrogen at the abovementioned time points for gene expression. The RNA extraction from the above samples was carried out using the TRIzol reagent (QIAGEN; Valencia, CA) following the procedures proposed by Sharma et al. (2022a). In brief, the total RNA concentration was determined using the NanoDrop-8 spectrophotometer (Thermo Fisher Scientific, Waltham, MA). It was later reverse-transcribed to cDNA after normalizing it to 2 µg/µL using a commercial kit (Applied Biosystems, Foster City, CA), and qRT-PCR was performed in duplicate. The ΔCt values of each marker gene from the NC group were used to calculate the ΔΔCt value. The expression levels of the NC group were then normalized to 1 using the $2^{-\Delta\Delta Ct}$ method, and the relative fold change of the mRNA expression levels was calculated using the $2^{-\Delta\Delta CT}$ relative quantification methods compared to the NC group. The list of primers for the target and housekeeping genes is shown in Table 3.

The oxidative status of the laying hens following the *Eimeria* challenge was measured from 1 hen per replicate at 6, 14, and 21 dpi. The concentration of the total antioxidant capacity (**TAC**) in the

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TABLE 3 List of the nucleotide sequences of the housekeeping and target genes used in this study.

Gene[a]	Accession number	Nucleotide sequence
Housekeeping gene		
ACTB	NM_205518.2	F: CAACACAGTGCTGTCTGGTGGTA R: ATCGTACTCCTGCTTGCTGATCC
Intestinal barrier functions		
JAM-2	XM_025149444.1	F: AGCCTCAAATGGGATTGGATT R: CATCAACTTGCATTCGCTTCA
ZO-2	XM_025144669.1	F: GGCAAATCATTGAGCAGGA R: ATTGATGGTGGCTGTAAAGAG
CLDN-1	NM_001013611.2	F: TGGAGGATGACCAGGTGAAGA R: CGAGCCACTCTGTTGCCATA
OCLN	NM_205128.1	F: ACGGCAGCACCTACCTCAA R: GGCGAAGAAGCAGATGAG
MUC-2	NM_001318434.1	F: ATGCGATGTTAACACAGGACTC R: GTGGAGCACAGCAGACTTTG
iNOS	NM_204961.2	F: GAGCACTCATGACCCCAAAG R: GGGCCAGGTGCTCTTCTATT
Nutrient transporter genes		
$b^{0,+}$ AT	NM_001199133.2	F: TTATCACCGCACCTGAAC R: AGCATCTGAAGGTGCATAG
b^0 AT	XM_040663289.2	F: GGGTTTTGTGTTGGCTTAGGAA R: TCCATGGCTCTGGCAGAGATTT
EAAT-3	XM_046936555.1	F: TGCTGCTTTGGATTCCAGTGT R: AGCAATGACTGTAGTGCA GAAGTAATATATG
pepT-1	NM_204365.2	F: CCCCTGAGGAGGATCACTGTT R: CAAAAGAGCAGCAGCAACGA
y^+ LAT-1	XM_046911929.1	F: AATGTGAAGTGGGGAACTCG R: CACCCTGCGTAGGAGAAGAG
SGLT-1	XM_046928028.1	F: GCCATGGCCAGGGCTTA R: CAATAACCTGATCTGTGCACCAGTA
GLUT-1	NM_205209.2	F: TCCTCCTGATCAACCGCAAT R: TGTGCCCCGGAGCTTCT
Inflammatory cytokines		
IL-1β	NM_204524.2	F: TGCCTGCAGAAGAAGCCTCG R: GACGGGCTCAAAAACCTCCT
IL-4	NM_001398460.1	F: CTTATGCAAAGCCTCCACAA R: TGGTGGAAGAAGGTACGTAGG
IL-10	NM_001004414.4	F: AGCAGATCAAGGAGACGTTC R: ATCAGCAGGTACTCCTCGAT
IL-17	NM_204460.2	F: TATCAGCAAACGCTCACTGG R: AGTTCACGCACCTGGAATG
TNF-α	MF000729.1	F: CGTGGTTCGAGTCGCTGTAT R: CCGTGCAGGTCGAGGTACT
IFN-γ	NM_205149.2	F: CACATATCTGAGGAGCTCTATAC F: GTTCATTCGCGGCTTTG

[a]ACTB, beta-actin; JAM-2, junctional adhesion molecule-2; ZO-2, zonula occludin-2; CLDN-1, claudin-1; OCLN, occludin; MUC-2, mucin-2; iNOS, inducible nitric oxide synthase; b0,+ AT (SLCA9), solute carrier family 7, member 9; b0 AT (SC6A19), solute carrier family 6, member 19; EAAT-3 (SLC1A1), excitatory amino acid transporter 3; pepT-1(SLC15A1); y^+ LAT-1 (SLC7A7), y^+ L amino acid transporter-1; SGLT-1 (SLC5A1), sodium-glucose transporter-1; GLUT-1 (SLC2A1), glucose transporter-1; VDR, vitamin D receptor; IL-1β, interleukin-1 β; IL-4, interleukin-4; IL-10, interleukin-10; IL-17, interleukin-17 A; TNF-α, tumor necrosis factor-α; IFN-γ, interferon-γ.

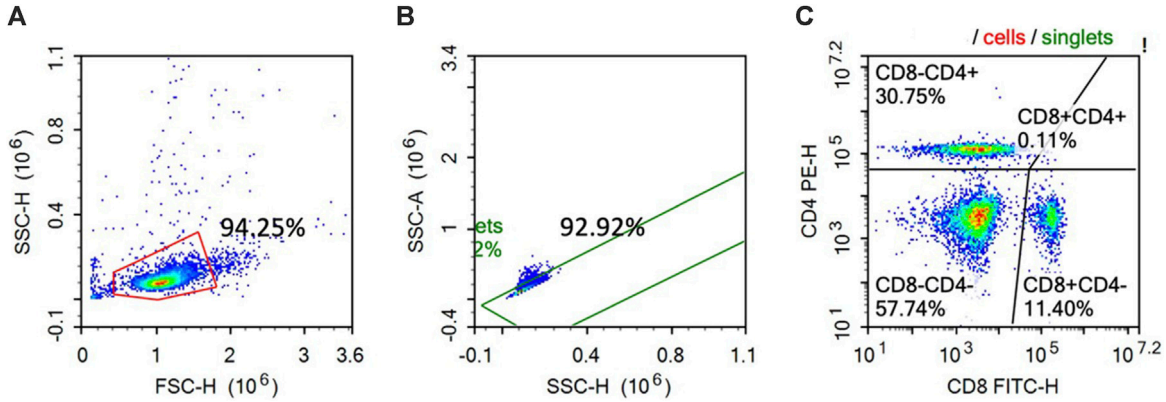

FIGURE 1
Representative histogram of the gating protocol used on the enriched chicken peripheral blood lymphocytes stained with PE-CD4 and FITC CD-8. The cells were first gated based on the size (FSC-H) and granularity (SSC-H) **(A)**. The single cell population was then gated from the histogram **(A)** based on the cell area (SSC-A) and the cell height (SSC-H) (Fig. 1B). Singlet cells identified in **(B)** were then analyzed for PE-CD4 and FITC-CD8 staining **(C)**.

serum was measured following the manufacturer's instructions (BioAssay Systems, Hayward, CA). In brief, blood was collected from the wing vein, serum was separated by centrifugation at 1,000 × *g* for 15 min, and the TAC concentration was measured using a plate reader (λ = 485 nm; SpectraMax ABS Plus, SoftMax Pro 7 software, Molecular Devices, San Jose, CA). The superoxide dismutase (**SOD**) and malondialdehyde (**MDA**) concentrations in the liver were analyzed as a marker of oxidative status using commercial kits (MDA: BioAssay Systems, Hayward, CA; SOD: Cayman Chemical assay kits, Ann Arbor, MI). Approximately 50–70 mg of the liver sample was homogenized in the respective solutions following the manufacturer's instructions, and the concentrations of MDA (λex/em = 530/550 nm) and SOD (λ = 450 nm) were measured using a plate reader (SpectraMax ABS Plus, SoftMax Pro 7 software, Molecular Devices, San Jose, CA). The concentrations of SOD and MDA were standardized for the protein concentration in the sample.

Flow cytometry

At 14 dpi, 10 mL of blood was withdrawn from 1 hen per replicate through the wing vein into the Na-heparin glass tubes. The peripheral mononuclear blood cells were isolated following the procedures proposed by Choi et al. (2022) to a final concentration of 4.0 × 10^6/mL. The $CD4^+$ and $CD8^+$ cell percentages were analyzed using flow cytometry following the procedures proposed by Krunkosky et al. (2020). In brief, 100 µL of the isolated cells were incubated with CD4 (mouse anti-chicken CD4-PE; SouthernBiotech, Birmingham, AL) and CD8 (mouse anti-chicken CD8a-FITC; SouthernBiotech, Birmingham, AL) antibodies. After incubation, the cells were washed with FACS-PBS and centrifuged at 250 *g* for 10 min, and the supernatant was discarded and resuspended in 100 µL FACS-PBS and 100 µL flow fixing solution (Thermo Fisher Scientific). Flow cytometry was conducted on a BD Accuri C6 Flow Cytometer (San Jose, CA). A total of 10,000 events were conducted per sample within the lymphocyte gate, and the expression percentages for each of the utilized markers were analyzed, as shown in Figure 1.

Statistics

The data related to laying hen performance, gene expression, oxidative status, and intestinal morphology were analyzed using one-way analysis of variance in SAS (SAS Institute, Cary, NC). The lesion score data were analyzed as nonparametric data using the Kruskal–Wallis test. In contrast, the feed intake and egg production data were analyzed as split plots over time using the PROC GLM procedure of SAS (SAS Institute, Cary, NC). Statistical significance was set at $p < 0.05$, and the means were compared using Fisher's LSD.

Results

Laying hen performance

The effects of feeding AA leaves on the growth performance of the laying hens following the *Eimeria* challenge are shown in Table 4. During the adaptation period, the BW of the hens fed 0.5% AA was reduced compared to the other treatment groups ($p = 0.0642$). At 6 dpi, the BW of hens infected with *Eimeria* spp. was reduced by at least 7% compared to 3% in the PF hens ($p < 0.0001$). However, at 14 dpi, the BW loss increased up to 8% in PF hens and at least 9% in the infected hens ($p < 0.0001$). The lower BW observed in the 0.5AA group during the adaptation period was exacerbated by the *Eimeria* infection, with the lowest BW observed at 14 dpi ($p < 0.0001$). By 21 dpi, the BW of the PF hens and challenged hens had recovered to levels comparable to those of NC hens ($p = 0.1926$).

At 23 weeks of age, the HDEP of the laying hens fed AA was reduced, but it recovered back to the level of the NC group by 24 weeks of age ($p < 0.0001$; Figure 2). Following the *Eimeria* challenge at 25 weeks of age, the HDEP was significantly reduced in *Eimeria*-challenged hens compared to PF and NC hens. The daily egg production of laying hens started to decrease in *Eimeria*-challenged groups from 5 dpi, peaked at 9 dpi, and was not able to recover by 21 dpi, except for those hens fed 1% AA (Figure 3). The laying hens fed 0.5% and 1% AA increased their overall egg

TABLE 4 Effects of the dietary inclusion of phytogenic feed additive *Artemisia annua* in laying hen's diets on the growth performance of the laying hens pre- and post-*Eimeria* challenge.

Items[a]	BW (initial)	BW (0 dpi)	BW (6 dpi)	BWG (0–6 dpi)	BW (14 dpi)	BWG (0–14 dpi)	BW (21 dpi)	BWG (0–21 dpi)
NC	1,571.5	1,568.9^{a}	1544.7^{a}	−24.17^{a}	1,573.9^{a}	5.07^{a}	1,562.4	−6.48
PF	1,574.1	1,566.5^{a}	1495.1^{b}	−71.40^{b}	1,448.6^{b}	−117.93^{b}	1,572.6	6.14
CC	1,569.3	1,571.7^{a}	1,433.4^{c}	−138.34^{c}	1,422.7^{bc}	−149.01^{b}	1,566.4	−5.38
0.5AA	1,574.1	1,539.3^{b}	1,401.2^{d}	−138.05^{c}	1,379.3^{c}	−159.96^{b}	1,521.6	−17.71
1AA	1,571.6	1,552.0^{ab}	1,436.8^{c}	−115.13^{c}	1,408.7^{bc}	−143.26^{b}	1,524.5	−17.71
SEM	3.77	8.43	10.21	11.42	19.98	20.58	18.83	14.78
p-value	0.8776	0.0642	<0.0001	<0.0001	<0.0001	<0.0001	0.1926	0.8424

[a-c]values within columns not sharing the same superscripts are significantly different at $p < 0.05$.

[a]BW, average body weight (g); BWG, average body weight gain (g/hen). At 6, 14, and 21 dpi, the number of laying hens per replicate was as follows: n = 9, n = 6, and n = 5, respectively. NC, non-challenged control; PF, pair-fed control; CC: challenged control (12,500 *E. maxima*; 12,500 *E. tenella*; and 62,500 *E. acervulina* oocysts per mL); 0.5AA, challenged control with the inclusion of 0.5% *Artemisia annua* in the diet; 1AA, challenged control with the inclusion of 1% *A. annua* in the diet; dpi, days post-inoculation of *Eimeria* spp.

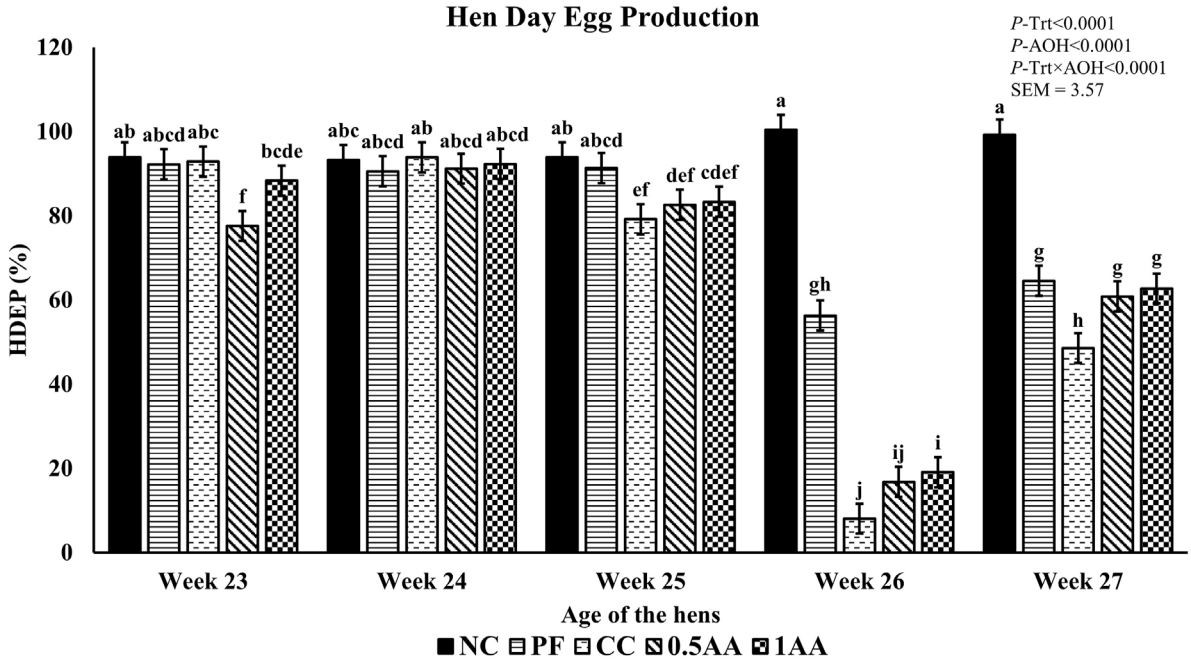

FIGURE 2
Effect of the inclusion of phytogenic feed additive *Artemisia annua* in the diets of laying hens on their weekly hen–day egg production from 23 weeks to 27 weeks of age. At 25 weeks of age, 12,500 *Eimeria maxima*, 12,500 *Eimeria tenella*, and 62,500 *Eimeria acervulina* oocysts were inoculated in laying hens grouped into challenged control, 0.5% *A. annua*, and 1% *A. annua*. NC: non-challenged control; PF: pair-fed control; CC: challenged control (12,500 *E. maxima*; 12,500 *E. tenella*; and 62,500 *E. acervulina* oocysts per mL); 0.5AA: challenged control with the inclusion of 0.5% *A. annua* in the diet; 1AA: challenged control with the inclusion of 1% *A. annua* in the diet. Before the *Eimeria* challenge at 25 weeks of age, treatments of NC, PF, and CC hens at 23 and 24 weeks of age were not different among themselves. Pair-feeding was carried out from 0–14 dpi after the *Eimeria* challenge. HDEP: hen–day egg production (%); Trt: treatment groups; AOH: age of the hens. Graphs not sharing the same superscripts (a–j) are significantly different at $p < 0.05$.

production (0–21 dpi) from 46.7% in CC hens to 52.9% and 54.8%, respectively. However, the HDEP was still lower than that of the PF (70.2%) and NC (98.0%) groups. The difference in the HDEP (0–21 dpi) between NC and PF hens was 27.8%, while it was observed to be 23.5% between PF and CC hens. The results for the ADFI following the *Eimeria* challenge at 25 weeks of age have a similar trend for the daily HDEP, as shown in Figure 4. An interaction was observed between different treatment groups and dpi for the ADFI ($p < 0.0001$). The ADFI started to decrease in challenged hens, beginning at 4 dpi, peaking at 7 dpi, and recovering to the same level as that of NC hens at 19 dpi. However, feeding AA did not have any beneficial effect on improving the ADFI. The FCR increased significantly in CC hens (7.93 kg feed/dozen of eggs), followed by 0.5AA (4.75 kg feed/dozen eggs) and 1AA (4.63 kg feed/

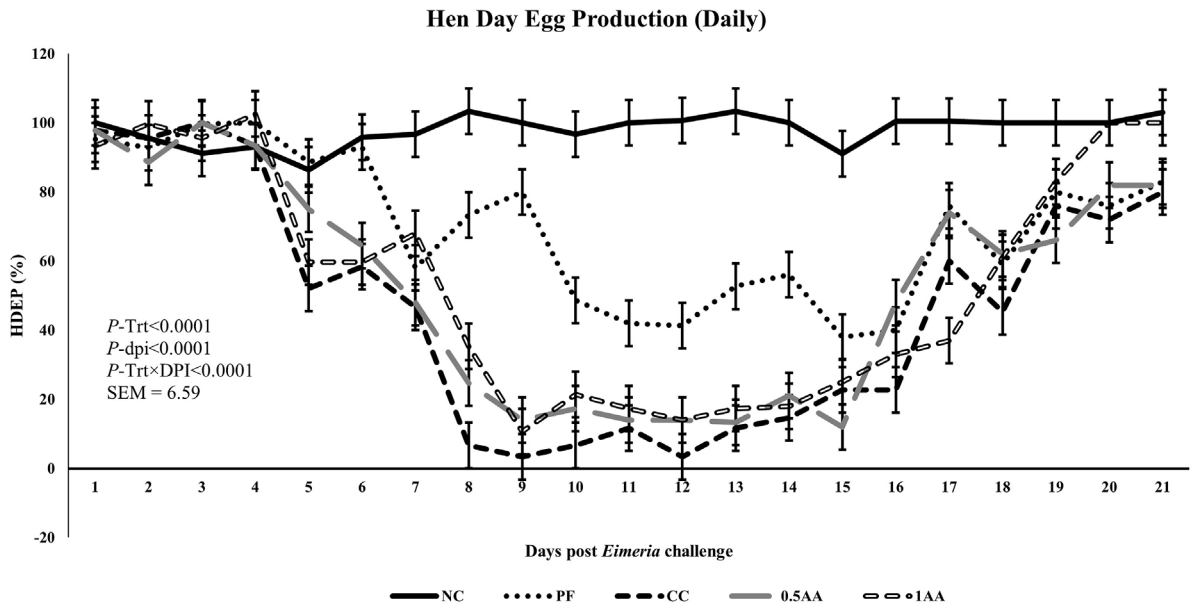

FIGURE 3
Effect of the inclusion of phytogenic feed additive *A. annua* in the diets of laying hens on their daily hen–day egg production from the day of a challenge until 21 days post-*Eimeria* challenge. At 25 weeks of age, 12,500 *E. maxima*, 12,500 *E. tenella*, and 62,500 *E. acervulina* oocysts were inoculated in laying hens grouped into challenged control, 0.5% *A. annua*, and 1% *A. annua*. NC: non-challenged control; PF: pair-fed control; CC: challenged control (12,500 *E. maxima*; 12,500 *E. tenella*; and 62,500 *E. acervulina* oocysts per mL); 0.5AA: challenged control with the inclusion of 0.5% *A. annua* in the diet; 1AA: challenged control with the inclusion of 1% *A. annua* in the diet. HDEP: hen–day egg production (%); Trt: treatment groups; dpi: days post-inoculation of *Eimeria* spp.

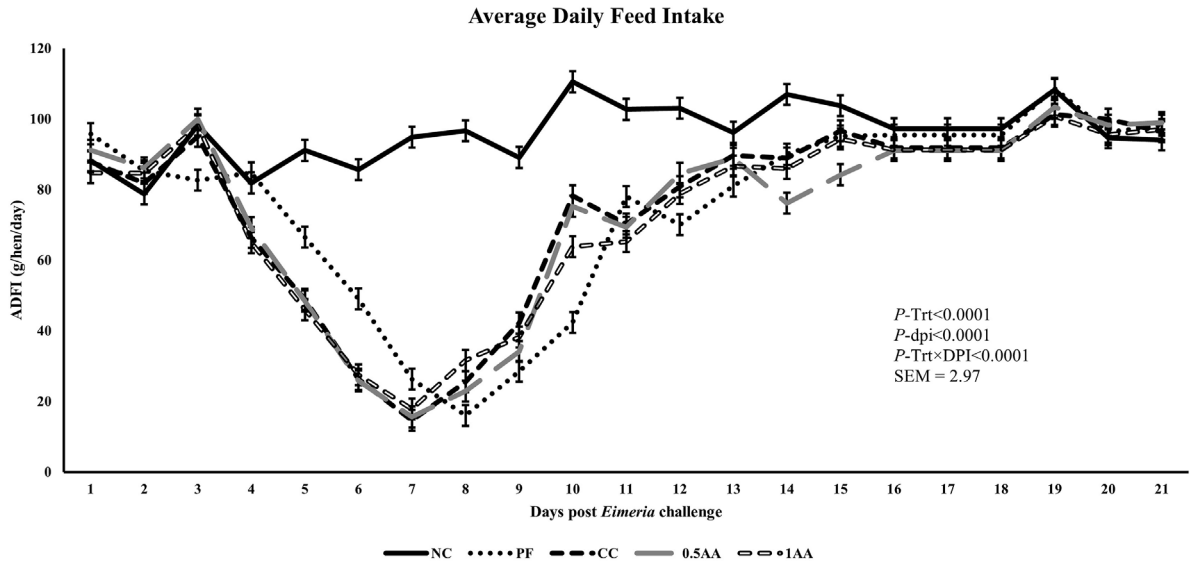

FIGURE 4
Effect of the inclusion of phytogenic feed additive *A. annua* in the diets of laying hens on their average daily feed intake from the day of a challenge until 21 days post-*Eimeria* challenge. At 25 weeks of age, 12,500 *E. maxima*, 12,500 *E. tenella*, and 62,500 *E. acervulina* oocysts were inoculated in laying hens grouped into challenged control, 0.5% *A. annua*, and 1% *A. annua*. NC: non-challenged control; PF: pair-fed control; CC: challenged control (12,500 *E. maxima*; 12,500 *E. tenella*; and 62,500 *E. acervulina* oocysts per mL); 0.5AA: challenged control with the inclusion of 0.5% *A. annua* in the diet; 1AA: challenged control with the inclusion of 1% *A. annua* in the diet. ADFI: average daily feed intake (g/hen/day); Trt: treatment groups; dpi: days post-inoculation of *Eimeria* spp.

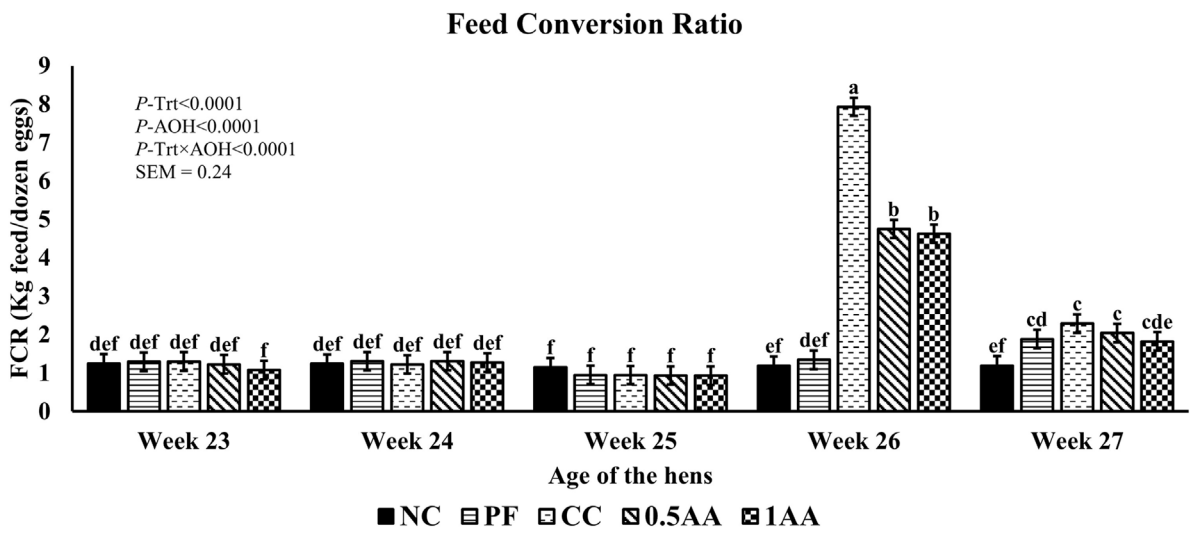

FIGURE 5
Effect of the inclusion of phytogenic feed additive *A. annua* in the diets of laying hens on their feed conversion ratio from 23 weeks to 27 weeks of age. At 25 weeks of age, 12,500 *E. maxima*, 12,500 *E. tenella*, and 62,500 *E. acervulina* oocysts were inoculated in laying hens grouped into challenged control, 0.5% *A. annua*, and 1% *A. annua*. NC: non-challenged control; PF: pair-fed control; CC: challenged control (12,500 *E. maxima*; 12,500 *E. tenella*; and 62,500 *E. acervulina* oocysts per mL); 0.5AA: challenged control with the inclusion of 0.5% *A. annua* in the diet; 1AA: challenged control with the inclusion of 1% *A. annua* in the diet. Before the *Eimeria* challenge at 25 weeks of age, treatments of NC, PF, and CC hens at 23 and 24 weeks of age were not different among themselves. Pair-feeding was carried out from 0–14 dpi after the *Eimeria* challenge. FCR: feed conversion ratio (kg feed per dozen eggs); Trt: treatment groups; AOH: age of the hens. Graphs not sharing the same superscripts (a–f) are significantly different at $p < 0.05$.

TABLE 5 Effects of the dietary inclusion of phytogenic feed additive *Artemisia annua* in the diets of laying hens on their gut permeability at 5 dpi and oocyst shedding of *Eimeria acervulina, Eimeria maxima*, and *Eimeria tenella* from 5–8 dpi.

Items[a]	Gut permeability (μg/mL)	*E. acervulina* (Log_{10})	*E. maxima* (Log_{10})	*E. tenella* (Log_{10})
NC	0.01[b]	0.00[c]	0.00[b]	0.00[c]
PF	0.01[b]	0.00[c]	0.00[b]	0.00[c]
CC	0.48[a]	5.43[a]	3.90[a]	4.40[b]
0.5AA	0.34[a]	5.22[b]	4.02[a]	5.22[a]
1AA	0.34[a]	5.29[a]	3.84[a]	4.57[ab]
SEM	0.06	0.07	0.07	0.09
p-value	<0.0001	<0.0001	<0.0001	<0.0001

[a–c]values within columns not sharing the same superscripts are significantly different at $p < 0.05$.
[a]NC, non-challenged control; PF, pair-fed control; CC, challenged control (12,500 *E. maxima*; 12,500 *E. tenella*; and 62,500 *E. acervulina* oocysts per mL); 0.5AA, challenged control with the inclusion of 0.5% *Artemisia annua* in the diet; 1AA, challenged control with the inclusion of 1% *A. annua* in the diet; dpi: days post-inoculation of *Eimeria* spp.

dozen eggs) hens compared to NC hens (1.19 kg feed/dozen of eggs; $p < 0.0001$) at 26 weeks of age, as shown in Figure 5. However, 0.5% AA and 1%AA inclusion reduced the overall FCR by 0.50 and 0.61 during the challenged period (25–27 weeks of age), respectively.

Gut permeability, lesion score, and oocyst shedding

The *Eimeria*-challenged hens showed higher gut permeability than non-challenged hens ($p < 0.0001$; Table 5). Among the challenged hens, the inclusion of AA reduced the gut permeability by 0.14 μg/mL compared to CC hens (0.48 μg/mL). The inclusion of AA in the diet resulted in a reduction in the severity of intestinal lesions, transitioning from higher to lower lesion scores across all three sections of the intestine (Figure 6). Oocyst shedding was only observed in *Eimeria*-challenged hens, confirming that the NC and PF hens were not infected. The inclusion of 0.5% AA in the diet increased the oocyst shedding of *E. tenella* and reduced the oocyst shedding of *E. acervulina* ($p < 0.05$; Table 5).

Gene expression

The effects of feeding AA leaves on the intestinal barrier function and nutrient transporter gene expression of the laying hens post-*Eimeria* challenge are shown in Table 6. At 6 and 14 dpi, the expression of JAM-2, OCLN, ZO-2, and MUC-2 was

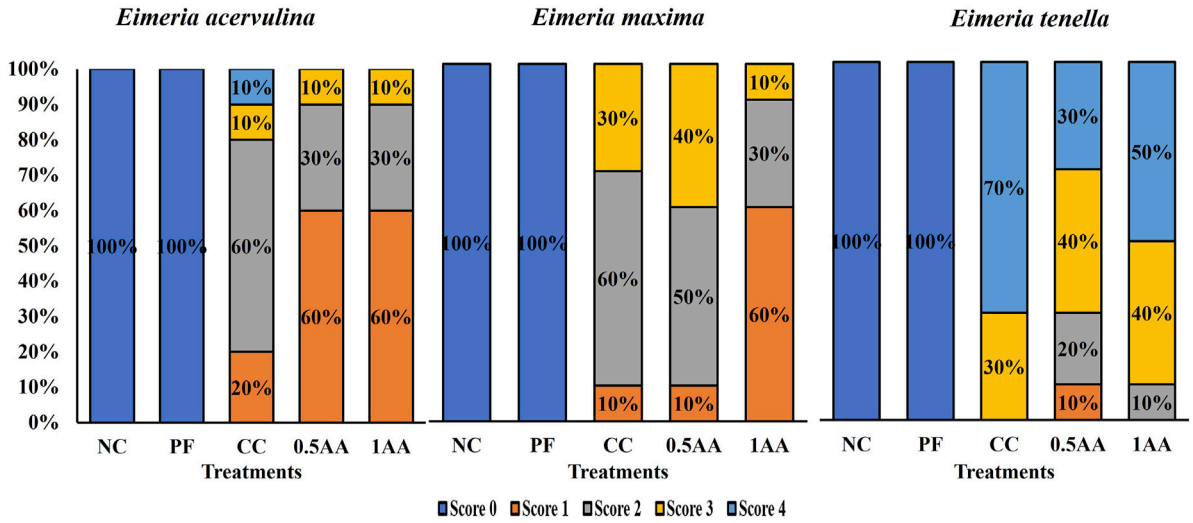

FIGURE 6
Effect of the inclusion of phytogenic feed additive *A. annua* in the diets of laying hens on their intestinal lesion score for *E. acervulina* (duodenum), *E. maxima* (part of the jejunum and ileum), and *E. tenella* (ceca) at 6 days post-*Eimeria* challenge. The lesion scores were ranked based on the severity of lesions from 0 to 4, with 0 indicating no lesions and 4 indicating extremely severe lesions. At 25 weeks of age, 12,500 *E. maxima*, 12,500 *E. tenella*, and 62,500 *E. acervulina* oocysts were inoculated in laying hens grouped into challenged control, 0.5% *A. annua*, and 1% *A. annua*. NC: non-challenged control; PF: pair-fed control; CC: challenged control (12,500 *E. maxima*; 12,500 *E. tenella*; and 62,500 *E. acervulina* oocysts per mL); 0.5AA: challenged control with the inclusion of 0.5% *A. annua* in the diet; 1AA: challenged control with the inclusion of 1% *A. annua* in the diet; dpi: days post-inoculation of *Eimeria* spp.

downregulated in *Eimeria*-challenged hens compared to non-challenged hens, whereas the expression of CLDN-1 was upregulated only at 6 dpi ($p < 0.05$). The inclusion of AA leaves upregulated the mRNA expression of iNOS at 6, 14, and 21 dpi ($p < 0.05$). The expression of nutrient transporter genes (b^{0+} AT, EAAT-3, y^+LAT, b^0AT, pepT-1, SGLT-1, and GLUT-1) was slightly upregulated in the PF group, whereas they were downregulated in the *Eimeria*-challenged hens at 6 dpi ($p < 0.05$). At 14 dpi, 1% AA inclusion upregulated the amino acid transporter genes compared to CC hens but remained lower than that of the NC and PF hens ($p < 0.05$). Furthermore, y^+LAT, GLUT-1, and SGLT-1 expression was downregulated in PF hens compared to NC hens ($p < 0.05$; Table 6). At 6 dpi, the expression of cytokines IL-10 and IFN-γ in *Eimeria*-challenged hens (CC and 1AA) was upregulated compared to non-challenged hens. However, this upregulation was lower in hens grouped in 0.5AA than that in CC and 1AA hens ($p < 0.05$; Table 7). On the other hand, the expression of IL-4 was downregulated by the inclusion of AA ($p = 0.0808$). No significant differences in any genes among the treatment groups were observed at 14 dpi ($p > 0.05$). However, at 21 dpi, all treatments exhibited lower INF-γ expression than the NC treatment ($p < 0.05$).

Small intestine histomorphology

The effect of dietary inclusion of AA leaves in a laying hen diet infected with *Eimeria* spp. is presented in Table 8. At 6 dpi, the VH of the duodenum and jejunum decreased, whereas CD increased by the *Eimeria* challenge, resulting in a lower VH-to-CD ratio ($p < 0.05$). Although no significant difference was observed in ileal VH, CD was significantly increased, lowering the VH-to-CD ratio in challenged hens ($p < 0.05$). The inclusion of 0.5% AA resulted in a higher VH in all three sections relative to CC at 6 dpi. At 14 and 21 dpi, the inclusion of AA reduced the CD in challenged hens in all three sections of the intestine compared to the CC hens ($p < 0.1$). However, VH of the jejunum increased at 6 dpi ($p < 0.05$), and as feed restriction progressed in the PF group, a slight reduction in jejunum VH was observed at 14 and 21 dpi ($p > 0.05$). Although a higher jejunum CD was observed in challenged hens at 14 dpi ($p < 0.05$), it became similar to that of NC hens by 21 dpi. In the ileum, the VH decreased and CD increased through 21 dpi in the PF group compared to the NC group ($p < 0.05$).

Flow cytometry

No significant differences were observed in the peripheral mononuclear blood cell count at 14 dpi ($p > 0.05$; Figure 7). However, the monocyte counts in the peripheral blood increased by 71% in hens grouped in CC and by 138% in those fed 1% AA compared to NC hens. Likewise, no significant differences were observed for $CD4^+$, $CD8^+$, and their ratio.

Oxidative status

The effect of the inclusion of AA leaves on the oxidative status of laying hens post-*Eimeria* challenge is shown in Figure 8. At 6 dpi, *Eimeria* challenges and pair feeding reduced the total antioxidant capacity; however, at 14 dpi, the TAC increased in challenged hens but not in the PF group ($p < 0.05$). Likewise, MDA levels increased in *Eimeria*-challenged hens at 6 dpi ($p < 0.05$) and became similar to that of NC hens by 14 dpi. The inclusion of AA did not have a beneficial effect on improving the oxidative status of the laying hens post-*Eimeria* challenge.

TABLE 6 Effects of the dietary inclusion of phytogenic feed additive *Artemisia annua* in the diet of laying hens on their intestinal barrier function and nutrient transporter genes 6, 14, and 21 dpi.

	6 dpi												
	CLDN-1	JAM-2	OCLN	ZO-2	iNOS	MUC-2	b^{0+}, AT	EAAT-3	y^+LAT	pepT-1	b^0 AT	GLUT-1	SGLT-1
NC	1.00^{b}	1.00^{ab}	1.00^{a}	1.00	1.00^{c}	1.00^{a}	1.00^{b}	1.00^{a}	1.00^{a}	1.00^{ab}	1.00^{a}	1.00^{a}	1.00^{a}
PF	1.27^{b}	1.24^{a}	1.18^{a}	1.09	1.26^{c}	1.08^{a}	1.30^{a}	1.08^{a}	1.12^{a}	1.18^{a}	1.44^{a}	1.09^{a}	1.20^{a}
CC	10.33^{a}	0.72^{c}	0.47^{b}	0.85	3.23^{bc}	0.40^{b}	0.21^{c}	0.38^{b}	0.10^{b}	0.62^{bc}	0.39^{b}	0.17^{b}	0.35^{b}
0.5AA	10.76^{a}	0.80^{bc}	0.51^{b}	0.87	6.66^{a}	0.36^{b}	0.11^{c}	0.31^{b}	0.04^{b}	0.34^{c}	0.41^{b}	0.04^{b}	0.25^{b}
1AA	11.05^{a}	0.78^{bc}	0.50^{b}	0.80	4.56^{ab}	0.32^{b}	0.20^{c}	0.36^{b}	0.06^{b}	0.60^{bc}	0.25^{b}	0.17^{b}	0.32^{b}
SEM	1.22	0.09	0.08	0.11	0.93	0.08	0.08	0.06	0.06	0.16	0.19	0.13	0.09
p-value	<0.0001	0.0042	<0.0001	0.3534	0.0018	<0.0001	<0.0001	<0.0001	<0.0001	0.0133	0.0008	<0.0001	<0.0001
14 dpi													
NC	1.00^{b}	1.00	1.00^{a}	1.00^{a}	1.00^{a}	1.00^{ab}	1.00^{ab}	1.00^{a}	1.00^{a}	1.00^{a}	1.00	1.00^{a}	1.00^{a}
PF	1.55^{a}	1.04	0.78^{a}	1.02^{a}	0.93^{a}	1.15^{a}	1.41^{a}	1.03^{a}	0.60^{b}	0.90^{a}	1.39	0.59^{b}	0.66^{b}
CC	0.48^{cd}	0.72	0.39^{b}	0.54^{b}	0.55^{c}	0.58^{b}	0.50^{b}	0.46^{b}	0.38^{b}	0.45^{b}	0.29	0.42^{b}	0.36^{c}
0.5AA	0.44^{d}	0.71	0.36^{b}	0.50^{b}	0.68^{bc}	0.53^{b}	0.48^{b}	0.38^{b}	0.33^{b}	0.22^{b}	0.2	0.23^{b}	0.34^{c}
1AA	0.77^{bc}	1.00	0.50^{b}	0.69^{b}	0.85^{ab}	0.78^{ab}	0.84^{ab}	0.71^{ab}	0.46^{b}	0.36^{b}	0.58	0.36^{b}	0.57^{b}
SEM	0.10	0.13	0.08	0.09	0.07	0.17	0.23	0.14	0.11	0.40	0.33	0.12	0.06
p-value	<0.0001	0.2022	<0.0001	0.0004	0.0019	0.0717	0.0538	0.0089	0.0029	0.0018	0.1068	0.0026	<0.0001
21 dpi													
NC	1.00	1.00^{b}	1.00	1.00	1.00^{c}	1.00	1.00	1.00	1.00	1.00	1.00	1.00	1.00
PF	0.97	1.07^{b}	1.11	1.13	1.30^{ab}	1.23	0.95	1.24	0.99	1.18	1.14	0.96	1.23
CC	1.25	1.32^{a}	1.28	1.20	1.28^{bc}	0.91	0.99	1.21	1.17	1.18	1.29	1.13	1.38
0.5AA	1.36	1.40^{a}	1.38	1.08	1.68^{a}	1.16	1.27	1.11	0.73	0.88	1.26	0.65	0.95
1AA	1.00	1.37^{a}	1.23	1.04	1.38^{ab}	1.03	1.03	1.22	1.10	0.83	1.18	1.00	1.14
SEM	0.22	0.07	0.10	0.09	0.12	0.10	0.09	0.09	0.18	0.16	0.17	0.21	0.12
p-value	0.656	0.001	0.1158	0.5379	0.0113	0.1695	0.1165	0.3472	0.508	0.387	0.7867	0.5943	0.124

[a-c]values within columns not sharing the same superscripts are significantly different at $p < 0.05$.

[a]NC: non-challenged control; PF: pair-fed control; CC: challenged control (12,500 *E. maxima*; 12,500 *E. tenella*; and 62,500 *E. acervulina* oocysts per mL); 0.5AA: challenged control with the inclusion of 0.5% *Artemisia annua* in the diet; 1AA: challenged control with the inclusion of 1% *A. annua* in the diet.

CLDN-1, claudin-1; ZO-2, zonula occludin-2; OCLN, occludin; JAM-2, junctional adhesion molecule-2; MUC-2, mucin-2; iNOS, inducible nitric oxide synthase; $b^{0,+}$ AT, solute carrier family 7, member 9; EAAT-3, excitatory amino acid transporter 3; pepT-1; b^0 AT, solute carrier family 6, member 19; y^+ LAT-1, y^+ L amino acid transporter-1; SGLT-1, sodium-glucose transporter-1; GLUT-1, glucose transporter-1; dpi, days post-inoculation of *Eimeria* spp.

Discussion

In this study, we investigated the role of phytogenic feed additive (*A. annua*) supplementation on the performance, intestinal barrier functions, oxidative status, and immune response of laying hens infected with mixed *Eimeria* species. Supplementing the phytogenic feed additive (*A. annua*) showed some beneficial effects on improving the laying hen performance, intestinal barrier functions, and, to some extent, immune responses against *Eimeria* spp. tested in the current study.

As expected, *Eimeria* infection reduced the egg-laying performance and feed intake of laying hens while increasing the feed conversion ratio over the 21-day period. Although the inclusion of AA did not improve the growth performance, it did improve the egg production and feed efficiency of the laying hens. The results of growth performance were different from those observed previously, where the inclusion of the AA leaves or its extract (artemisinin) at various levels prevented weight loss in broilers compared to *Eimeria*-challenged hens (Allen et al., 1997; Hady and Zaki, 2012; Pop et al., 2015). However, in this study, AA inclusion was ineffective in preventing BW loss. As the hens were not growing, they might have utilized their fat reserve to lay eggs during the acute phase (0–6 dpi) while simultaneously fighting the infection (Sharma et al., 2024b). In the current study, the distinct smell and bitter taste of AA led to strong feed refusal in the lower AA-inclusion group compared to the higher inclusion group. However, the exact reason for strong

10.3389/fphys.2024.1381548

TABLE 7 Effects of the dietary inclusion of phytogenic feed additive *Artemisia annua* in the diets of laying hens on their expression of inflammatory cytokines at 6, 14, and 21 dpi.

Items	6 dpi					
	IL-1β	IL-4	IL-10	IL-17	IFN-γ	TNF-α
NC	1.00	1.00^{ab}	1.00^{b}	1.00	1.00^{c}	1.00^{a}
PF	0.96	1.22^{a}	1.15^{b}	0.56	1.36^{c}	1.09^{a}
CC	1.35	1.14^{ab}	4.07^{a}	0.86	10.40^{a}	0.67^{b}
0.5AA	1.41	0.53^{b}	1.69^{b}	0.92	5.70^{b}	0.62^{b}
1AA	1.30	0.50^{b}	4.01^{a}	0.81	10.04^{a}	0.59^{b}
SEM	0.23	0.22	0.59	0.26	1.29	0.09
p-value	0.5231	0.0808	0.0016	0.8000	<0.0001	0.0021
14 dpi						
NC	1.00	1.00	1.00	1.00	1.00	1.00
PF	1.23	0.98	0.82	1.41	0.74	1.16
CC	1.43	0.84	0.8	2.54	0.84	1.14
0.5AA	1.31	1.41	0.94	1.20	1.33	1.13
1AA	1.51	0.85	0.95	1.39	1.18	0.97
SEM	0.26	0.22	0.19	0.57	0.35	0.11
p-value	0.6935	0.3784	0.9338	0.3757	0.7434	0.6847
21 dpi						
NC	1.00	1.00	1.00	1.00^{a}	1.00^{a}	1.00
PF	0.72	1.35	0.59	0.34^{b}	0.35^{b}	0.83
CC	0.91	1.04	0.91	0.52^{b}	0.45^{b}	0.92
0.5AA	0.99	0.83	0.77	0.68^{ab}	0.47^{b}	0.83
1AA	1.15	1.00	0.80	0.65^{ab}	0.33^{b}	0.99
SEM	0.14	0.18	0.16	0.15	0.11	0.09
p-value	0.3253	0.3497	0.4867	0.0642	0.0031	0.6405

[a-c]values within columns not sharing the same superscripts are significantly different at $p < 0.05$.

[a]NC, non-challenged control; PF, pair-fed control; CC, challenged control (12,500 *E. maxima*; 12,500 *E. tenella*; and 62,500 *E. acervulina* oocysts per mL); 0.5AA, challenged control with the inclusion of 0.5% *Artemisia annua* in the diet; 1AA, challenged control with the inclusion of 1% *A. annua* in the diet.

IL-1β, interleukin-1 β; IL-4, interleukin-4; IL-10, interleukin-10; IL-17, interleukin-17 A; TNF-α, tumor necrosis factor-α; IFN-γ, interferon-γ; dpi, days post-inoculation of *Eimeria* spp.

feed refusal in the lower inclusion group remains unclear. Consequently, this resulted in a decrease in body weight and egg production in the 0.5% AA-inclusion group during the adaptation period (Engberg et al., 2012). The literature on the effect of AA on laying hen performance is scarce; however, the increase in egg production in hens during the challenge period might be due to the antioxidant, antimicrobial, and antiprotozoal properties of artemisinin (Allen et al., 1997; Del Cacho et al., 2010; Engberg et al., 2012; Baghban-Kanani et al., 2019). The difference between NC and CC hens (51.3%) for the HDEP was attributed to the reduced feed intake and symptoms associated with disease during the infection period. Additionally, the difference in the HDEP between PF and CC hens (23.5%) might be attributed to the nutritional redistribution associated with inflammation and immune responses, whereas 27.8% was associated with reduced feed intake following coccidiosis.

Gastrointestinal health is of great importance in poultry as it affects nutrient digestion, absorption, and utilization, and, most importantly, if compromised, affects the health and welfare of the chickens (Choi et al., 2023a; Tompkins et al., 2023; Sharma et al., 2024a; 2024b). The findings from the current study confirmed that *Eimeria* species damage the intestinal tract with mild-to-severe intestinal lesions and structural damage to the intestinal architecture of the villi, ultimately affecting the performance of the laying hens. However, the inclusion of the AA in the diet reduced the severity of intestinal lesions in the duodenum, jejunum, ileum, and ceca. Previously, it had been observed that either the AA leaves or its extract (artemisinin) reduced the lesion score, oocyst shedding, and symptoms related to coccidiosis (Allen et al., 1997; Almeida et al., 2012; Hady and Zaki, 2012; Kaboutari et al., 2014; Mo et al., 2014; Jiao et al., 2018). Unlike the findings obtained by Allen et al. (1997), who reported a significant

TABLE 8 Effects of the dietary inclusion of phytogenic feed additive *Artemisia annua* in the diets of laying hens on their small intestinal histomorphology at 6, 14, and 21 dpi.

	Duodenum								
	6 dpi			14 dpi			21 dpi		
	VH	CD	VHCD	VH	CD	VHCD	VH	CD	VHCD
NC	1,827.0^{a}	121.4^{b}	15.78^{a}	1,752.7	121.8^{d}	14.46^{a}	1,845.1	130.1^{c}	14.43
PF	1,560.8^{b}	145.9^{b}	11.14^{b}	1,790.6	159.2^{d}	11.32^{b}	1,730.9	142.1^{bc}	12.50
CC	1,110.8^{c}	448.8^{a}	2.55^{c}	1,620.8	379.4^{a}	4.33^{d}	1,900	186.1^{a}	10.86
0.5AA	1,233.1^{c}	383.0^{a}	3.32^{c}	1,775.0	299.8^{b}	6.15^{cd}	1,886.4	176.3^{ab}	10.91
1AA	1,229.9^{c}	406.1^{a}	3.09^{c}	1,753.0	233.8^{c}	7.71^{c}	1,692.3	151.1^{abc}	11.43
SEM	62.01	26.75	0.93	76.52	17.42	0.63	65.12	14.71	1.02
p-value	<0.0001	<0.0001	<0.0001	0.5501	<0.0001	<0.0001	0.1211	0.0714	0.1116
Jejunum									
NC	1,100.2^{ab}	97.1^{c}	11.35^{a}	1,294.2	113.6^{c}	11.70^{a}	1,056.5	110.0	9.88
PF	1,277.5^{a}	133.5^{c}	9.66^{b}	1,152.1	144.4^{b}	8.09^{b}	938.0	109.5	9.21
CC	932.3^{b}	416.1^{a}	2.29^{d}	1,157.5	183.4^{a}	6.43^{b}	1,084.7	135.1	8.36
0.5AA	966.5^{b}	397.3^{b}	3.44^{c}	1,198.2	183.2^{a}	6.61^{b}	1,054.1	143.0	7.70
1AA	912.5^{b}	351.2^{ab}	2.70^{cd}	1,117.1	149.5^{b}	7.63^{b}	1,001.9	121.0	8.29
SEM	80.16	28.34	0.38	60.15	10.35	0.68	47.99	13.97	0.78
p-value	0.0222	<0.0001	<0.0001	0.3105	0.0005	0.0001	0.2505	0.362	0.3359
Ileum									
NC	686.1	84.4^{c}	8.23^{a}	760.4^{a}	81.5^{c}	9.68^{a}	759.2^{ab}	79.2^{c}	8.79
PF	707.3	107.0^{c}	6.78^{b}	616.8^{b}	96.8^{bc}	6.61^{b}	607.1^{b}	86.7^{bc}	8.04
CC	739.5	335.4^{a}	2.26^{c}	807.1^{a}	133.0^{a}	6.33^{b}	864.5^{a}	122.3^{a}	7.13
0.5AA	772.1	356.7^{b}	3.04^{c}	794.8^{a}	111.7^{ab}	7.15^{b}	784.1^{a}	113.7^{ab}	7.22
1AA	694.3	246.5^{b}	2.88^{c}	869.5^{a}	126.7^{a}	6.34^{b}	859.3^{a}	96.0^{abc}	9.10
SEM	41.15	19.88	0.34	31.0	9.83	0.71	53.1	10.38	0.69
p-value	0.6523	<0.0001	<0.0001	0.0022	0.0082	0.0164	0.0173	0.0414	0.1972

[a-c]values within columns not sharing the same superscripts are significantly different at $p < 0.05$.
[a]NC, non-challenged control; PF, pair-fed control; CC, challenged control (12,500 *E. maxima*; 12,500 *E. tenella*; and 62,500 *E. acervulina* oocysts per mL); 0.5AA, challenged control with the inclusion of 0.5% *Artemisia annua* in the diet; 1AA, challenged control with the inclusion of 1% *A. annua* in the diet.
VH, villus height (μm); CD, crypt depth (μm); VHCD, villus height-to-crypt depth ratio; dpi, days post-inoculation of *Eimeria* spp.

reduction in the oocyst shedding of *E. tenella*, our study observed only a slight decrease in the oocyst shedding of *E. acervulina*. The reduced oocyst shedding observed may be attributed to the artemisinin derived from the AA leaves, which alters the formation of the oocyst wall, resulting in both death and reduced sporulation rate of oocysts (Del Cacho et al., 2010; Fatemi et al., 2015; Pop et al., 2015). The variation in oocyst shedding observed between the studies might be due to differences in the source of artemisinin used, such as extracted artemisinin or artemisinin released from the AA leaves. In this study, the differences observed in *E. tenella* and *E. acervulina* oocyst shedding between the 0.5% and 1% AA inclusion groups might be associated with the environments where they reside. Unlike *E. acervulina*, *E. tenella* develops under relatively anaerobic conditions in the ceca, filled with cecal contents. This anaerobic environment in the ceca may require a higher concentration of artemisinin to exert its effects (Allen et al., 1997). Furthermore, it was reported that artemisinin from AA leaves induces apoptotic events in *Eimeria*-infected cells, resulting in increased apoptosis of merozoites and protozoal host cells. This ultimately reduces the second-generation merozoites released into the intestinal lumen (Del Cacho et al., 2010; Mo et al., 2014). Consequently, this reduces the intestinal lesion score and gut permeability in the infected hosts, as observed in this study.

In this study, the oxidative status of the laying hens following the *Eimeria* challenge among the infected groups was not different. It

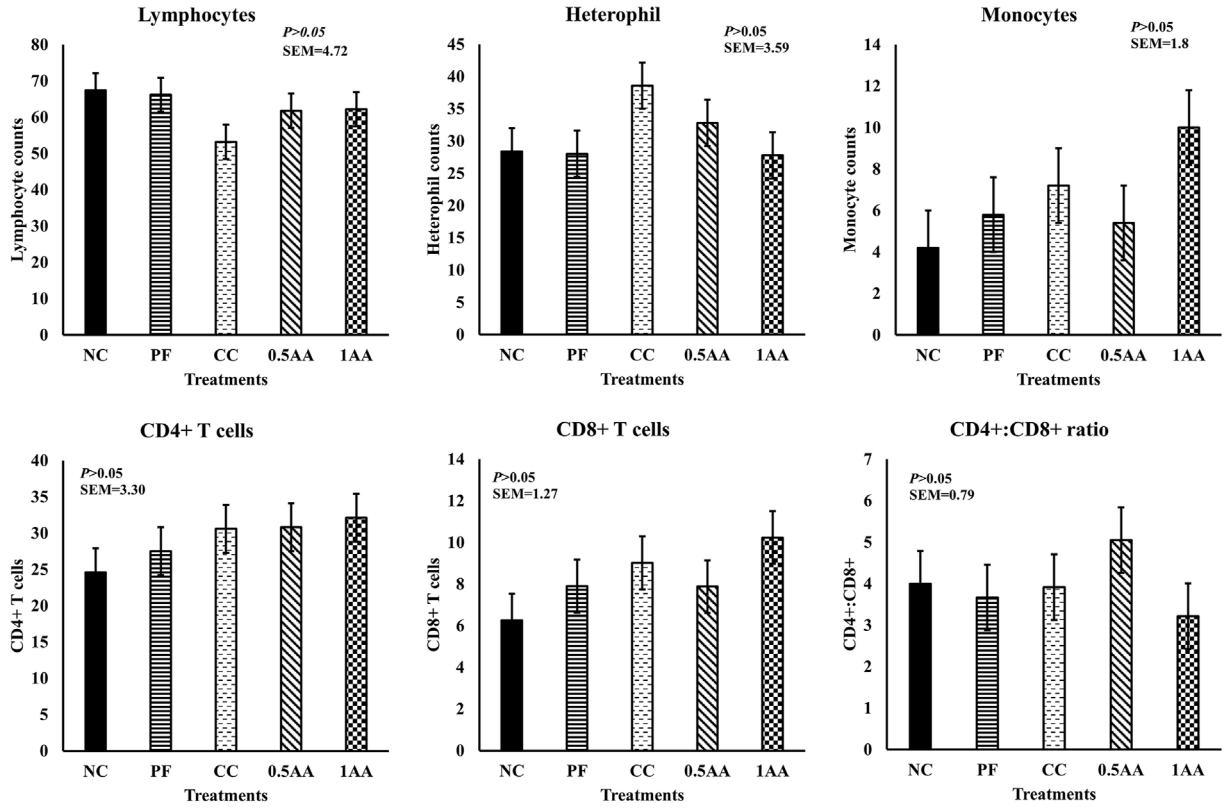

FIGURE 7
Effect of the inclusion of phytogenic feed additive *A. annua* in the diets of laying hens on their peripheral mononuclear blood cell count and $CD4^+$ and $CD8^+$ T cell population 14 days post-*Eimeria* challenge. At 25 weeks of age, 12,500 *E. maxima*, 12,500 *E. maxima*, and 62,500 *E. tenella* oocysts were inoculated in laying hens grouped into challenged control, 0.5% *A. annua*, and 1% *A. annua*. NC: non-challenged control; PF: pair-fed control; CC: challenged control (12,500 *E. maxima*; 12,500 *E. tenella*; and 62,500 *E. acervulina* oocysts per mL); 0.5AA: challenged control with the inclusion of 0.5% *A. annua* in the diet; 1AA: challenged control with the inclusion of 1% *A. annua* in the diet; dpi: days post-inoculation of *Eimeria* spp.

has been observed that artemisinin is capable of inducing oxidative stress by increasing free radicals in specific host cells where *Eimeria* resides. This increased production of free radicals is suggested to kill the intracellular parasite, in this case, *Eimeria* (Meshnick et al., 1989; Kaboutari et al., 2014; Jiao et al., 2018). Moreover, the inducible nitric oxide synthase (iNOS) expression was upregulated in hens in 0.5% AA and 1% AA groups compared to CC hens. Inducible nitric oxide synthase helps in the production of nitric oxide, which induces oxidative stress, thereby limiting *Eimeria* multiplication in the host cells (Castro et al., 2020). Although artemisinin has an antioxidant capacity to prevent free radicals and lipid peroxidation in non-infected birds (Drăgan et al., 2014), the *Eimeria* infection may have changed its functions to specifically increase oxidative stress in infected host cells to cause apoptosis of the infected host cells and merozoites residing in them (Meshnick et al., 1989; Kaboutari et al., 2014; Jiao et al., 2018).

As previously observed, *Eimeria* infection significantly influenced duodenal, jejunal, and ileal morphology by decreasing the VH and increasing the CD of laying hens (Sharma et al., 2024a; 2024b). Conversely, the dietary inclusion of AA partially alleviated the negative effect of coccidiosis on the small intestinal morphometric architecture during the recovery phase (8–21 dpi). The selective apoptosis of *Eimeria*-infected host cells by artemisinin might have facilitated the quick recovery of intestinal villi (Mo et al., 2014; Jiao et al., 2018). As observed in this study, the beneficial effect of AA on intestinal villus recovery may depend on multiple factors, including intestinal location, dpi, and dosage. For instance, 0.5% AA inclusion increased the VH in duodenal and jejunal regions, while 1% AA reduced the CD. However, 1% AA increased the VH and reduced the CD at 21 dpi in the ileum. In PF hens, a dietary restriction similar to that of CC hens from 0–14 dpi might be one of the reasons for the regression in intestinal villus height and increased crypt depth at 21 dpi. The regression in the villi may contribute to the downregulation of glucose transporters (GLUT-1 and SGLT-1) and y^+LAT amino acid transporters at 14 dpi in the PF hens. This regression in villi and downregulation of specific nutrient transporters might have also played a role in the prominent production loss observed in PF groups by 14 dpi.

The breach in the intestinal mucosa by *Eimeria* triggers several signaling cascades, leading to the production of cytokines involved in inflammation (Lillehoj and Lillehoj, 2000; Yun et al., 2000; Hong et al., 2006a). Following the *Eimeria* infection, the expression of pro-inflammatory (IFN-γ) and anti-inflammatory cytokine (IL-10) increased in challenged

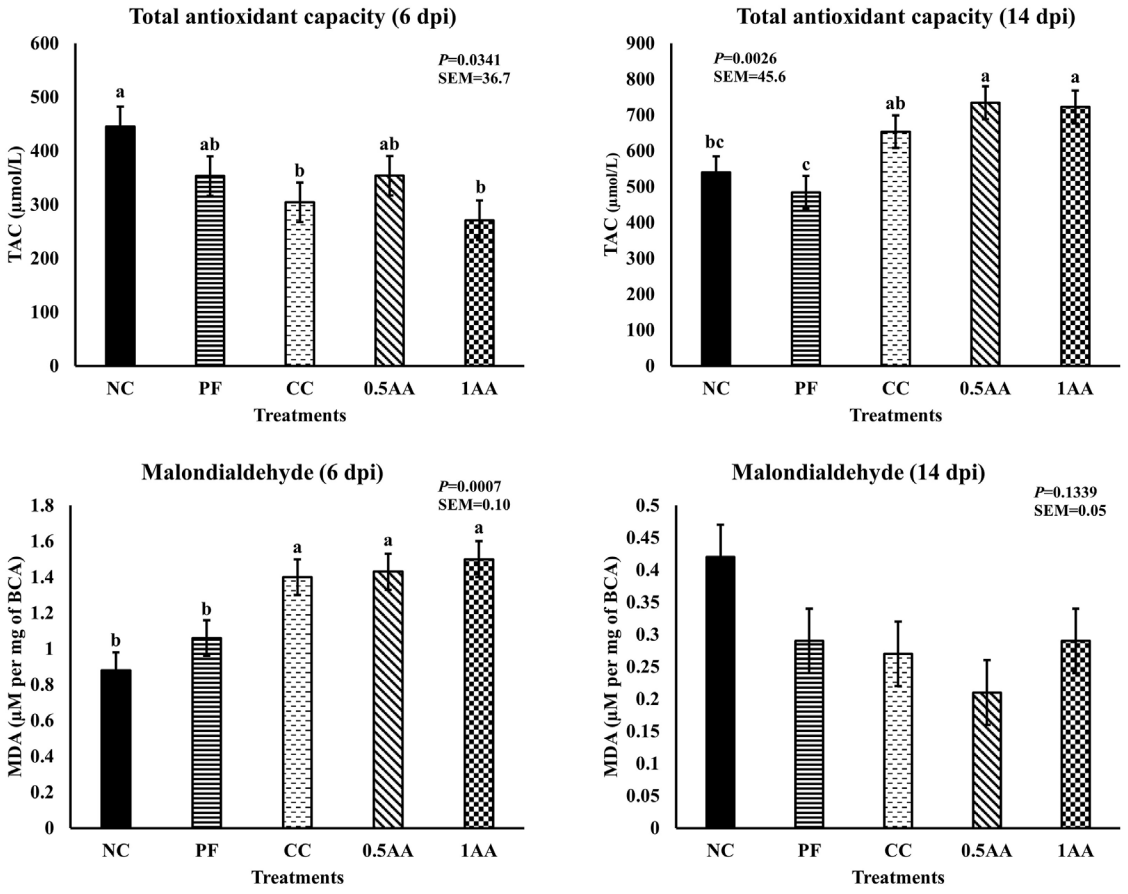

FIGURE 8
Effect of the inclusion of phytogenic feed additive *A. annua* in the diets of laying hens on their oxidative status at 6 and 14days post-*Eimeria* challenge. At 25 weeks of age, 12,500 *E. maxima*, 12,500 *E. maxima*, and 62,500 *E. tenella* oocysts were inoculated in laying hens grouped into challenged control, 0.5% *A. annua*, and 1% *A. annua*. NC: non-challenged control; PF: pair-fed control; CC: challenged control (12,500 *E. maxima*; 12,500 *E. tenella*; and 62,500 *E. acervulina* oocysts per mL); 0.5AA: challenged control with the inclusion of 0.5% *A. annua* in the diet; 1AA: challenged control with the inclusion of 1% *A. annua* in the diet; TAC: total antioxidant capacity (µmol/L); MDA: malondialdehyde (µM per mg of BCA); dpi: days post-inoculation of *Eimeria* spp. Graphs not sharing the same superscripts (a–b) are significantly different at $p < 0.05$.

birds, consistent with the findings from previous studies (Hong et al., 2006b; Sharma et al., 2024a; 2024b). However, 0.5% AA inclusion reduced the expression of both IFN-γ and IL-10 compared to the CC, suggesting that AA can reduce the inflammatory response of chickens by various means and help the normal functioning and upregulation of humoral responses (Song et al., 2017; Yang et al., 2021). Artemisinin is known to suppress the production of these cytokines by inhibiting the activation of NF-κB; however, the reason why the 1% inclusion of AA did not have the same effect as 0.5% AA inclusion remains unclear (Jiao et al., 2018). Furthermore, the inclusion of 1% AA upregulated cell-mediated immunity by increasing the monocyte counts and $CD8^+$ cells, which are responsible for clearing intracellular pathogens (Liu et al., 2023; Shah et al., 2023). The partial reduction in inflammation and increased cell-mediated immunity to kill the intracellular *Eimeria* parasite, along with reduced intestinal lesions and permeability in AA-fed hens, might contribute to reducing energy allocation for host defense. This could potentially improve the performance of the hens by increasing the available energy for production, consequently increasing the HDEP by at least 6.25% in the AA groups.

Conclusion

Based on the findings from the current study, it can be concluded that the adverse effects of the *Eimeria* challenge are associated with reduced feed intake and redistribution of the nutritional reserve of the host for inflammation and oxidative stress rather than the performance. However, dietary supplementation of the phytogenic feed additive (*A. annua* leaves) partially helped restore the egg production and intestinal integrity of the laying hens infected with *Eimeria* spp. Furthermore, it also helped modulate the immune response to alleviate the negative effect of coccidiosis on the performance. However, further studies need to be conducted to find the optimal dose of AA leaves to prevent feed refusal and improve the performance of laying hens.

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 approved by the Institutional Animal Care and Use Committee of the University of Georgia. The study was conducted in accordance with the local legislation and institutional requirements.

Author contributions

MS: conceptualization, formal analysis, investigation, methodology, and writing–original draft. GL: formal analysis, investigation, methodology, and writing–review and editing. VC: formal analysis, investigation, methodology, and writing–review and editing. HR-N: formal analysis, investigation, methodology, and writing–review and editing. FM: formal analysis, investigation, methodology, and writing–review and editing. BM: investigation, methodology, and writing–review and editing. RG: formal analysis, investigation, methodology, and writing–review and editing. WK: conceptualization, data curation, funding acquisition, investigation, project administration, supervision, and writing–review and editing.

Funding

The author(s) declare that no financial support was received for the research, authorship, and/or publication of this article.

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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frontiers | Frontiers in Physiology

TYPE Original Research
PUBLISHED 07 June 2024
DOI 10.3389/fphys.2024.1400611

OPEN ACCESS

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RECEIVED 13 March 2024
ACCEPTED 16 May 2024
PUBLISHED 07 June 2024

CITATION
Whittle RH, Kiarie EG, Ma DWL and Widowski TM (2024), Feeding flaxseed to chicken hens changes the size and fatty acid composition of their chicks' brains.
Front. Physiol. 15:1400611.
doi: 10.3389/fphys.2024.1400611

Feeding flaxseed to chicken hens changes the size and fatty acid composition of their chicks' brains

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Diets fed to commercial chicken breeders are high in n-6 fatty acids (n-6 FAs) and low in n-3 fatty acids (n-3 FAs). N-3 FAs are essential for embryonic brain development. In precocial birds, like chickens, brain development and brain n-3 FA accrual occur primarily before hatching. In two experiments, broiler and layer breeders were fed diets with or without flaxseed as the source of n-3 FAs from plant-based alpha-linolenic acid. Day-old broiler (n = 80) and layer (n = 96) offspring were dissected to calculate the percentage brain-to-body weight. Brain FA analyses from total lipid extracts were determined in the broiler (n = 24) and layer (n = 24) offspring brains, and the percentage FA composition and concentration (µg FAs per g brain) were calculated for each n-3 and n-6 FA. The brain size was only increased in broiler offspring from mothers fed flaxseed ($\chi 2$ = 9.22, p = 0.002). In layer offspring only, the maternal flaxseed diet increased the brain concentration and percentage of n-3 FAs and decreased n-6 FAs (p < 0.05). We showed that feeding flaxseed to mothers increased the brain size in broiler offspring and altered brain FA composition in layer offspring. These results may have implications for poultry and other captive bird species fed diets low in n-3 FAs.

KEYWORDS

chicken, broiler, layer, maternal diet, omega-3 fatty acids, brain size

1 Introduction

Long-chain omega-3 polyunsaturated fatty acids (n-3 FAs) are key structural components of neural, muscle, and retinal tissue in vertebrate species, including fish (Závorka et al., 2022), mammals (Carlson and Neuringer, 1999), and birds (Speake et al., 1998). The two predominant n-3 FAs are docosahexaenoic acid (DHA) from marine dietary sources such as fish oil and its precursor alpha-linolenic acid (ALA) from plant sources, including flaxseed (Innis, 2008; Bradbury, 2011). While n-3 FAs are important, omega-6 FA (n-6 FA) cannot be overlooked. n-3 FA and n-6 FA compete for the same metabolic enzymes. When n-6 FA and n-3 FA consumption is imbalanced (high n-6 FA-to-n-3 FA ratio), more n-6 FAs than n-3 FAs are found in cellular membranes (Schmitz and Ecker, 2008). Dietary n-3 FAs, particularly DHA, are essential in maternal and postnatal diets for embryonic brain and central nervous system development. Perinatal n-3 FA nutrition affects both brain size and fatty acid composition of the tissues (Coti Bertrand et al., 2006). For example, higher consumption of prey rich in n-3 FAs in wild brown trout resulted in increased muscle DHA concentration, positively correlated with relative brain size (Závorka et al., 2022). A study on neurogenesis in embryonic rats found that n-3 FA deficiency during

development resulted in reduced brain size due to reductions in the size of the cortical plate, primordial hippocampus, and dentate gyrus. In addition to neurogenesis, other processes in the brain rely on DHA, including neurotransmission and protection against oxidative stress, with n-3 FA deprivation affecting the membrane stability and gene expression (Innis, 2007). In rats, dietary n-3 FAs are involved in the upregulation and downregulation of genes affecting synaptic plasticity, signal transduction, energy metabolism, and neurotransmitter receptors (Kitajka et al., 2004; Kuperstein et al., 2005). In one study, for example, n-3 FA-deficient rat dams produced offspring with impaired dopaminergic regulatory systems (Kuperstein et al., 2008). Furthermore, perinatal n-3 FA has been linked to cognitive ability in many species, including mammals (Fedorova and Salem, 2006; Chung et al., 2008) and birds (Lamarre et al., 2021). For example, mice (Carrié et al., 2000) and rats (Chung et al., 2008) from mothers deficient in n-3 FAs had longer escape latencies in the Morris water maze test. Furthermore, red-legged partridges that were n-3 FA-deficient produced offspring with decreased discrimination ability (Fronte et al., 2008).

In birds, the maternal n-3 FA status influences offspring development through the egg nutrient content and, most importantly, the egg yolk (Cherian, 2011). The nutrient content of bird eggs varies, with altricial birds laying eggs with small dilute yolks compared to precocial birds, whose eggs have large yolks consisting of a high percentage of lipids and proteins (as reviewed in (Speake and Wood, 2005)). In precocial birds, such as chickens, the developmental period within the egg is when DHA accumulation in the brain and other tissues is the most significant (Speake, 2005). Facilitating brain development through dietary n-3 FAs may positively influence cognitive ability. Commercial chickens are fed cost-effective diets primarily composed of grains, including corn and soybean. However, these diets have a high n-6-to-n-3 FA ratio, resulting in eggs with n-6:n-3 FA ratios averaging between 9:1 (EU) and 19.5:1 (USA) (De Meester, 2008). These ratios exclude eggs from commercial hens fed n-3 FA-rich diets for omega-3 egg niche markets. The current recommendations for chicken breeder diets are based on optimizing production outcomes (i.e., poultry meat and egg) with least-cost diet formulations, where brain health and development are not considered. However, adequate levels of n-3 FAs in the egg may be necessary. The ideal ratio of n-6 to n-3 FAs in chicken eggs to promote embryonic brain development is unknown. Research shows that free-ranging chickens eating a wide variety of vegetation lay eggs with a ratio close to 1:1 (Simopoulos and Salem, 1989). Considering the importance of n-3 FAs in embryonic development, feeding potentially n-3 FA-deficient commercial diets to breeder chickens may have long-term negative implications for the behavior and welfare of their offspring.

To the best of our knowledge, only one study has examined the effect of maternal-fed n-3 FAs (from marine oils) on chicken brain size at hatching and did not show increased brain size in broiler chicken offspring from supplemented mothers (Ajuyah et al., 2003). Broiler chickens, raised for meat, and egg-laying chickens are phenotypically very different due to their selection for different production traits. Broiler chickens have been genetically selected for large appetites, fast growth, muscle gain, and feed efficiency to rapidly produce meat for human consumption (Bradshaw et al., 2002). On the other hand, egg-laying chickens have been selected for reproductive efficiency, producing many eggs with characteristics desirable for human consumption (Anderson et al., 2013). Selection for these traits has resulted in broiler and egg-laying chickens differing physically, behaviorally, and metabolically. For example, mothers of broiler chickens lay eggs with larger yolks and higher energy availability than layer breeders (Nangsuay et al., 2015). Broiler-chick embryos also grow faster and have larger organ weights at hatching than egg-layer chicks, likely due to their selection for growth and increased nutrient availability in the eggs (Nangsuay et al., 2015). In egg-laying chickens, there are differences between genetic strains, primarily brown *versus* white egg-laying phenotypes, which also typically correspond with feather color. Strain-dependent effects have been found for n-3 FA deposition in hatching eggs. Shaver White hens deposited a higher percentage of DHA into the phospholipid fraction of the egg yolk than ISA Brown hens, 40.8% *versus* 19%, respectively (Akbari Moghaddam Kakhki et al., 2020b). Akbari Moghaddam Kakhki et al. (2020b) also found that white-strain chick embryos utilized 11% more phospholipids during embryonic development, with chicks from breeders fed diets enriched with ALA utilizing less ALA and more DHA than chicks from control-fed breeders. These findings suggest that phylogeny affects FA metabolism in chickens.

This study aimed to investigate the effects of maternal diets supplemented with plant-based n-3 FAs on offspring brain size and brain FA composition using two different avian models. To accomplish this, we conducted two experiments. We fed one strain of broiler breeder (experiment 1) and two strains of layer breeders (experiment 2), one brown and one white egg-producing, diets supplemented with n-3 FAs and compared brain measures in their offspring with those of chicks from mothers fed standard commercial poultry diets. We hypothesized that mothers supplemented with n-3 FAs would produce offspring with altered brain FA composition and brain size.

2 Materials and methods

All animal use and procedures in this study were considered and approved by the University of Guelph Animal Care Committee and followed the Canadian Council on Animal Care guidelines (Animal Utilization Protocol #4246).

2.1 Breeders and diet

Two successive experiments were performed to assess the effect of maternal flaxseed diets on the brain size and n-3 FA composition of broiler (experiment 1) and layer (experiment 2) offspring.

In experiment 1, eight pens of Ross 708 broiler breeder hens (n = 213) were fed a control (commercial diet formulated for broiler breeders) *versus* n-3 FA-supplemented diet during rearing and a control or n-3 FA-supplemented diet during laying, resulting in a 2 × 2 factorial design. The n-3 FA-supplemented diets contained 2.57% (wt/wt%) of an ALA-rich co-extruded full-fat flaxseed product (LinPRO, O&T Farms, Regina, SK, Canada). The n-6:n-3 FA ratio of the control diet during the laying period was 27.6:1 compared to the flaxseed diet ratio of 4.2:1. These dietary treatments resulted in four rearing–laying maternal diet combinations (MDCs): control–control, control–flaxseed, flaxseed–control, and flaxseed–flaxseed. Eggs were collected for incubation from each MDC at 30 and 33 weeks of age (WoA).

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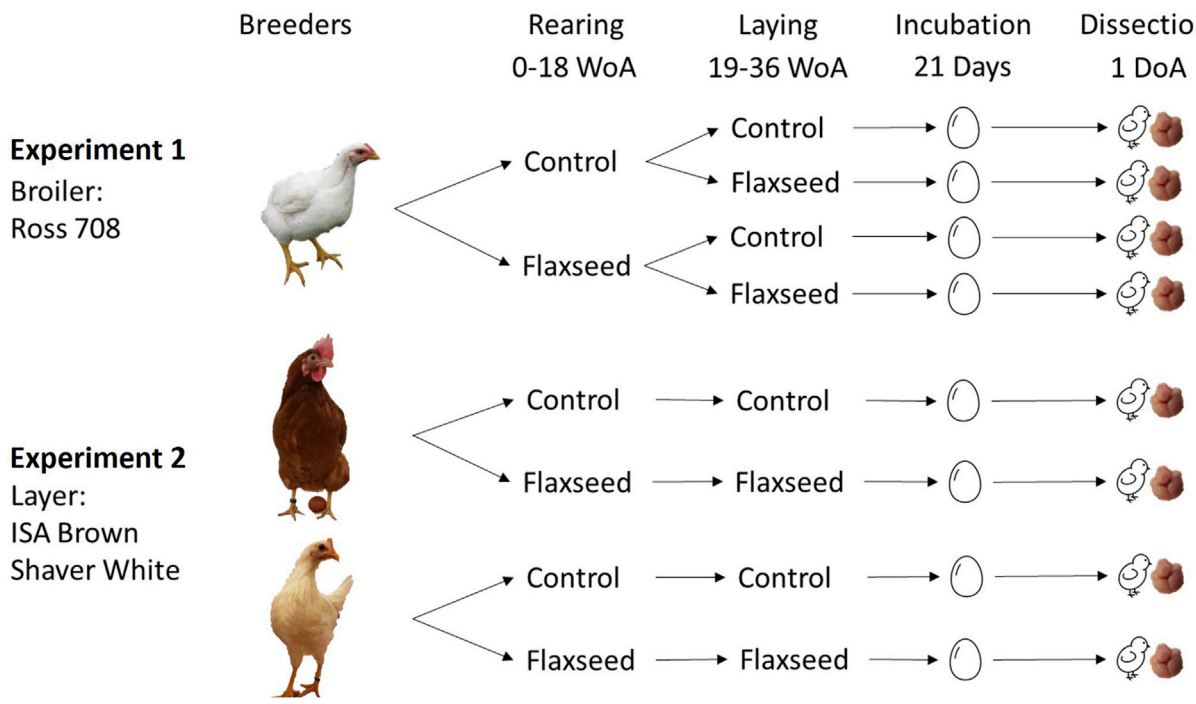

FIGURE 1
Experimental design for experiments 1 and 2. Experiment 1 used one strain of broiler breeder (Ross 708) fed control or flaxseed-supplemented diets in the rearing (0–18 WoA) and/or laying period (19–36 WoA), resulting in four rearing–laying diet combinations. Experiment 2 used two strains of layer breeders, one that produces brown-feathered offspring (ISA Brown) and one that produces white-feathered offspring (Shaver White), that were fed either control or flaxseed-supplemented diets during both the rearing and laying periods. Eggs were collected from the breeders at two different ages and incubated for 21 days. Upon hatching, unsexed chicks were euthanized for brain dissections.

TABLE 1 Yolk fatty acid analyses for eggs from broiler breeder hens fed either a control- or flaxseed-enriched diet during the rearing or laying period. Data are shown as % of total fatty acids. Significant pairwise comparisons ($p < 0.05$) between eggs laid by hens fed different rearing and laying diets are indicated with differing superscript letters.

Rearing diet Fatty acid↓/laying diet	Control Control	Flaxseed Control	Control Flaxseed	Flaxseed Flaxseed	Rearing	ANOVA Laying	Rearing*laying
Alpha-linolenic acid (C18:3 n-3)	1.14 ± 0.12^{a}	1.32 ± 0.13^{a}	2.20 ± 0.15^{b}	3.07 ± 0.05^{c}	***F = 13.18, p = 0.007***	***F = 94.40, p<0.001***	***F = 5.69, p<0.001***
Eicosatrienoic acid (C20:3 n-3)	0.08 ± 0.05	0.02 ± 0.00	0.08 ± 0.04	0.06 ± 0.01	*F = 1.16, p = 0.31*	*F = 0.31, p = 0.59*	*F = 0.31, p = 0.59*
Eicosapentaenoic acid (C20:5 n-3)	0.01 ± 0.004	0.03 ± 0.007	0.05 ± 0.003	0.08 ± 0.016	***F = 9.33, p = 0.02***	***F = 32.19, p<0.001***	*F = 0.19, p = 0.67*
Docosapentaenoic acid (C22:5 n-3)	0.18 ± 0.005	0.22 ± 0.019	0.32 ± 0.025	0.45 ± 0.077	*F = 2.74, p = 0.14*	***F = 13.00, p = 0.007***	*F = 0.77, p = 0.41*
Docosahexaenoic acid (C22:6 n-3)	0.87 ± 0.07^{a}	1.10 ± 0.02^{a}	1.46 ± 0.10^{b}	1.89 ± 0.03^{c}	***F = 44.96, p<0.001***	***F = 203.01, p<0.001***	***F = 5.31, p = 0.05***
Σ n-3	2.31 ± 0.15^{a}	2.69 ± 0.30^{a}	4.12 ± 0.24^{b}	5.55 ± 0.04^{c}	***F = 19.97, p = 0.002***	***F = 131.00, p<0.001***	***F = 6.62, p = 0.03***
Linoleic acid (C18:2 n-6)	25.70 ± 0.74	24.90 ± 0.39	23.66 ± 0.20	24.57 ± 0.70	*F = 0.01, p = 0.93*	*F = 3.08, p = 0.12*	*F = 1.59, p = 0.24*
Gamma-linolenic acid (C18:3 n-6)	0.55 ± 0.15	0.43 ± 0.07	0.40 ± 0.09	0.39 ± 0.36	*F = 0.28, p = 0.61*	*F = 0.59, p = 0.46*	*F = 0.18, p = 0.24*
Eicosadienoic acid (20:2 n-6)	0.55 ± 0.17	0.40 ± 0.06	0.43 ± 0.14	0.36 ± 0.06	*F = 0.69, p = 0.43*	*F = 0.39, p = 0.55*	*F = 0.09, p = 0.77*
Eicosatrienoic acid (20:3 n-6)	0.41 ± 0.19	0.22 ± 0.02	0.36 ± 0.13	0.22 ± 0.04	*F = 1.35, p = 0.28*	*F = 0.03, p = 0.86*	*F = 0.04, p = 0.84*
Arachidonic acid (20:4 n-6)	2.87 ± 0.09	2.68 ± 0.09	2.62 ± 0.14	2.31 ± 0.02	*F = 6.48, p = 0.06*	***F = 0.83, p = 0.03***	*F = 0.26, p = 0.63*
Docosadienoic acid (22:2 n-6)	0 ± 0	0.003 ± 0.003	0.04 ± 0.03	0 ± 0	*F = 0.83, p = 0.39*	*F = 0.83, p = 0.39*	*F = 1.17, p = 0.31*
Σ n-6	30.08 ± 0.81	28.64 ± 0.27	27.51 ± 0.25	27.86 ± 0.57	*F = 0.72, p = 0.42*	***F = 6.77, p = 0.03***	*F = 1.94, p = 0.20*
Σ n-6:Σ n-3	13.16 ± 0.67	10.78 ± 0.79	6.75 ± 0.40	5.02 ± 0.08	***F = 9.12, p = 0.02***	***F = 79.81, p<0.001***	*F = 0.22, p = 0.65*

Bold values represent significant values at $p \leq 0.05$.

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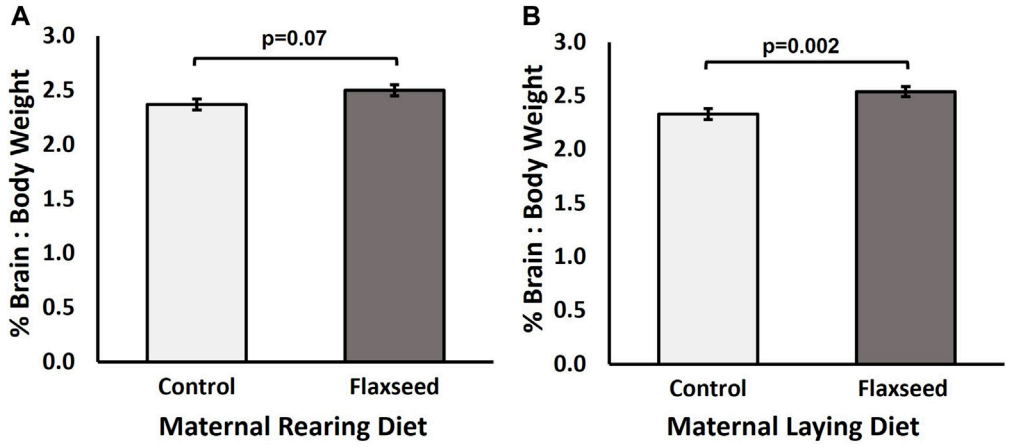

FIGURE 2
Experiment 1. Estimated mean (±SE) brain-to-body weight percentage [(brain/body weight) * 100] of day-old Ross 708 broiler chicks from breeders fed either a control or flaxseed diet during the rearing or laying period. **(A)** Maternal rearing diet tended to affect the brain-to-body weight percentage of the offspring at hatching (χ^2 = 3.29, p = 0.07). **(B)** Maternal laying diet significantly affected the brain-to-body weight percentage of the offspring at hatching (χ^2 = 9.22, p = 0.002).

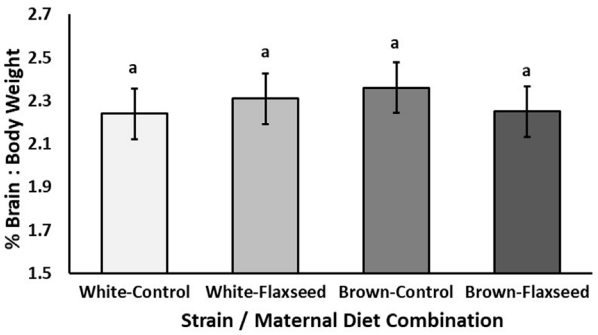

FIGURE 3
Experiment 2. Estimated mean (±SE) brain-to-body weight percentage [(brain/body weight) * 100] of day-old Shaver White and ISA Brown layer chicks from breeders fed either a control or flaxseed diet. The interaction between the strain and maternal diet tended to explain the brain-to-body weight percentage of the offspring (χ^2 = 3.65, p = 0.056), but there were no significant pairwise comparisons (p > 0.28).

In experiment 2, two strains of layer breeders, Shaver White (n = 192) and ISA Brown (n = 192), were raised in 16 pens (4 per strain/treatment) and fed either a control (commercial diet formulated for laying hens) or a flaxseed-supplemented diet using the same product as in the broiler breeder diets. The n-6:n3 FA ratio of the control diet during the laying period was 14.7:1 compared with 5.3:1 for the flaxseed diet. These treatments resulted in four strain and maternal diet combinations: brown–control, brown–flaxseed, white–control, and white-flaxseed. At 30 and 36 WoA, eggs from each strain and maternal diet combination were collected for incubation.

Figure 1 shows the experimental designs and the different diet combinations for broilers and layer breeders. All diets were isocaloric, isonitrogenous, and specifically formulated to only differ in fatty acid composition. Detailed ingredients and calculated nutrients for broiler and layer breeders, as well as breeder body weight, egg weight, and chick weight, are found in the study by Whittle et al. (2024). There was no reported effect of the maternal flaxseed diet on breeder body weight, egg weight, or chick hatching weight.

2.2 Egg yolk fatty acid analysis

In experiment 1, three eggs were collected from each broiler breeder parent pen at 28 WoA. The egg yolks were combined and homogenized to take a pooled sample from each pen. Thus, one homogenized sample from each parent pen was assayed. Samples were sent to Activation Laboratories Ltd. (Ancaster, ON, Canada) for fatty acid–total lipid analysis, according to O'Fallon et al. (2007). Yolk fatty acids are reported as the mean percentage composition and standard error of the mean. The yolk n-6-to-n-3 FA ratio was calculated as Σ n-6 FA divided by Σ n-3 FA.

Extensive fatty acid analyses of eggs from broiler breeders and layer breeders of the same strains and fed the same product and identical diet formulations as in the current study have been previously reported (Akbari Moghaddam Kakhki and Kiarie, 2021; Thanabalan, 2023).

2.3 Offspring brain collection

Live hatch weight was taken for 40 male and 40 female broiler chicks from each MDC (N = 80) in experiment 1 and 108 female layer chicks from each strain by maternal diet combination (N = 108) in experiment 2. The sample size was calculated to determine the number of individuals needed for dissections by comparing the sum of the degrees of freedom to the 3R reduction (minimum number) and refinement (optimal number) principles. The minimum number of individuals was used (reduction) whilst still allowing analyses of population variation (Eisenhauer, 2008; Pandey and Bright, 2008). Ten unsexed broiler chicks and six unsexed layer chicks from each parent pen and parental age were euthanized using

TABLE 2 Experiment 1. Brain fatty acid concentrations (μg/1 g) and composition (% of total fatty acids) from total lipid analysis. Brain tissue was sampled from the offspring of broiler breeder hens fed with and without flaxseed during the rearing or laying periods. Significant pairwise comparisons ($p < 0.05$) between maternal rearing and laying diets are indicated with different superscript letters.

Rearing diet Fatty acid↓/laying diet	Unit	Control Control	Flaxseed Control	Control Flaxseed	Flaxseed Flaxseed	ANOVA Rearing	ANOVA Laying	ANOVA Rearing*laying
Alpha-linolenic acid (C18: 3 n-3)	μg/1 g %	8.70 ± 2.29 0.11 ± 0.02^{ab}	8.44 ± 1.43 0.16 ± 0.02^{a}	5.55 ± 1.35 0.11 ± 0.03^{ab}	3.48 ± 1.12 0.06 ± 0.02^{b}	*F = 0.79, p = 0.38* *F = 0.11, p = 0.75*	***F = 5.86, p = 0.02*** ***F = 4.20, p = 0.05***	*F = 0.28, p = 0.60* ***F = 4.18, p = 0.05***
Stearidonic acid (C18:4 n-3)	μg/1 g %	4.88 ± 0.43 0.07 ± 0.01	6.25 ± 2.32 0.10 ± 0.03	5.78 ± 2.25 0.09 ± 0.03	9.00 ± 2.79 0.13 ± 0.07	*F = 1.00, p = 0.38* *F = 1.37, p = 0.26*	*F = 0.57, p = 0.46* *F = 0.89, p = 0.36*	*F = 0.14, p = 0.72* *F = 0.01, p = 0.92*
Eicosatrienoic acid (C20:3 n-3)	μg/1 g %	5.33 ± 0.77 0.07 ± 0.01	4.10 ± 0.32 0.08 ± 0.03	3.83 ± 0.40 0.07 ± 0.03	4.71 ± 1.17 0.07 ± 0.04	*F = 0.02, p = 0.89* *F = 0.03, p = 0.86*	*F = 0.17, p = 0.69* *F = 0.49, p = 0.49*	*F = 1.33, p = 0.71* *F = 0.06, p = 0.81*
Eicosapentaenoic acid (C20: 5 n-3)	μg/1 g %	16.06 ± 6.70 0.24 ± 0.10	5.26 ± 0.50 0.11 ± 0.45	4.97 ± 1.32 0.10 ± 0.02	8.77 ± 3.21 0.12 ± 0.04	*F = 0.65, p = 0.43* *F = 0.72, p = 0.86*	*F = 0.67, p = 0.42* *F = 1.00, p = 0.33*	*F = 3.27, p = 0.08* *F = 1.85, p = 0.19*
Docosapentaenoic acid (C22: 5 n-3)	μg/1 g %	133.74 ± 48.09 1.63 ± 0.45	74.23 ± 16.90 1.57 ± 0.44	72.54 ± 15.21 1.37 ± 0.35	67.16 ± 12.84 1.20 ± 0.29	*F = 1.37, p = 0.25* *F = 0.12, p = 0.74*	*F = 1.32, p = 0.26* *F = 0.58, p = 0.45*	*F = 0.92, p = 0.35* *F = 0.02, p = 0.89*
Docosahexaenoic acid (C22: 6 n-3)	μg/1 g %	872.64 ± 96.85 11.51 ± 0.56	683.52 ± 64.81 12.67 ± 0.57	658.11 ± 57.18 11.69 ± 0.71	810.61 ± 144.21 12.24 ± 0.63	*F = 0.01, p = 0.94* *F = 1.63, p = 0.22*	*F = 0.08, p = 0.79* *F = 0.05, p = 0.83*	*F = 2.12, p = 0.16* *F = 0.22, p = 0.64*
Σ n-3	μg/1 g %	1041.35 ± 134.03 12.63 ± 0.57	781.80 ± 64.81 14.68 ± 2.26	750.79 ± 61.05 13.44 ± 0.97	903.73 ± 144.21 13.82 ± 0.63	*F = 0.12, p = 0.73* *F = 0.61, p = 0.45*	*F = 0.30, p = 0.59* *F = 0.41, p = 0.53*	*F = 2.55, p = 0.13* *F = 0.12, p = 0.70*
Linoleic acid (C18:2 n-6)	μg/1 g %	157.60 ± 26.83 1.98 ± 0.10	118.63 ± 18.15 2.13 ± 0.14	113.64 ± 12.89 1.98 ± 0.08	124.05 ± 15.95 1.94 ± 0.04	*F = 0.46, p = 0.51* *F = 0.21, p = 0.65*	*F = 0.73, p = 0.40* *F = 0.99, p = 0.33*	*F = 1.43, p = 0.24* *F = 0.89, p = 0.36*
Gamma-linolenic acid (C18: 3 n-6)	μg/1 g %	0.91 ± 0.55 0.01 ± 0.007	0.88 ± 0.40 0.02 ± 0.007	0.68 ± 0.40 0.01 ± 0.007	1.75 ± 0.92 0.02 ± 0.01	*F = 0.64, p = 0.43* *F = 0.84, p = 0.37*	*F = 0.24, p = 0.63* *F = 0.17, p = 0.69*	*F = 0.56, p = 0.11* *F = 0.56, p = 0.46*
Eicosadienoic acid (20:2 n-6)	μg/1 g %	60.04 ± 8.74 0.77 ± 0.02	46.46 ± 6.81 0.83 ± 0.04	45.02 ± 5.26 0.78 ± 0.08	49.10 ± 5.91 0.77 ± 0.55	*F = 0.39, p = 0.54* *F = 0.64, p = 0.34*	*F = 0.58, p = 0.46* *F = 0.58, p = 0.46*	*F = 1.44, p = 0.24* *F = 1.47, p = 0.24*
Eicosatrienoic acid (20:3 n-6)	μg/1 g %	39.96 ± 3.87 0.53 ± 0.03	28.34 ± 2.25 0.53 ± 0.04	28.83 ± 3.64 0.50 ± 0.03	37.36 ± 7.97 0.55 ± 0.03	*F = 0.03, p = 0.87* *F = 0.59, p = 0.45*	*F = 0.01, p = 0.94* *F = 0.05, p = 0.83*	*F = 2.66, p = 0.12* *F = 0.42, p = 0.53*
Arachidonic acid (20:4 n-6)	μg/1 g %	656.29 ± 99.62 8.38 ± 0.29	485.33 ± 64.52 8.72 ± 0.22	496.53 ± 59.74 8.61 ± 0.18	551.80 ± 83.70 8.52 ± 0.24	*F = 0.37, p = 0.55* *F = 0.18, p = 0.68*	*F = 0.20, p = 0.66* *F = 0.01, p = 0.98*	*F = 1.66, p = 0.22* *F = 0.68, p = 0.42*
Docosadienoic acid (22:2 n-6)	μg/1 g %	65.22 ± 24.30 0.83 ± 0.23^{a}	92.52 ± 58.41 1.57 ± 0.88^{a}	91.19 ± 33.56 1.66 ± 0.60^{a}	26.52 ± 7.93 0.41 ± 0.13^{a}	*F = 0.41, p = 0.53* *F = 0.34, p = 0.56*	*F = 0.40, p = 0.53* *F = 0.17, p = 0.68*	*F = 1.58, p = 0.22* *F = 3.10, p = 0.09*
Adrenic acid (C22:4 n-6)	μg/1 g %	213.06 ± 37.58 2.68 ± 0.15	152.74 ± 27.47 2.65 ± 0.21	177.71 ± 23.27 3.13 ± 0.31	158.60 ± 13.00 2.16 ± 0.18	*F = 2.03, p = 0.17* *F = 1.41, p = 0.25*	*F = 0.23, p = 0.63* *F = 0.64, p = 0.43*	*F = 0.55, p = 0.47* *F = 1.07, p = 0.31*
Docosapentaenoic acid (C22: 5 n-6)	μg/1 g %	206.72 ± 71.22 2.43 ± 0.73	91.90 ± 24.89 1.48 ± 1.03	127.22 ± 32.65 2.14 ± 0.38	105.13 ± 23.08 1.90 ± 0.52	*F = 2.34, p = 0.14* *F = 1.00, p = 0.34*	*F = 0.46, p = 0.51* *F = 0.02, p = 0.88*	*F = 1.09, p = 0.31* *F = 0.40, p = 0.53*
Σ n-6	μg/1 g %	1399.80 ± 241.79 17.61 ± 0.96	1016.81 ± 159.85 17.93 ± 1.03	1080.83 ± 129.75 18.82 ± 0.38	1054.12 ± 126.12 16.74 ± 0.72	*F = 1.25, p = 0.28* *F = 1.06, p = 0.32*	*F = 0.51, p = 0.48* *F = 0.01, p = 0.94*	*F = 0.96, p = 0.34* *F = 1.62, p = 0.22*
Σ n-6:Σ n-3	μg/1 g %	1.32 ± 0.13 1.32 ± 0.13	1.27 ± 0.14 1.27 ± 0.14	1.46 ± 0.14 1.46 ± 0.14	1.25 ± 0.10 1.25 ± 0.10	*F = 0.94, p = 0.34* *F = 0.94, p = 0.34*	*F = 0.14, p = 0.71* *F = 0.14, p = 0.71*	*F = 0.36, p = 0.56* *F = 0.36, p = 0.56*

Bold values represent significant values at $p \leq 0.05$.

CO_2 for dissections (broiler N = 80 and layer N = 96). The whole body and brain weights were recorded. Dissected brains were placed in labeled sample bags and flash-frozen over dry ice within 10 min of euthanasia. The samples were transferred to a −20°C freezer until analysis. Brain size was calculated as the brain-to-body weight percentage (brain weight divided by body weight) multiplied by 100, for both experiments.

2.4 Offspring brain fatty acid analysis

For experiment 1, six chick brains were collected from each broiler breeder maternal diet combination balanced over breeder age (i.e., 30 and 33 WoA) and parental pen (N = 24). Similarly, for experiment 2, six brains were collected from each layer breeder strain and maternal diet combination balanced over breeder age (30 and 36 WoA) and parental pen (N = 24). For both experiments, brain fatty acids were analyzed by gas–liquid chromatography (Akbari Moghaddam Kakhki et al., 2020a). Each brain was homogenized in 1 mL of 0.1 M of KCl, and 100 μL of the homogenized brain was further diluted in 900 μL 0.1 M of KCl. A known concentration of C17:0 (50 μg) was added as an internal standard. Then, 4 mL of $CHCl_3$:MeOH (2:1) was added and vortexed, and tubes were flushed with N_2 and refrigerated at 4°C overnight. Samples were centrifuged at 357 *x g* (RCF) for 10 min, the chloroform layer was drawn off and dried down under a stream of N_2, and then, 2 mL of 0.5 M KOH in MeOH was added before being saponified at 100°C for 1 h. To the cooled samples, 2 mL of hexane

and 2 mL of 14% BF3-MeOH were added, and the samples were then methylated at 100°C for 1 h. Two mL of double-distilled water was added to the cooled samples to stop methylation. The samples were vortexed and centrifuged at 357 *x g* (RCF) for 10 min. The hexane layer was drawn off, dried under N_2, and reconstituted in 500 µL of hexane for gas chromatography analysis and compared to the known standard (C17:0 50 µg) (Akbari Moghaddam Kakhki et al., 2020a). The percentage of fatty acids in the brain was calculated by (FA area/total area) * 100. Brain FA concentrations were calculated by determining the µg of FAs in 100 µL of the sample (FA area * standard (50 µg))/area standard. Values were then used to calculate (µg of FAs in 100 µL of sample/brain weight in 1 mL of solution) µg of FAs per g brain tissue accounting for different amounts of brain tissue added at the beginning of the process. The n-6-to-n-3 FA ratio was calculated by summing the area under the curve of all n-6 FAs and then all n-3 FAs. The ratio was determined by dividing the sum µg of n-6 FAs by the sum µg of n-3 FAs.

2.5 Statistics

Statistical analyses were conducted in R v4.1.2 and R Studio using the "stats," "lme4," and "emmeans" packages. Broiler breeder yolk FA content was analyzed using two-way ANOVA with maternal rearing and maternal laying diet as fixed effects. Significant interactions between fixed effects were explored using *post hoc* Tukey's test. Brain-to-body weight ratio data were analyzed using linear mixed-effects models using the individual chick as the experimental unit. Maternal rearing and maternal laying diet were the fixed effects in experiment 1 for broiler offspring, and maternal diet and strain were the fixed effects in experiment 2 for layer offspring. Individual brain n-3 and n-6 fatty acids, Σ n-3 and Σ n-6 fatty acids, and n-6-to-n-3 FA ratio were analyzed with two-way ANOVA using maternal rearing and laying diet as fixed effects in experiment 1 for broiler offspring, and maternal diet and strain as fixed effects in experiment 2 for layer offspring and using the individual chick as the experimental unit. Significant interactions between main effects were analyzed using *post hoc* Tukey's tests using Bonferroni correction for multiple testing.

3 Results

3.1 Broiler breeder yolk fatty acids

There was an interaction between maternal rearing and maternal laying diet for ALA ($F = 5.69$, $p < 0.001$), DHA ($F = 5.31$, $p = 0.05$), and Σ n-3 ($F = 6.62$, $p = 0.03$) (Table 1). Eggs from hens fed flaxseed in both the rearing and laying periods deposited the highest ALA, DHA, and total n-3 FAs; control–control hens deposited the least. Significant pairwise comparisons between rearing and laying maternal diet are given in Table 1. The maternal laying diet significantly affected the percentage of Σ n-6 ($F = 6.77$, $p = 0.03$). Eggs from hens fed the control diet (29.36% ± 0.52%) during the laying period had a higher percentage of n-6 FAs than those fed flaxseed (27.68% ± 0.32%). There was a significant effect of rearing ($F = 9.13$, $p = 0.02$) and laying diet ($F = 79.81$, $p < 0.001$) on the n-6-to-n-3 FA ratio in egg yolks. The ratio of n-6 to n-3 FAs was lower in hens fed flaxseed in the rearing (7.89 ± 1.24) and laying period (5.88 ± 0.41) compared with those fed control diets in the rearing (9.96 ± 1.37) and laying period (11.97 ± 0.71). A complete list of results for broiler breeder yolk fatty acids is given in Table 1.

3.2 Brain size

Experiment 1: Broiler chicks from the flaxseed maternal rearing diet tended to have a larger brain-to-body weight percentage than chicks from the control maternal rearing diet treatment ($\chi^2 = 3.29$, $p = 0.07$; Figure 2A). The maternal laying diet significantly affected the brain-to-body weight percentage ($\chi^2 = 9.22$, $p = 0.002$) of the broiler offspring, with chicks from the flaxseed maternal laying diet having a larger brain-to-body weight percentage than chicks from the control maternal laying diet (Figure 2B). There was no interaction between maternal rearing and maternal laying diets ($\chi^2 = 0.35$, $p = 0.55$). The means and standard error for each MDC are as follows: control–control, 2.25% ± 0.06%; flaxseed–control, 2.41% ± 0.08%; control–flaxseed, 2.50% ± 0.06%; and flaxseed–flaxseed, 2.58% ± 0.05%.

Experiment 2: For layer chicks, there tended to be an interaction between the strain and maternal diet ($\chi^2 = 3.65$, $p = 0.056$) for the brain-to-body weight percentage of layer offspring. However, there were no significant pairwise comparisons (Figure 3; $p > 0.28$). The means and standard error for each strain and diet combination are Brown–control, 2.36% ± 0.04%, Brown–flaxseed, 2.22% ± 0.05%, White–control, 2.24% ± 0.04%, and White–flaxseed, 2.31% ± 0.04%. There was no effect of strain ($\chi^2 = 0.48$, $p = 0.49$) or maternal diet ($\chi^2 = 0.21$, $p = 0.65$) alone on the brain-to-body weight percentage of layer chicks.

3.3 Brain fatty acids

Experiment 1: Analysis of brain fatty acids showed a significant effect of the laying diet on the µg ALA/g brain in broiler offspring brains. Broiler offspring from the control laying diet had a significantly higher concentration of ALA in the brain than offspring from the flaxseed laying diet ($F = 5.86$, $p = 0.02$). There was also an interaction between maternal rearing and laying diet of the percentage of ALA in the brain ($F = 4.18$, $p = 0.05$). *Post hoc* analyses showed that offspring from the flaxseed–flaxseed maternal diet combination had a significantly lower percentage of ALA in the brain than offspring from the flaxseed–control maternal diet (CIL = −0.19, CIH = −0.004, $p = 0.04$). The maternal diet had no other significant effects on brain fatty acid concentrations or percentage composition. Complete brain fatty acid analysis for broiler offspring is given in Table 2.

Experiment 2: In the layer offspring, there was a significant effect of the maternal diet on the percentage of DHA ($F = 20.97$, $p < 0.001$), Σ n-3 ($F = 26.91$, $p < 0.001$), and Σ n-6 ($F = 79.20$, $p < 0.001$), with offspring from flaxseed-fed mothers having a higher percentage of DHA and Σ n-3, a lower percentage of Σ n-6, and a lower n-6:n-3 ratio. Table 3 shows that the maternal

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TABLE 3 Brain fatty acid concentrations (μg/1 g) and composition (% of total fatty acids) from total lipid analysis. Brain tissue was sampled from the offspring of ISA Brown and Shaver White layer breeder hens fed with and without flaxseed. Significant pairwise comparisons ($p < 0.05$) between strain and maternal diet treatment are indicated with different superscript letters.

Strain Fatty acid↓/maternal diet	Unit	Brown Control	Brown Flaxseed	White Control	White Flaxseed	Strain	*ANOVA* Diet	Strain*diet
Alpha-linolenic acid (C18:3 n-3)	μg/1 g %	7.03 ± 1.08 0.16 ± 0.02	8.60 ± 1.42 0.16 ± 0.01	6.93 ± 0.75 0.15 ± 0.02	8.83 ± 1.79 0.17 ± 0.04	*F = 0.00, p = 0.96* *F = 0.00, p = 0.98*	*F = 1.44, p = 0.22* *F = 0.36, p = 0.56*	*F = 0.01, p = 0.91* *F = 0.11, p = 0.74*
Stearidonic acid (C18:4 n-3)	μg/1 g %	3.09 ± 0.42 0.07 ± 0.005	3.97 ± 0.61 0.07 ± 0.008	3.34 ± 0.77 0.07 ± 0.013	3.56 ± 0.49 0.08 ± 0.008	*F = 0.01, p = 0.91* *F = 0.05, p = 0.84*	*F = 0.74, p = 0.40* *F = 0.19, p = 0.67*	*F = 0.26, p = 0.61* *F = 0.08, p = 0.78*
Eicosatrienoic acid (C20:3 n-3)	μg/1 g %	2.30 ± 0.47 0.05 ± 0.01	4.89 ± 1.08 0.09 ± 0.01	3.43 ± 0.77 0.07 ± 0.02	4.49 ± 0.31 0.09 ± 0.01	*F = 0.24, p = 0.63* *F = 0.48, p = 0.50*	***F = 5.97, p = 0.02*** ***F = 10.25, p = 0.004***	*F = 1.04, p = 0.32* *F = 0.67, p = 0.42*
Eicosapentaenoic acid (C20:5 n-3)	μg/1 g %	4.36 ± 0.23 0.10 ± 0.01[a]	6.41 ± 0.52 0.13 ± 0.01[b]	5.00 ± 0.83 0.10 ± 0.02[a]	7.99 ± 0.43 0.16 ± 0.01[c]	*F = 3.42, p = 0.08* ***F = 5.52, p = 0.03***	***F = 17.75, p<0.001*** ***F = 47.37, p<0.001***	*F = 0.61, p = 0.44* ***F = 5.68, p = 0.03***
Docosapentaenoic acid (C22:5 n-3)	μg/1 g %	18.20 ± 1.09 0.40 ± 0.02	26.71 ± 1.44 0.54 ± 0.04	23.57 ± 3.44 0.46 ± 0.02	25.97 ± 1.21 0.52 ± 0.02	*F = 1.08, p = 0.31* *F = 0.51, p = 0.48*	***F = 6.00, p = 0.02*** ***F = 13.27, p = 0.002***	*F = 1.98, p = 0.18* *F = 1.89, p = 0.19*
Docosahexaenoic acid (C22:6 n-3)	μg/1 g %	601.29 ± 28.18 13.24 ± 0.22	722.48 ± 71.64 14.06 ± 0.28	689.35 ± 96.56 13.40 ± 0.29	749.49 ± 26.34 14.92 ± 0.08	*F = 0.69, p = 0.42* *F = 4.00, p = 0.06*	*F = 1.72, p = 0.21* ***F = 20.97, p<0.001***	*F = 0.20, p = 0.66* *F = 1.88, p = 0.19*
Σ n-3	μg/1 g %	636.25 ± 29.04 14.01 ± 0.21	773.06 ± 76.09 15.05 ± 0.31	731.62 ± 101.43 14.24 ± 0.29	800.33 ± 29.33 15.92 ± 0.09	*F = 0.71, p = 0.41* *F = 4.36, p = 0.05*	*F = 1.98, p = 0.18* ***F = 26.91, p<0.001***	*F = 0.22, p = 0.65* *F = 1.88, p = 0.23*
Linoleic acid (C18:2 n-6)	μg/1 g %	90.16 ± 5.40 1.98 ± 0.04	112.37 ± 17.20 2.13 ± 0.17	105.23 ± 13.01 2.08 ± 0.07	102.97 ± 4.45 2.05 ± 0.05	*F = 0.05, p = 0.82* *F = 0.02, p = 0.88*	*F = 0.64, p = 0.43* *F = 0.97, p = 0.34*	*F = 0.97, p = 0.34* *F = 1.99, p = 0.17*
Gamma-linolenic acid (C18:3 n-6)	μg/1 g %	0.61 ± 0.35 0.01 ± 0.007	1.70 ± 0.39 0.03 ± 0.006	0.83 ± 0.34 0.02 ± 0.008	1.24 ± 0.36 0.02 ± 0.007	*F = 0.10, p = 0.76* *F = 0.00, p = 0.99*	*F = 3.59, p = 0.07* *F = 2.23, p = 0.15*	*F = 0.74, p = 0.40* *F = 1.09, p = 0.31*
Eicosadienoic acid (20:2 n-6)	μg/1 g %	38.62 ± 2.40 0.85 ± 0.02	42.52 ± 5.77 0.81 ± 0.02	41.34 ± 5.92 0.80 ± 0.03	38.79 ± 1.17 0.77 ± 0.01	*F = 0.01, p = 0.92* *F = 3.14, p = 0.09*	*F = 0.02, p = 0.89* *F = 1.84, p = 0.19*	*F = 0.46, p = 0.51* *F = 0.00, p = 0.97*
Eicosatrienoic acid (20:3 n-6)	μg/1 g %	25.90 ± 1.46 0.57 ± 0.01	33.99 ± 3.82 0.66 ± 0.03	22.29 ± 2.52 0.44 ± 0.01	26.06 ± 0.67 0.52 ± 0.01	***F = 4.72, p = 0.04*** ***F = 49.16, p<0.001***	***F = 4.98, p = 0.04*** ***F = 19.99, p<0.001***	*F = 0.66, p = 0.43* *F = 0.09, p = 0.77*
Arachidonic acid (20:4 n-6)	μg/1 g %	412.64 ± 24.95 9.04 ± 0.11	443.60 ± 52.79 8.54 ± 0.07	481.52 ± 59.98 9.46 ± 0.07	451.61 ± 14.06 9.00 ± 0.05	*F = 0.68, p = 0.42* ***F = 26.44, p<0.001***	*F = 0.00, p = 0.99* ***F = 32.30, p<0.001***	*F = 0.43, p = 0.52* *F = 0.06, p = 0.82*
Docosadienoic acid (22:2 n-6)	μg/1 g %	8.07 ± 0.22 0.18 ± 0.01	8.98 ± 1.51 0.17 ± 0.01	6.22 ± 0.92 0.13 ± 0.02	5.63 ± 0.61 0.11 ± 0.01	***F = 6.35, p = 0.02*** ***F = 10.93, p = 0.004***	*F = 0.02, p = 0.88* *F = 0.54, p = 0.47*	*F = 0.53, p = 0.48* *F = 0.01, p = 0.93*
Adrenic acid (C22:4 n-6)	μg/1 g %	115.85 ± 6.16 2.54 ± 0.03	120.68 ± 15.00 2.32 ± 0.03	123.86 ± 16.44 2.42 ± 0.03	110.69 ± 3.55 2.21 ± 0.03	*F = 0.01, p = 0.94* ***F = 15.12, p<0.001***	*F = 0.11, p = 0.75* ***F = 52.40, p<0.001***	*F = 0.50, p = 0.49* *F = 0.04, p = 0.84*
Docosapentaenoic acid (C22:5 n-6)	μg/1 g %	80.23 ± 7.42 1.75 ± 0.11	42.69 ± 5.42 0.82 ± 0.02	70.00 ± 7.79 1.40 ± 0.06	31.06 ± 1.85 0.62 ± 0.04	*F = 2.68, p = 0.12* ***F = 15.10, p<0.001***	***F = 32.80, p<0.001*** ***F = 144.0, p<0.001***	*F = 0.01, p = 0.92* *F = 1.19, p = 0.29*
Σ n-6	μg/1 g %	772.09 ± 46.33 16.92 ± 0.23	806.53 ± 101.19 14.48 ± 0.12	851.33 ± 104.83 16.74 ± 1.18	768.05 ± 23.21 15.30 ± 0.08	*F = 0.06, p=0.81* *F = 1.24, p = 0.28*	*F = 0.08, p = 0.78* ***F = 79.20, p<0.001***	*F = 0.48, p = 0.50* *F = 0.00, p = 1.00*
Σ n-6:Σ n-3	μg/1 g %	1.21 ± 0.03 1.21 ± 0.03	1.03 ± 0.02 1.03 ± 0.02	1.18 ± 0.03 1.18 ± 0.03	0.96 ± 0.01 0.96 ± 0.01	*F = 3.62, p = 0.07* *F = 3.62, p = 0.07*	***F = 55.12, p<0.001*** ***F = 55.12, p<0.001***	*F = 0.53, p = 0.48* *F = 0.53, p = 0.48*

Bold values represent significant values at $p \leq 0.05$.

flaxseed diet increased the concentration and percentage of n-3 FAs with a corresponding decrease in n-6 FAs in layer offspring brains.

4 Discussion

We used two avian models, commercial broiler and commercial layer chickens, to determine whether maternal n-3 FAs transfer to the egg and accumulate differentially in the brain tissue of offspring from mothers fed flaxseed-supplemented diets *versus* mothers fed the control diet. We hypothesized that maternal-fed n-3 FAs would alter offspring brain FA composition and brain size. Our hypotheses were partially supported in that supplementing the maternal diet with flaxseed significantly increased brain size in broiler offspring but not layer offspring. However, we found that a maternal flaxseed diet significantly increased the percentage of DHA and reduced the n-6-to-n-3 ratio in the brains of layer chicks but not broilers. The current study highlighted distinct differences in development and nutrient utilization between meat and egg-producing chickens, suggesting differences in how maternal-fed flaxseed diets influence the offspring brain.

Layer and broiler breeders have divergent phenotypes. Compared to layer strains selected for high egg production, broiler strains have been selected for fast growth and muscle development. The management of breeder flocks varies to accommodate these selection criteria. One key difference in the management is that layer breeders are fed *ad libitum*. In contrast, broiler breeders are fed a quantitatively restricted diet to maintain growth targets and ensure that breeders reach sexual maturity. For

this reason, it is logistically challenging to conduct an experiment on broiler and layer breeders simultaneously and in a manner that allows for direct comparisons.

The experimental design of these two experiments also differed. Broiler breeders were fed a flaxseed or control diet in the rearing and laying periods, so some breeders only experienced an n-3 FA diet during rearing or laying. The layer breeders, in comparison, were fed the n-3 FA or control diet throughout the experiment and experienced no switching of diets for the laying period. The decision to simplify the feeding regime in the layer breeder experiment was to account for using two strains of layer breeders. This difference in experimental design is another reason why the two experiments could not be directly compared.

There appeared to be value in feeding some broiler breeders flaxseed in only the rearing or laying period. The broiler breeder yolk FA analysis results suggest an accumulative effect of feeding n-3 FAs to breeders. The breeders fed flaxseed throughout rearing and laying deposited the highest percentage of DHA in the egg. We also showed that although broiler breeder diets were switched at 19 WoA, breeders fed n-3 FAs in the rearing period only still deposited a higher percentage of n-3 FAs in the egg at 30 WoA than those that were never fed flaxseed. This carry-over effect suggests that broiler breeder hens may deposit n-3 FAs from stores in their body for at least 10 weeks after switching to a control diet. This n-3 FA accumulation could add some cost-effective value to feeding flaxseed, where it may not be required to feed n-3 FAs consistently to maintain different n-3 FA profiles in the egg.

In broiler chicks, we found that maternal flaxseed rearing and maternal laying diet increased the brain-to-body weight ratio (larger brain size) in chicks. This finding supports our hypothesis that maternal-fed n-3 FAs would alter offspring brain size; however, the same result was not observed in layer offspring. These results contradicted those obtained by Ajuyah et al. (2003), who did not find differences in brain size due to feeding broiler breeders marine n-3 FAs. Research on neurogenesis in rats has shown negative impacts on brain size due to n-3 FA deficiency during development (Coti Bertrand et al., 2006). In this study, feeding broiler breeders diets supplemented with flaxseed, particularly in the laying diet, increased the brain-to-body weight ratio. This result is interesting because, across species, the brain-to-body weight ratio of birds correlates with cognition and intelligence (Lefebvre et al., 2004). In layer offspring, we found that a maternal flaxseed diet altered the brain n-6-to-n-3 FA ratio, increasing the concentration of n-3 FA and decreasing n-6 FA concentrations, thus supporting our hypothesis. The differences in brain size and n-6:n-3 FA ratio results between broiler and layer chick may be due to metabolic differences. Cherian (2015) summarized that early nutrition with n-3 FAs provides extra energy for developing embryos and could positively affect broiler health status and allow for resources to be allocated toward the development of the brain. Altering the brain characteristics of chicks could result in potential changes in behavior, gene expression, or neuroendocrine responses (Schmitz and Ecker, 2008; Calder, 2011; Vinot et al., 2011).

Corn and soybean commercial diets commonly fed to chickens are low in n-3 FAs. Diets of populations of feral chickens consist of a range of vegetation, seeds, insects, and human food waste, unlike the diets of commercially housed chickens (Ferrario et al., 2017). Populations of range-fed chickens in Greece, observed to eat fallen fruit, vegetation, and insects, produce eggs with an n-6-to-n-3 ratio of 1.30:1 (Simopoulos and Salem, 1989; De Meester, 2008). Additionally, the development of Columbus® eggs by Belovo (Sint-Eloois-Vijve, Belgium) heralded the production of commercial "wild-type" eggs with an n-6-to-n-3 ratio of 1.03:1 (De Meester, 2008). These ratios close to unity (1:1) are similar to those found in the yolks of many species of wild precocial birds (Surai and Speake, 2008). They could reflect the ideal ratios of n-6 to n-3 FAs required for developing chicken embryos. It has even been suggested that an n-6 to n-3 FA ratio of 1:1 is ideal for human brain health (Simopoulos, 2011). Species of wild precocial birds have been shown to have higher n-6-to-n-3 FA ratios in the egg than captive counterparts fed grain-based diets (Surai and Speake, 2008). Grain-based diets result in egg n-6-to-n-3 FA ratios of 6.8:1 for pheasants, 27.2:1 for partridges, and 10.7:1 for ducks compared to their wild counterparts having ratios of 0.4:1, 3.6:1, and 1.9:1, respectively (Surai and Speake, 2008). Our results could translate to other precocial birds bred in captivity that are fed grain-based diets, and the implications for offspring brain development may not be limited to commercial chickens.

Extensive analyses of broiler and layer breeder egg yolks conducted by Akbari Moghaddam Kakhki and Kiarie (2021) and Thanabalan (2023), respectively, showed that diet formulations enriched with flaxseed fed to the broiler and layer breeders altered yolk fatty acid profiles. More specifically, the same flaxseed diet fed to the same strain of broiler breeders at an identical n-6-to-n-3 FA ratio as in our study (4.17:1) resulted in a yolk ratio of 4.86:1 in the egg, compared with the control diet (26:1) resulting in a ratio of 15.6:1 in the egg (Thanabalan, 2023). The layer breeder diets in our study may not have resulted in a large enough difference in the yolk fatty acid ratios to yield differences in brain size between the flaxseed fed (n-6-to-n-3 FA ratio of 5.31:1) and control fed (n-6-to-n-3 FA ratio of 14.71:1) breeders. Using diets identical to those fed in the study reported here, Akbari Moghaddam Kakhki and Kiarie (2021) reported that ISA Brown breeders fed flaxseed produced eggs with an n-6-to-n-3 FA ratio of 3.42:1 compared with those fed the control diet producing eggs with a ratio of 5.23:1. Similarly, the Shaver White breeders fed flaxseed in that experiment produced eggs with an n-6-to-n-3 FA ratio of 2.64:1 compared to those fed the control diet producing eggs with a ratio of 3.40:1 (Akbari Moghaddam Kakhki and Kiarie, 2021). However, the layer offspring from mothers fed flaxseed did have an altered brain n-6:n-3 FA ratio, which was not observed in the broilers. Strain differences in the amount of n-3 FA deposited in the egg and embryo uptake and utilization of n-3 FAs have been found, with Shaver White embryos performing better than ISA Brown embryos (Akbari Moghaddam Kakhki and Kiarie, 2021). In this study, we also found that the maternal flaxseed diet tended to have an effect on the brain n-6:n-3 FA ratio between ISA Brown and Shaver White offspring. Therefore, observing differences between layer and broiler chickens would be unsurprising.

The current study adds to the scarce fundamental knowledge about n-3 FAs and chicken brain development. To date, research on relative brain size has focused on the effects of domestication or the effect of selection for tameness (Agnvall et al., 2017; Gjøen et al., 2023) and tends to not assess the effect of maternal nutrition. Feeding flaxseed to breeders increases broiler offspring brain size and alters the FA composition of layer offspring. These results suggest that egg- and meat-type chickens may utilize maternal-fed nutrients differently. Future research should assess the effects of maternal n-3 FA supplementation on the behavior of their offspring,

given that brain morphology and the composition of FAs can be influenced through maternal diets. These results might have important implications for chicken breeders fed diets typically low in n-3 FAs. However, broader implications may also exist for all captive bird species fed diets low in n-3 FAs that are not typical of the self-selecting diet of their wild counterparts.

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: Borealis, the Canadian Dataverse Repository https://doi.org/10.5683/SP3/WSZBAS.

Ethics statement

The animal study was approved by the Animal Care Committee, University of Guelph (Animal Utilization Protocol #4246). The study was conducted in accordance with the local legislation and institutional requirements.

Author contributions

RW: data curation, formal analysis, investigation, methodology, visualization, writing–original draft, and writing–review and editing. EK: conceptualization, funding acquisition, resources, supervision, and writing–review and editing. DM: investigation, methodology, supervision, and writing–review and editing. TW: conceptualization, funding acquisition, methodology, supervision, visualization, writing–original draft, and writing–review and editing.

Funding

The author(s) declare that financial support was received for the research, authorship, and/or publication of this article. This research was funded by the Ontario Agri-Food Innovation Alliance (grant number: 27320), Natural Sciences and Engineering Research Council of Canada-CRD Program (grant number: 401329), Egg Farmers of Canada (grant number: 053529), Egg Farmers of Ontario (grant number: 053445), Alltech Canada (grant number: 053530), and O&T Farms, Regina, SK, Canada (grant number: 053057). The funders were not involved in the study design, collection, analysis, interpretation of data, the writing of this article, or the decision to submit it for publication.

Acknowledgments

The authors acknowledge the contributions of the Arkell Poultry Research Staff for maintaining the breeder flocks throughout the experiment. The authors thank the helpers on this project, including Ana Rentsch, Linda Caston, Amanda Pufall, Anna Laszczuk, Joey Chan, Emily Croft, Em Brooker, Blossom Chen, Rachel Strassburger, and McKenna Lessard. The authors thank Lyn Hillyer for technical support during laboratory analyses and Dr Kiarie's graduate students for helping produce the experimental diets.

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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frontiers | Frontiers in Physiology

TYPE Brief Research Report
PUBLISHED 12 June 2024
DOI 10.3389/fphys.2024.1409211

OPEN ACCESS

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RECEIVED 29 March 2024
ACCEPTED 22 May 2024
PUBLISHED 12 June 2024

CITATION
Sulaiman U, Vaughan R, Siegel P, Liu D, Gilbert ER and Cline MA (2024), Oleuropein has hypophagic effects in broiler chicks.
Front. Physiol. 15:1409211.
doi: 10.3389/fphys.2024.1409211

Oleuropein has hypophagic effects in broiler chicks

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Oleuropein, a phenolic compound derived from olives, has known glucoregulatory effects in mammalian models but effects in birds are unknown. We investigated effects of dietary supplementation and exogenous administration of oleuropein on broiler chick feed intake and glucose homeostasis during the first 7 days post-hatch. One hundred and forty-eight day-of-hatch broiler chicks were randomly allocated to one of four dietary treatments with varying oleuropein concentrations (0, 250, 500, or 1,000 mg/kg). Body weight and breast muscle and liver weights were recorded on day 7. In the next experiment, chicks received intraperitoneal (IP) injections of oleuropein at doses of 0 (vehicle), 50, 100, or 200 mg/kg on day 4 post-hatch, with feed intake and blood glucose levels measured thereafter. Lastly, chicks fed a control diet were fasted and administered intracerebroventricular (ICV) injections of oleuropein at doses of 0, 50, 100, or 200 µg, after which feed intake was recorded. Results indicated that IP and ICV injections led to decreased feed intake, primarily at 60 min post-injection, with effects diminishing by 90 min in the IP study. Blood glucose levels decreased 1-h post-IP injection at higher oleuropein doses. These findings suggest that oleuropein acts as a mild appetite suppressant and influences energy metabolism in broiler chickens.

KEYWORDS

oleuropein, broiler, feed intake, dietary supplement, blood glucose

1 Introduction

Polyphenolic phytochemicals, naturally occurring metabolites found in plants, have garnered increasing attention due to their potential to elicit a wide range of beneficial physiological effects in both humans and animals, including anti-inflammatory, anti-oxidative, pro-gut health, and metabolic responses (Starčević et al., 2015; Shimao et al., 2019). As a result, plant extracts and plant-derived polyphenolic compounds have emerged as novel feed ingredients with the capacity to modulate growth and enhance overall health across several species. Given the phasing out of antibiotics at sub-therapeutic levels in poultry diets, identifying alternatives that exert health- and growth-promoting properties is important to the poultry industry and consumers of poultry products.

Oleuropein, a key component of extra virgin olive oil (EVO) with higher phytochemical content than pure olive oil (POO), has demonstrated health benefits in both human subjects and animals (Alkhatib et al., 2018). It was suggested that the beneficial metabolic effects of EVO, distinguished by its nearly identical fatty acid composition to POO, but substantially higher phenolic content, are in part due to its phenolic components (Bogani et al., 2007). Among these components, oleuropein, a non-toxic glycosylated seco-iridoid phenol extracted from olives, holds particular promise in modulating physiological processes or

functions. Oleuropein's abundance in olives and olive leaves, coupled with its high bioavailability, rapid absorption, and maximal plasma concentrations within 2 h after oral administration in humans, underscore its potential as a novel food ingredient (Omar, 2010).

Oleuropein's physiological impact has been investigated in several mammalian species including humans and mice, demonstrating a spectrum of benefits including antioxidant, antimicrobial, anticancer, hypoglycemic, hypolipidemic, anti-inflammatory, antiatherogenic, and antiviral effects (Omar, 2010; Haidari et al., 2021). In addition, oleuropein has shown the potential to prevent hepatic steatosis in rodent models (Park et al., 2011), a condition of particular concern in the poultry layer industry, where fatty liver disease affects laying hens (Lin et al., 2021).

In light of the potential benefits of oleuropein, this study seeks to investigate its effects on broiler chickens, specifically focusing on breast muscle and liver weight, as well as variations in feed intake and blood glucose levels. Our research aims to contribute insights into the physiological impacts of oleuropein in broilers, with potential implications for poultry production and animal welfare.

2 Methods

2.1 Animals

All animal protocols were approved by the Institutional Animal Care and Use Committee (IACUC) at Virginia Tech. One hundred and forty-eight Hubbard x Cobb-500 broiler chicks were obtained from a nearby commercial hatchery on the day of the hatch. Upon arrival at our facility, they were maintained at 30°C ± 2°C and 50% ± 5% relative humidity with free access to feed and water.

2.2 Experiment 1: dietary oleuropein supplementation

Forty-four chicks were assigned to one of four diets that contained oleuropein at 0, 250, 500, or 1,000 mg/kg. Dietary inclusion levels were based on a previous study involving plant compound supplementation, with a higher "high" dose to identify the effective dose range (Xiao et al., 2021). Oleuropein was purchased from Xi'An Yile Bio-Tech Company, China, and was tested for purity using high-performance liquid chromatography (HPLC, purity ≥90%).

Daily body weight data were collected for all broiler chicks. On day 7 post-hatch, breast muscle and liver weights were recorded. To assess organ weight relative to body weight, we computed the ratio of organ weight to the corresponding body weight. Data are reported for male chicks.

2.3 Experiment 2: peripheral oleuropein administration

Fifty-two day-of-hatch chicks were fed the control diet and on day 2 post-hatch, they were housed in individual cages that provided visual and auditory contact with other chicks and permitted measurement of individual feed intake. On day 4 post-hatch, 48 chicks (n = 12 per treatment) were randomly assigned to receive an intraperitoneal (IP) injection of one of four doses of oleuropein diluted in saline (0, 50, 100, or 200 mg/kg of body weight) and delivered in a volume of 150 µL with insulin syringes (BD Biosciences). The injection technique was described by Rice et al. (2014). Doses were based on previous studies with plant compounds, with a higher dose used to identify the effective dose range (Xiao et al., 2021). Feed was withheld before injection for 3 h. After injection, chicks were returned to individual cages, and feed intake was measured (to the nearest 0.1 g) in 30-min intervals for 90 min. Chicks were then euthanized by cervical dislocation, and sex was determined via gonadal inspection.

2.4 Experiment 3: central oleuropein administration

Housing and diets were the same as in Experiment 2. On day 4 post-hatch, 52 chicks were randomly assigned to receive an intracerebroventricular (ICV; in the left lateral ventricle of the brain) injection of one of four doses of oleuropein diluted in artificial cerebrospinal fluid (0, 50, 100, and 200 µg). Forty-eight chicks (n = 12 per treatment) were injected using a method adapted from Davis et al. (1979) that does not appear to induce physiological stress and is used routinely by our group. The heads of the chicks were briefly inserted into a restraining device that left the cranium exposed and allowed for freehand injection with a Hamilton syringe. Injection coordinates were 3 mm anterior to the coronal suture, 1 mm lateral from the sagittal suture, and 2 mm deep targeting the left lateral ventricle. Anatomical landmarks were determined visually and by palpation. Injection depth was controlled by placing a plastic tubing sheath over the needle. The needle remained at injection depth in the un-anesthetized chick for 5 s post-injection to reduce backflow. The total injection volume was 5 µL and contained 0.06% Evans Blue dye to facilitate injection site localization after the experiment. After injection, chicks were returned to their individual cages, and feed intake (to the nearest 0.1 g) was recorded in 30-min intervals for 90 min. After data collection, the chicks were decapitated and their heads sectioned along the frontal plane to determine the site of injection. Any chick without dye present in the lateral ventricle system was eliminated from the analysis. Sex was determined via gonadal inspection.

2.5 Experiment 4: blood glucose

On day 4 post-hatch, 40 chicks fed a standard starter diet were administered oleuropein intraperitoneally using four doses (0, 50, 100, and 200 mg/kg of body weight). Blood glucose levels were measured at 1-h post-injection using hand-held glucometers (Sumners et al., 2014).

2.6 Data analysis

Body weight gain, organ weight, blood glucose, and feed intake data were analyzed by ANOVA using the Fit Model platform of JMP Pro 16 (SAS Institute Inc., Cary, NC, 1989–2023). The model included the main effect of treatment within time and Tukey's test was used for *post hoc* pairwise comparisons and results with a p-value ≤0.05 were

TABLE 1 The effect of dietary oleuropein supplementation on body weight gain and organ weights at day 7 post-hatch in Hubbard × Cobb-500 broilers[a].

Oleuropein dose	Average daily weight gain to day 7 (g)	Liver weight as % of body weight	Breast muscle weight as % of body weight
0	29.54	4.00	11.60
250	30.38	4.01	11.88
500	28.83	3.78	12.22
1,000	32.80	3.93	12.82
SEM	1.63	0.13	0.13
p-value	0.34	0.57	0.22

[a]Values represent least squares means and pooled standard errors of the means with associated *p*-values for the effect of dietary treatment ($n = 10$/group; males). Treatments are dietary inclusion level of oleuropein (mg/kg).

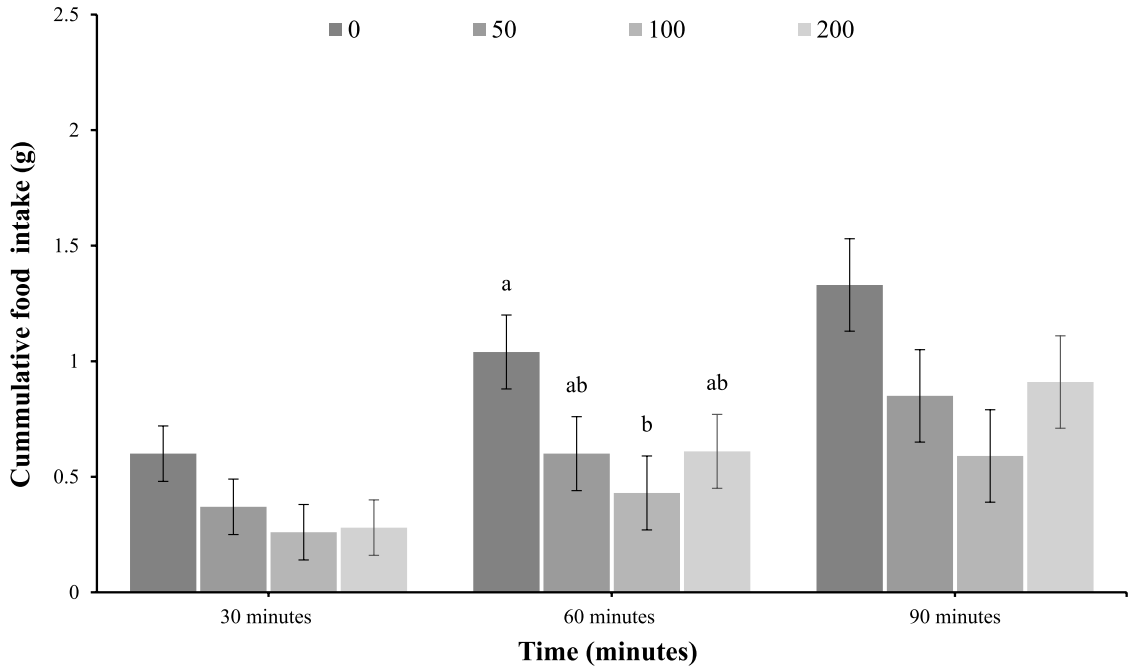

FIGURE 1
Feed intake of 4-day-old broiler chicks after intraperitoneal (IP) injection with 0 (vehicle), 50, 100, or 200 mg/kg of body weight oleuropein. Chicks were fed a standard starter diet *ad lib* after hatch ($n = 12$ per group; mixed sex). Different letters within each time indicate a difference at $p < 0.05$; Tukey's test (main effect of treatment at 60 min; $p = 0.05$).

considered significant. Initial ANOVAs for Experiments 2 and 3 included sex in the model; however, sex was not significant and removed as a variable from subsequent models.

3 Results

3.1 Growth performance and organ weights

There was no effect on body weight gain during the first-week post-hatch. Average daily gain throughout the experiment did not differ among groups (data not shown) and was not different at day 7 (Table 1). Similarly, breast muscle and liver were similar among treatment groups on day 7.

3.2 Feed intake after peripheral and central administration

As shown in Figure 1, feed intake was not different among IP-injected groups at 30 min ($p = 0.22$) but was reduced in response to the 100 mg/kg of oleuropein dose at 60 min (main effect of treatment; $p = 0.05$), and was not different at 90 min post-injection ($p = 0.11$).

In contrast, there was a reduction in feed intake in response to the highest ICV dose (200 mg/kg) at 30 min post-injection (main effect of treatment; $p = 0.02$), while the 100 and 200 mg/kg doses were effective at 60 (main effect; $p = 0.002$) and 90 (main effect; $p = 0.0005$) minutes (Figure 2).

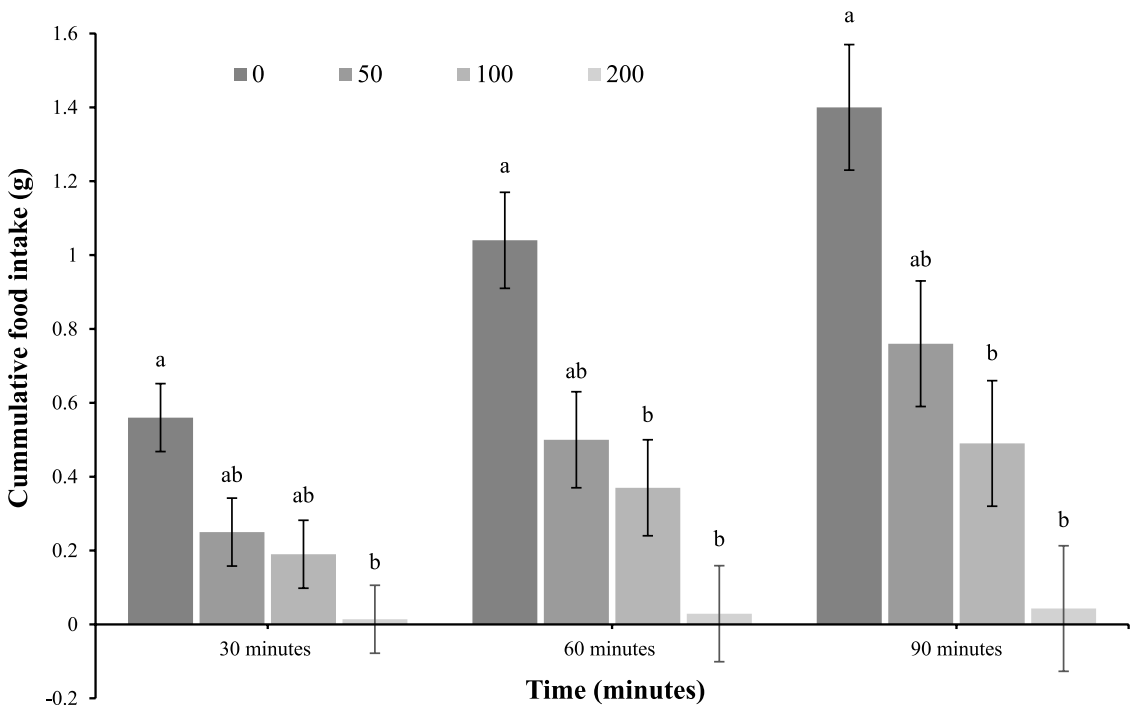

FIGURE 2
Feed intake of 4-day-old broiler chicks after intracerebroventricular (ICV) injection with 0 (vehicle), 50, 100, or 200 μg oleuropein. Chicks were fed a standard starter diet *ad lib* after hatch (n = 12 per group; mixed sex). Different letters within each time indicate a difference at $p < 0.05$; Tukey's test (main effect of treatment at each time point; $p < 0.05$).

3.3 Blood glucose

At 1-h post-IP injection, 100 and 200 mg/kg of oleuropein doses were efficacious (main effect; $p = 0.04$) at lowering blood glucose concentrations in chicks (Figure 3). The control and the 50 mg/kg of oleuropein dose values were similar.

4 Discussion

The objective of this experiment was to determine the effects of oleuropein on broiler chick growth and feed intake during the first week post-hatch. Body weight data were consistent with those of Shimao et al. (2019) who reported that dietary oleuropein supplementation did not impact final body weights at day 22 and that there was no difference in body weight gain and feed efficiency between days 8–22 in Ross 308 broilers. However, it is noteworthy that the liver weight was not affected by dietary oleuropein. This suggests that oleuropein's impact may not necessarily extend to the liver which is a repository for fat, particularly in older chickens.

Two experiments were designed to directly determine the effects of oleuropein on feed intake. Oleuropein was administered intraperitoneally and intracerebroventricularly because of variability in feed consumption (timing and amount) in an *ad lib* feeding setting. We injected oleuropein peripherally–a route that bypasses the effects of the gastrointestinal tract and can assess the contributions of peripheral factors to central appetite regulation in the animal. The chicks were fasted for 3 h prior to injection to intensify their hunger so that differences were more easily detected using fewer chicks. The hypophagic effects were relatively mild and delayed, with reductions observed in response to the low dose at 60 min post-injection.

Because of the peripheral route coupled with the time taken to observe an effect (>30 min), it is possible that effects on feed intake via the IP route were indirect, with oleuropein affecting some secondary pathway that caused the reduced feed intake. Also, it is possible that oleuropein is bio-transformed after IP injection (e.g., in the liver) and its metabolites exert an appetite regulatory effect. In humans, oleuropein is very bioavailable, however, it is metabolized to tyrosol and hydroxytyrosol in the blood or liver, which are excreted in the urine (Vissers et al., 2002). Thus, to determine whether oleuropein can exert direct effects on appetite, and to avoid confounds from peripheral injection, in the next experiment, oleuropein was intracerebroventricularly (ICV; in the left lateral ventricle of the brain) injected into the chick. Via this route, the ventricle system of the brain then delivers the oleuropein directly to the hypothalamus, the brain region primarily responsible for homeostatic feed intake. We found that when oleuropein was injected directly into the brain, feed intake was acutely suppressed within 30 min post-injection, with almost complete cessation in response to the highest dose. These findings support the notion that oleuropein directly influences appetite regulation in broilers. This is in contrast to the findings of Amini et al. (2019) who reported that dietary-supplemented olive leaf extract (OL) did not significantly affect feed intake across treatment groups. However, they reported that chickens receiving the 10,000 mg/kg OL ate less than those that received the 2,500 mg/kg OL dose. The contrasting results of Amini et al. (2019) can be attributed to differences in compound composition and purity (i.e., extract vs pure compound),

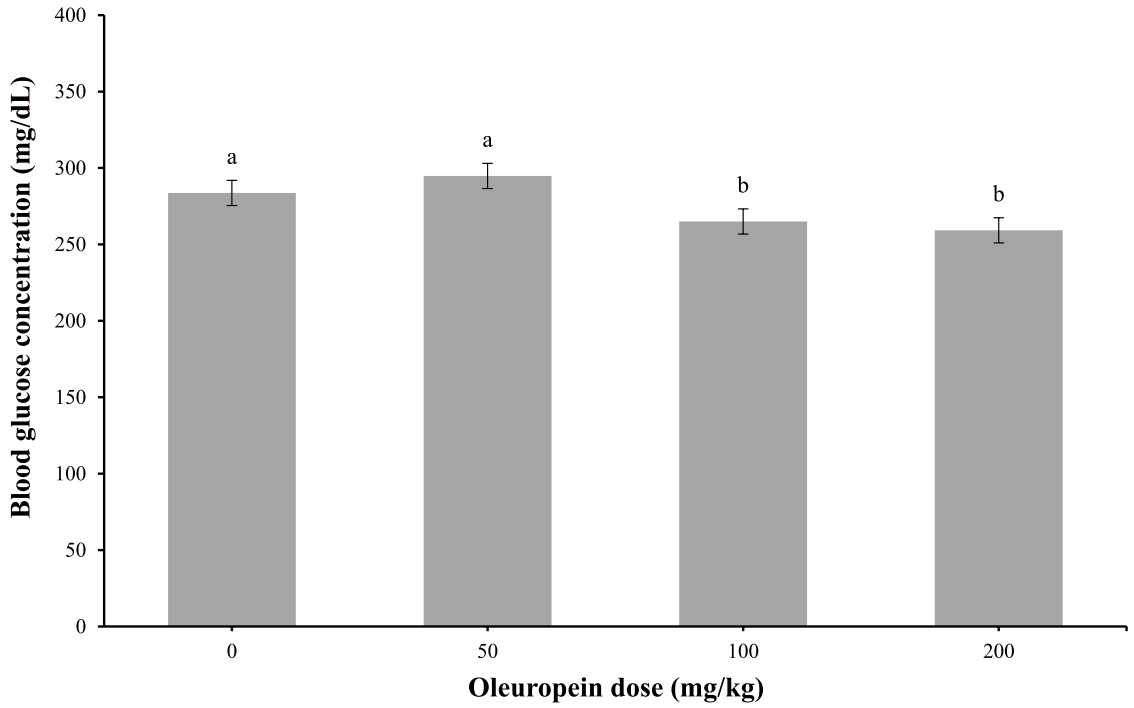

FIGURE 3
Whole blood glucose in fasted chicks at 1-h post-intraperitoneal injection with 0 (vehicle), 50, 100, or 200 mg/kg of body weight oleuropein. Chicks were fed a standard starter diet *ad lib* (n = 10 per group; mixed sex). Main effect of treatment, p = 0.0479. Different letters within each sampling measurement indicate a significant difference at $p < 0.05$; Tukey's test.

dosage levels, administration routes, and the potential variations in compound bioavailability and experimental conditions.

Comparing the two routes of administration, the percentage reduction in feed intake was more pronounced and immediate with ICV than IP injections. The ICV injections led to a nearly complete cessation of feed intake at the highest dose within 30 min, whereas the IP injections induced a milder reduction, primarily observed at 60 min post-injection. This suggests that the central administration of oleuropein has a stronger and faster impact on suppressing appetite compared to peripheral administration. The difference in effect could be due to the primary mode of action of the chemical (e.g., binding sites in central nervous system vs periphery) and whether the peripherally-injected chemical crosses the blood-brain barrier which may not be completely closed at the ages studied, and potential biotransformation of oleuropein in the liver as described above. That both routes induced a reduction in feed intake suggests that oleuropein affects appetite-regulatory pathways in the bird.

While the direct appetite-regulating effects of oleuropein are novel in any species, our glucoregulatory results align with the findings of Jung et al. (2019) who indicated a blood glucose-lowering and insulin sensitivity-improving effect of olive leaf extract in a mouse model. In our experiment, we observed that the two higher doses of oleuropein significantly lowered blood glucose concentrations in broiler chicks at 1-h post-injection. Importantly, this glucose-lowering effect was not a consequence of reduced feed intake, as the chicks were fasted before injection, and feed was withheld after injection. As chickens, like other avians, are inherently hyperglycemic with blood glucose levels that are particularly robust in response to fasting (Rice et al., 2014), the acute effect of oleuropein on blood glucose in broiler chicks is striking.

Although beneficial health effects are attributed to the consumption of olive oil and olive oil extracts, the chemicals that confer these bioactivities are still unclear and the mechanisms underlying effects on appetite and metabolism are also unknown. Thus, this research can further the use of oleuropein as a novel feed ingredient and dietary supplement across a range of species. In particular, this is the first report to our knowledge of a direct appetite-regulatory effect of oleuropein. Collectively, our results suggest that in chickens, oleuropein is a mild appetite suppressant that affects metabolic pathways related to glucose homeostasis, and thus may act to redirect energy during the post-absorptive state from the adipose tissue to the skeletal muscle, which should be addressed in the future studies. Further research is needed to elucidate the underlying mechanisms of the appetite- and glucose-regulatory effects and to optimize oleuropein supplementation strategies for practical application in poultry production.

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 the Virginia Tech Institutional Animal Care and Use Committee. The study was conducted in accordance with the local legislation and institutional requirements.

Author contributions

US: Conceptualization, Formal Analysis, Investigation, Methodology, Writing–original draft, Writing–review and editing. RV: Investigation, Methodology, Writing–review and editing. PS: Formal Analysis, Writing–review and editing. DL: Formal Analysis, Investigation, Resources, Writing–review and editing. EG: Conceptualization, Data curation, Funding acquisition, Investigation, Methodology, Project administration, Resources, Supervision, Validation, Visualization, Writing–review and editing. MC: Conceptualization, Formal Analysis, Funding acquisition, Investigation, Project administration, Resources, Supervision, Writing–review and editing.

Funding

The author(s) declare that financial support was received for the research, authorship, and/or publication of this article. This work was supported by The John Lee Pratt Endowment at Virginia Tech.

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.

The author(s) declared that they were an editorial board member of Frontiers, at the time of submission. This had no impact on the peer review process and the final decision.

Publisher's note

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frontiers | Frontiers in Physiology

TYPE Original Research
PUBLISHED 21 June 2024
DOI 10.3389/fphys.2024.1392968

OPEN ACCESS

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RECEIVED 28 February 2024
ACCEPTED 24 May 2024
PUBLISHED 21 June 2024

CITATION
Atallah E, Trehiou S, Alquier-Bacquie V, Lasserre F, Arroyo J, Molette C and Remignon H (2024), Development of hepatic steatosis in male and female mule ducks after respective force-feeding programs.
Front. Physiol. 15:1392968.
doi: 10.3389/fphys.2024.1392968

Development of hepatic steatosis in male and female mule ducks after respective force-feeding programs

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Male and female mule ducks were subjected to a force-feeding diet to induce liver steatosis as it is generally done only with male ducks for the production of foie gras. The different biochemical measurements indicated that the course of hepatic steatosis development was present in both sexes and associated with a huge increase in liver weight mainly due to the synthesis and accumulation of lipids in hepatocytes. In livers of male and female ducks, this lipid accumulation was associated with oxidative stress and hypoxia. However, certain specific modifications (kinetics of lipid droplet development and hepatic inflammation) indicate that female ducks may tolerate force-feeding less well, at least at the hepatic level. This is in contradiction with what is generally reported concerning hepatic steatosis induced by dietary disturbances in mammals but could be explained by the very specific conditions imposed by force-feeding. Despite this, force-feeding female ducks seems entirely feasible, provided that the final quality of the product is as good as that of the male ducks, which will remain to be demonstrated in future studies.

KEYWORDS

fatty liver, cellular stress, mule duck, sexual dimorphism, liver steatosis

Introduction

Foie gras is a typically renowned French dish and is part of French gastronomy included on the UNESCO's list of intangible heritage of humanity since 2010. It comes from the force-feeding of waterfowls, mainly ducks. Force-feeding is carried out over a short period (around 10 days) but includes very large quantities of a very caloric meal, almost exclusively composed of corn, which quickly induces hepatic steatosis. Indeed, under these conditions, the liver of birds quickly directs its metabolism toward a very intense production of lipids which are only partially exported and therefore accumulated in the hepatocytes (Heraut et al., 2010; Bax et al., 2012; Bonnefont et al., 2019; Lo et al., 2020). The result of the force-feeding of male ducks is similar, at least at the hepatic level, to what is also described in humans for non-alcoholic fatty liver disease (NAFLD) and/or non-alcoholic steatohepatitis (NASH) (Raza et al., 2021).

For the production of duck foie gras, only male ducks are generally used due to the sexual dimorphism that exists in birds (Brun et al., 2015): male ducks are larger than female

ducks and are therefore easier to force-feed. Male ducks are also said to be calmer than female ducks and therefore easier to handle during periods of rearing and force-feeding (Basso et al., 2014). In addition, a significant portion (20%–40%) of female ducks has a liver with a large and superficial venous network, which ultimately makes the product unattractive to consumers (Marie-Etancelin et al., 2015). The origin of this visual defect is not yet documented even if it seems to be of genetic origin and independent of the force-feeding operations themselves.

In recent years, global poultry production has been faced with numerous successive avian influenza crises which have had a significant impact on production and breeding flocks (Spackman, 2020). Among all the consequences of this massive destruction of several million wild and domestic birds, the reduced availability of male ducks for the production of foie gras has sparked interest in the possible substitute use of female ducks. However, until now, no specific information has been published on possible differences in the development of steatotic liver in male or female ducks, while it has been widely documented in mammals of various species (Lornardo et al., 2019; Lefebvre and Staels, 2021).

The aim of this article was therefore to describe separately the evolution of hepatic steatosis in male and female ducks subjected to two different force-feeding programs adapted to their original body sexual dimorphism.

Material and methods

Animals and liver sampling

A flock of approximately 1,000, 50% males + 50% females, mule ducks (*Caïrina moschata* x *Anas platyrhynchos*) was reared, on the same farm, for 12 weeks, from hatching, according to usual commercial rules. At 12 weeks of age, 12 birds from both sexes (live weights similar to the average live weight of their respective breeding flocks) were randomly selected and slaughtered to constitute the group of ducks (0 meal) before the start of force-feeding. At each stage of sampling, venous livers (exclusively seen in female ducks at a percentage of 20%–30%) were discarded to retain only the non-venous livers from both sexes. The remaining birds were subjected to two different gender-specific force-feeding programs of 21 meals (twice daily for 10 days) using moistened corn flour (97.5% corn) on independent farms and poultry houses. At the start of the force-feeding period, male and female ducks received an amount of 225 g of dry flour/meal, which was gradually, but differently, increased in both sexes to reach a final value of 510 g and 480 g for the last meal in male and female ducks, respectively. In total, during the entire force-feeding period, male and female ducks were forced to ingest different quantities of feed (8.4 kg and 8.0 kg, respectively) depending on their different body sizes. Therefore, this experiment is not a true comparison of the effect of a given force-feeding program on the development of fatty livers in male and female ducks. Rather, it is an analysis of the development of hepatic steatosis in male and female ducks in response to two force-feeding programs best suited to their respective sizes. After 8 and 16 meals of force-feeding, 12 animals of both sexes were randomly selected (in the same manner as described above for ducks at 0 meals) and slaughtered to constitute the 8- (8 meals of force-feeding) and 16-meal groups (16 meals of force-feeding). The remaining birds continued the force-feeding programs for five additional meals. At this time, all the birds were slaughtered, and 24 of them (12 males and 12 females) were selected to constitute the 21-meal group (end of the force-feeding period). At this final stage, because the data provided by the slaughterhouse allowed it, the selection of livers after 21 force-feeding meals was carried out according to the average weights of the livers observed for each sex in the remaining birds (mean ± SD = 566 g ± 93 and 517 g ± 92 for males and females, respectively). At each stage (0, 8, 16, and 21 meals), the ducks were slaughtered 12 h h after the last meal in a commercial slaughterhouse according to its standardized slaughter operations (electronarcosis, bleeding, scalding, and plucking). At the end of the slaughtering line, 20 min *post mortem*, the livers were harvested and weighed. Then, 50 g of tissue was collected from the median lobe and directly frozen in liquid nitrogen before storage at -80°C. Another piece of the liver was also collected from the same location and stored in paraformaldehyde (4%) buffer for histological observations.

All biochemical measures were performed in duplicate after grinding the tissues in liquid nitrogen.

Gross biochemical composition of livers

The dry matter (DM) content was determined by drying the ground liver in an oven at 105°C for 24 h. The total lipid content was measured according to Folch et al. (1957) after extraction with chloroform:methanol (2:1). The total protein content was determined according to the procedure described by the manufacturer (Pierce™ BCA Protein Assay Kit) after an extraction with a phosphate-buffered saline solution. The hydroxyproline (OH-Pro) content was determined according to Woessner (1961) on the delipidated and dry residue obtained after the extraction of the total lipids.

Oxidative status

GSH/GSSG analysis: The reduced glutathione/oxidized glutathione (GSH/GSSG) ratio was determined according to the protocol described by the manufacturer (catalog #: 239709, Abcam, Cambridge, United Kingdom).

The activities of the enzymes superoxide dismutase (SOD, catalog #: 19160, Sigma, St Louis, MO, United States) and catalase (Cat, catalog #: KB03012, BioQuoChem, Llanera-Asturias, Spain) were determined according to the procedures described by the manufacturers.

The results are expressed in U/mg of proteins.

ELISA tests

The contents of hypoxia-inducible factor 1 alpha (HIF1α) and hypoxia-inducible factor 2 alpha (HIF2α) were determined with ELISA tests on the proteins extracted from livers by using assay kits from MyBioSource (San Diego, CA, United States) according to the manufacturer's protocols.

Results are expressed in pg/mg of proteins.

Histology

Paraformaldehyde-fixed and paraffin-embedded liver tissue sections (3 µm) were stained with hematoxylin and eosin (H&E) for histopathological analysis. The stained liver sections were analyzed (magnification ×100) blindly according to a score ranging from 0 = no visible lipid droplets, 1 = only small lipid droplets, 2 = majority of small lipid droplets, 3 = majority of large lipid droplets, to 4 = almost only large lipid droplets in hepatocytes. Mean score values were obtained from three independent, trained observers.

Gene expression

Total cellular RNA was extracted from liver samples using the TRI reagent (Molecular Research Center Inc., Cincinnati, Ohio, United States). RNA was quantified using a NanoPhotometer (N60, Implen). Total RNA samples (2 µg) were reverse-transcribed using the High-capacity cDNA Reverse Transcription Kit (Applied Biosystems, Foster City, California, United States) for real-time quantitative polymerase chain reaction (qPCR) analyses. Primers were designed in two consecutive exons to avoid amplification of genomic DNA using PrimerQuest™ Tool (Integrated DNA Technologies, Coralville, Iowa, USA), and primers for SYBR Green assays are presented in Supplementary Table S1. Amplifications were performed on the AriaMx Real-time PCR System (Agilent, Santa Clara, California, United States). RT-qPCR data were normalized to the level of the GAPDH (glyceraldehyde-3 phosphate dehydrogenase) messenger RNA (mRNA) and analyzed by LinRegPCR (v2021.2). This program determines the PCR efficiency per sample and accounts for it in a linear regression approach to correct the cycle threshold value for mRNA level quantification. The initial concentration (N0) for each sample is calculated using N0 = threshold/(Effmean x Cq), with Effmean representing the mean PCR efficiency and Cq representing the quantification cycle.

For each analyzed transcript, the value at the beginning of the force-feeding period was set to 1, in each sex, to facilitate the comparisons.

Statistics

Statistical analyses were performed with SAS software, version 9.4, of the SAS System for Windows. Analysis of variance were performed with the general linear model (Proc GLM) completed with the Student–Newman–Keuls *post hoc* test to compare the means obtained after each meals in each of the two groups (sexes) independently. Where necessary, to satisfy normality and homoscedasticity conditions, variables were transformed before analysis (Log_2 for RT-PCR and OH-Pro analysis). The percentages of the respective scores from the histological analysis were compared independently for each gender according to Fischer's exact test. Values are expressed as the means ± standard deviation (SD). We set the significant level at $p < 0.05$.

Results and discussion

Throughout the experiment, total mortality (number of animals dead at the end of the rearing–feeding periods/number of animals at the beginning) was less than 1.5% in both sexes, indicating that almost all ducks were able to withstand the rearing conditions imposed. As expected, the force-feeding programs induced a huge increase in liver weight (Table 1). If, at the beginning of the force-feeding period, the weight of the liver of all the birds was approximately 125 g, nevertheless with a slightly higher value (+2.3%) in male ducks, after 21 meals, the weight of the livers increased by 4.51 and 4.23 in male and female ducks, respectively. These values were identical to those expected, at least in male ducks, for which references under close force-feeding conditions are available (Gabarrou et al., 1996, 695 g for 12.5 d of force-feeding; Bax et al., 2012, 660 g for 12 d of force-feeding; and Bonnefont et al., 2019, 600 g for 10 d of force-feeding). In both sexes, lipid levels increased (from 5.0% to 5.3% at meal 0 in males and females, respectively, to 58.3% and 60.5% at meal 21), while protein levels decreased throughout the overfeeding period. The percentage of lipids was then multiplied by more than 11 between 0 and 21 forced meals in both sexes. This indicates a spectacular accumulation of lipids and, therefore, the development of clear hepatic steatosis. This is due to the rapid transformation by the liver of the large quantities of carbohydrates provided by the successive corn meals (containing 62% starch) imposed by the force-feeding programs. It must therefore be concluded that male and female ducks both retain this capacity to transform sugars from the diet into lipids, which accumulate in the liver during the force-feeding period. This ability to accumulate lipids under these particular conditions has already been described in several experiments conducted with male ducks (Herault et al., 2010; Lo et al., 2020; Pioche et al., 2020; Tavernier et al., 2020). The liver's hydroxyproline (OH-Pro) content reflects its capacity to develop connective tissues, and this indicator has previously been used (Arai et al., 2022; Montefusco et al., 2022) to illustrate the shift from the simple steatosis associated with non-alcoholic fatty liver disease (NAFLD) to non-alcoholic steatohepatitis (NASH). In mice, an increase in the liver's OH-Pro content was reported by Matsumoto et al. (2013) and Hartimath et al. (2019) and considered a sign of the development of fibrosis characteristic of the NASH condition. In the present experiment, we report a significant increase (+179% and +236%, $p < 0.05$, in males and females, respectively) in the OH-Pro contents of livers only between 16 and 21 meals of force-feeding. This indicates that only at the very end of the force-feeding period, a fibrogenesis process could take place in both sexes. This is, however, contradictory to what had previously been reported in male ducks by Remignon and Burgue (2023), who had not observed such an increase. However, this last observation was the first published in force-fed ducks, and it is therefore difficult to recognize whether the current results are atypical or not, even if the presence of hepatic inflammation attested by an increase in its fibrogenesis seems rather logical in light of what is generally observed in severe cases of hepatitis steatosis in mammals (Schuppan et al., 2018; Schwabe et al., 2020).

Histological observations (Figure 1) confirm the biochemical measurements regarding lipid accumulation in hepatocytes. In both sexes, lipids were observed to accumulate in hepatocytes in the form of increasingly present and larger droplets throughout the force-

TABLE 1 Chemical composition of the livers according to the sex of the mule ducks and the number of meals during the force-feeding period (n = 12/sex/stage). Values are the means ± SD.

Gender	Parameter	0 meal	8 meals	16 meals	21 meals	P[3] <
Male	Liver weight (g)	127^{a} ± 15	276^{b} ± 25	426^{c} ± 65	573^{d} ± 14	0.0001
	Humidity[1]	66.9^{a} ± 0.8	50.7^{b} ± 2.6	36.1^{c} ± 2.3	31.7^{d} ± 2.3	0.0001
	Lipids[1]	5.0^{d} ± 0.8	31.6^{c} ± 3.0	50.7^{b} ± 2.2	58.3^{a} ± 4.9	0.0001
	Proteins[1]	7.9^{a} ± 1.2	5.9^{b} ± 0.7	4.4^{c} ± 0.9	3.4^{d} ± 0.3	0.0001
	OH-Pro[2]	160^{b} ± 22	104^{b} ± 8	144^{b} ± 19	258^{a} ± 41	0,0016
Female	Liver weight (g)	124^{a} ± 21	257^{b} ± 25	422^{c} ± 56	525^{d} ± 17	0.0001
	Humidity[1]	67.1^{a} ± 1.0	48.3^{b} ± 2.0	37.0^{c} ± 2.2	28.8^{d} ± 1.9	0.0001
	Lipids[1]	5.3^{d} ± 1.3	35.3^{c} ± 4.7	51.0^{b} ± 6.3	60.5^{a} ± 3.5	0.0001
	Proteins[1]	9.4^{a} ± 2.9	7.2^{b} ± 0.9	5.0^{c} ± 1.1	3.1^{d} ± 0.4	0.0001
	OH-Pro[2]	189^{b} ± 32	139^{b} ± 11	177^{b} ± 16	417^{a} ± 60	0.0001

Means with the same superscripts are not different ($p < 0.05$). Within a line, mean values with different superscripts are different ($p < 0.05$).
[1]: % of raw liver.
[2]: Hydroxyproline (OH-Pro) content in mg/g of delipidated and dry liver.
[3]: p-values are related to the effect of the number of meals.

feeding period. However, this accumulation seems to be more gradual and less intense in female ducks. At the final stage (after 21 meals of force-feeding), female ducks still presented a low percentage (10%) of hepatocytes with a majority of small droplets (score 2), while male ducks presented only liver cells with a majority of large lipid droplets (score 3 = 90%) or with almost only large lipid droplets (score 4 = 10%). In mice subjected for long periods to unbalanced diets (high-fat, Western, cafeteria, and fructose-enriched diets), male ducks were also found to be more susceptible to hepatic steatosis and had higher histological scores values than female ducks (Spruss et al., 2012; Gasparin et al., 2018; Smati et al., 2022). According to these authors, the prevention of the development hepatic steatosis development in female ducks could be linked to their particular hormonal status, which could also be the case in female ducks submitted to force-feeding (Tramunt et al., 2021).

Corn force-feeding, containing more than 60% starch, induces significant intakes of carbohydrates which mainly arrive in the liver, where they are transformed into lipids. These lipids are initially dedicated to export by lipoproteins (Hermier et al., 2003; Tavernier et al., 2018). However, due to the very large quantities of lipids synthetized, the storage capacity of peripheral tissues, mainly adipose tissues, is quickly exceeded, leading to lipid accumulation in the liver itself. There is therefore an enormous accumulation of complex lipids and free fatty acids in the hepatocytes. This abnormal accumulation of lipids has been described in mammals (Chen et al., 2019) as favoring lipotoxic species such as reactive oxygen species (ROS), which are highly toxic to the cell. However, cells have different mechanisms for neutralizing these ROS, among which the coupling activity of the enzymes superoxide dismutase (SOD) and catalase (Cat) is very effective (Perlemuter et al., 2005) in neutralizing the superoxide ion O_2^-, one of the most common ROS. In the present study, SOD activities increased rapidly from eight meals and beyond throughout the force-feeding period in both sexes (Figure 2). On the contrary, Cat activities decrease slightly during the same period in both sexes. An increase in SOD and Cat activities has often been described under different conditions (Zeng et al., 2014; Liu et al., 2015; Du et al., 2017; Zhang et al., 2017; Remignon and Burgues, 2023) to be associated to fight against oxidative stress due to the accumulation of lipids in hepatocytes. However, in mice fed a cafeteria diet, Gasparin et al. (2018) reported decreased Cat activity in the liver and indicated that this suggests impaired H_2O_2 neutralization that could contribute to lipid peroxidation. In the present study, this possible accumulation of H_2O_2 is highlighted by the decrease in the CAT/SOD ratio (Figure 2) during the force-feeding period in both sexes.

Other enzymatic antioxidant systems may act to catalyze the reduction of H_2O_2 to H_2O, as is primarily the case of the glutathione system (for review, see Engin, 2017). Reduced glutathione (GSH) has been described (Forman et al., 2009) to play a very effective role in maintaining redox homeostasis and, therefore, protects cells from oxidative damage (Sahoo et al., 2017). Associated with the action of specific peroxidases, the coupling activities of reduced and oxidized glutathione (GSSG) represent the most powerful cellular antioxidant system: the decrease in the GSSG/GSH ratio is therefore considered a major sign of cellular dysfunctions (Chen et al., 2013; Alkazemi et al., 2021). In the present study (Figure 3), the levels of GSH and GSSG in the liver decreased during the force-feeding period in both sexes. However, the GSSG/GSH ratio remained constant during this period, indicating that the renewal mechanisms of this powerful antioxidant continue to be functional. When large quantities of lipids are synthetized rapidly in hepatocytes, as observed during the development of hepatic steatosis, this also induces an increase in the activity of the ß-oxidation pathway and consequently a great leak of electrons which are at the origin of ROS (Svegliati-Baroni et al., 2019; Geng et al., 2021). If produced in large quantities, these ROS will clearly induce mitochondrial dysfunctions (Guo et al., 2013). To cope with this, hepatocytes will activate specific antioxidant mechanisms involving SOD, Cat, and glutathione, but if the ROS level remains high, these defense mechanisms against oxidative stress may prove insufficient, as illustrated by their strong reductions observed in severe syndromes of NAFLD or NASH

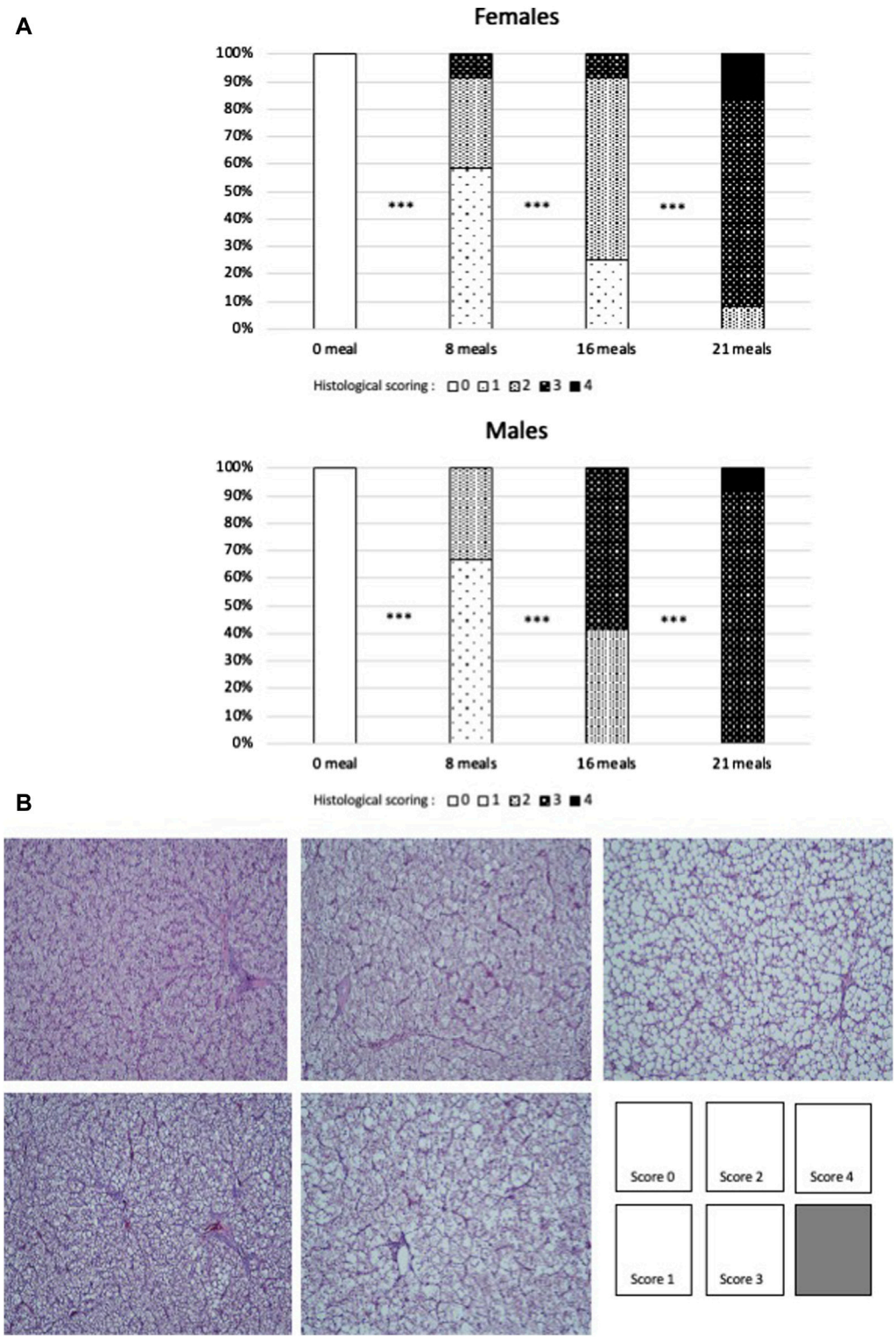

FIGURE 1
(A) Percentages of each score of histological sections of livers of female or male mule ducks according to the number of meals during the force-feeding period. (n = 12/sex/stage). Significance between two consecutive stages drawn according to Fisher's exact test (*** = $p < 0.001$). **(B)** Examples of scoring grades (hematoxylin–eosin staining), with the same magnification for all pictures.

(Sumida et al., 2013). In the present study, while the activity level of SOD increased, to neutralize superoxide ions, during the force-feeding period in both sexes, the activity level of CAT, GSH, and GSSG contents decreased, indicating that the liver cells of male and female ducks have to face uncontrolled oxidative stress during the rapid development of hepatic steatosis.

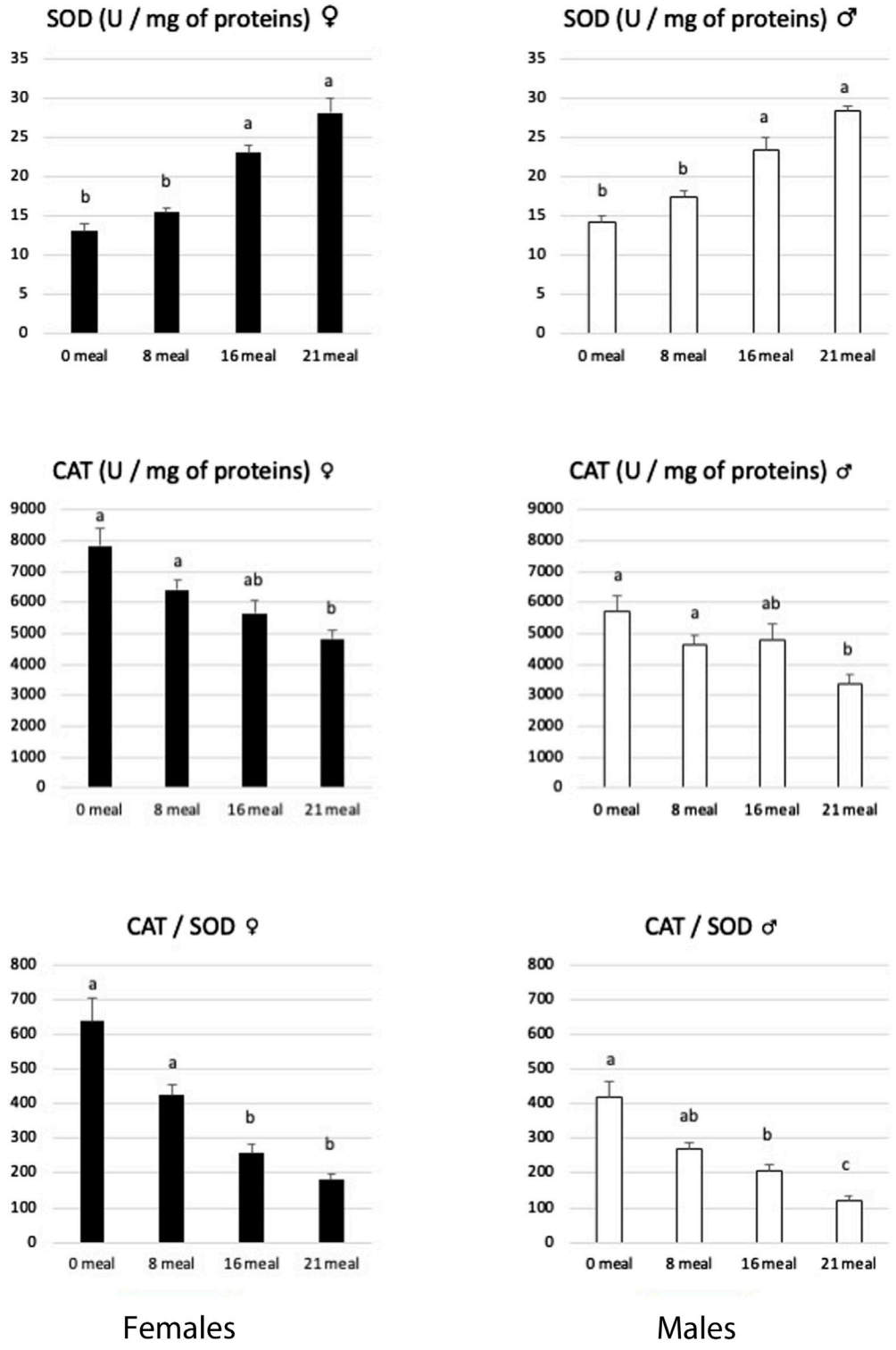

FIGURE 2
Activities of superoxide dismutase (SOD) and catalase (CAT) enzymes in livers of female and male mule ducks according to the number of meals during the force-feeding period. (n = 12/sex/stage). Values are the means ± SD. Values with different superscripts are different ($p < 0.05$).

The development of hepatic steatosis impairs metabolism and tissue remodeling (Suzuki et al., 2014), mainly through severe hypoxia that disrupts hepatic oxygen homeostasis. This activates the production of transcription factors HIF1α and HIF2α, which are involved in the response to initial or acute hypoxia, respectively (Downes et al., 2018). For example, Morello et al. (2018) reported that HIF2 is overexpressed in humans or mice developing hepatic steatosis of various origins, which leads to a hypoxic

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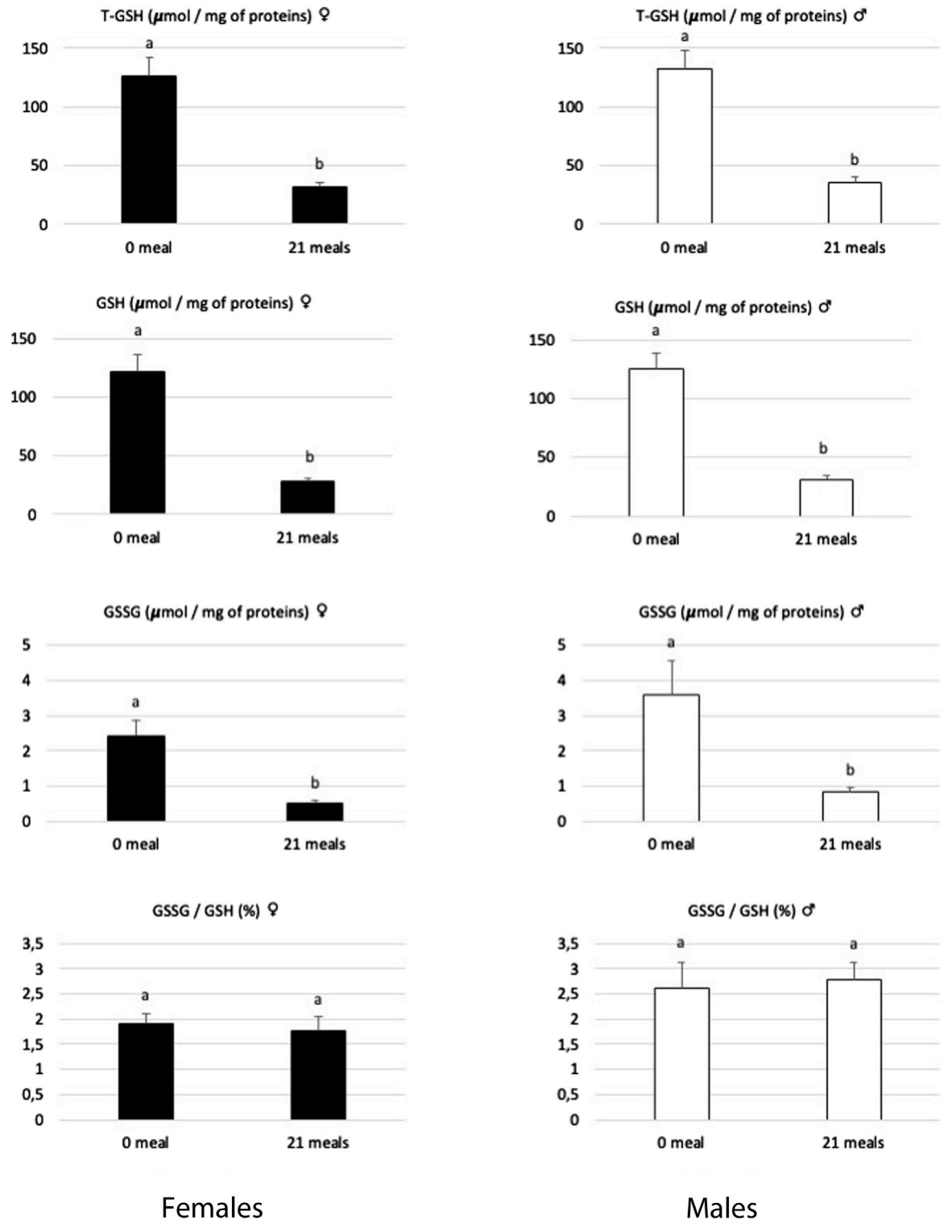

FIGURE 3
Biochemical contents of total glutathione (T-GSH), reduced glutathione (GSH), oxidized glutathione (GSSG), and the GSSG/GSH ratio in livers of female and male mule ducks before (0 meal, $n = 10$) and after (21 meals, $n = 10$ for T-GSH and $n = 5$ for GSH and GSSG) the force-feeding period. ($n = 12$/sex/stage). Values are the means ± SD. Values with different superscripts are different (SNK, $p < 0.05$).

microenvironment and mitochondrial dysfunctions. Remignon and Burgues (2023) previously reported an increase in HIF1α and HIF2α factors in the livers of force-fed male mule ducks. In the present study, the evolution of these two hypoxic factors follows the same pattern in male and female ducks (Figure 4). These increases indicate that during the force-feeding period, the livers of

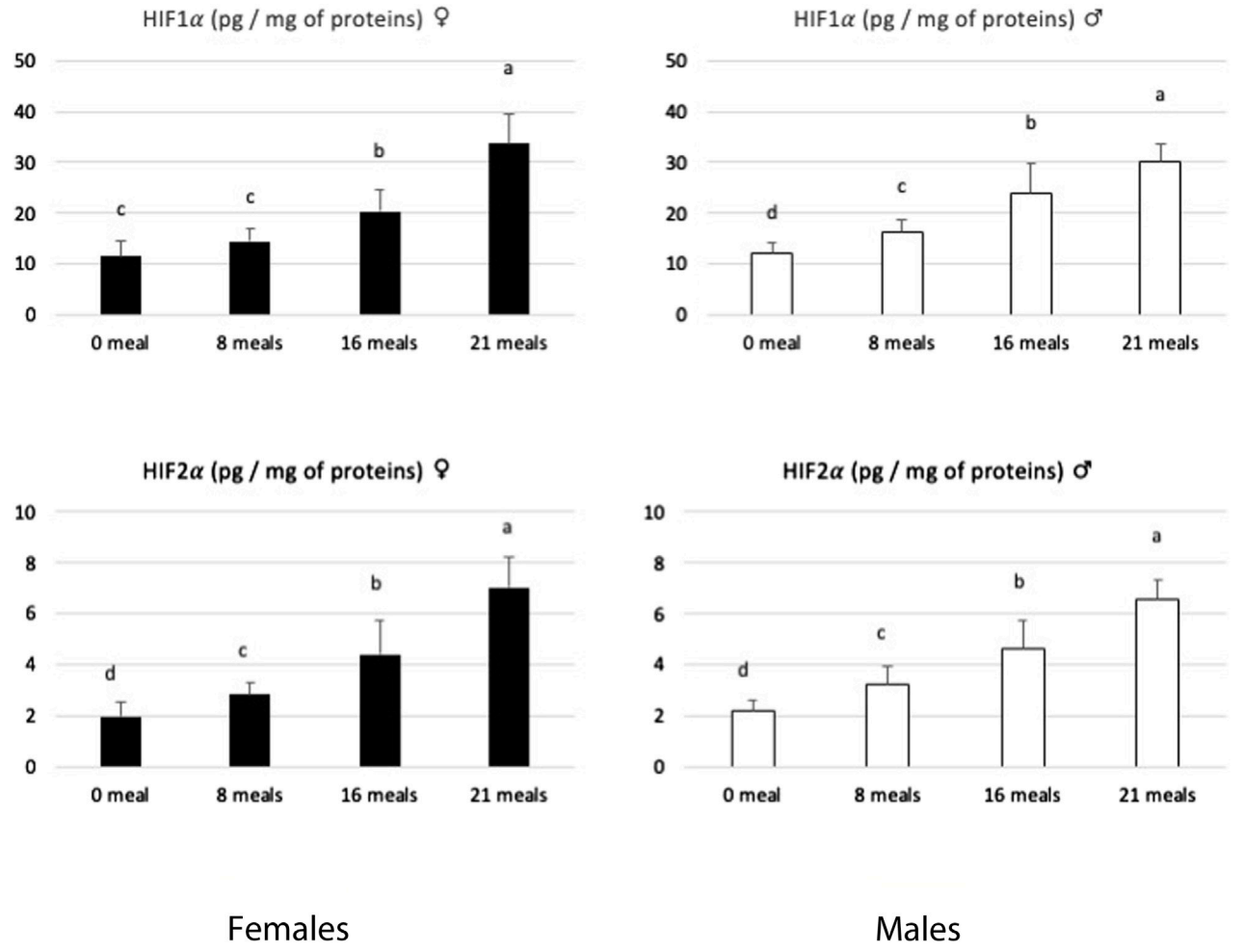

FIGURE 4
Biochemical contents of hypoxia-inducible factors 1 and 2 (HIF1α and HIF2α) in livers of female and male mule ducks according to the number of meals during the force-feeding period. (n = 12/sex/stage). Values are the means ± SD. Values with different superscripts are different (SNK, $p < 0.05$).

both male and female ducks experienced hypoxic conditions that could be related to physical disability due to liver enlargement and/or circulatory problems related to the elevated lipemia typically associated with the development of fatty liver. According to McGarry et al. (2018), an increase in ROS may result in a reduction in the efficiency of oxygen delivery. This could be progressively the case throughout the force-feeding period for both sexes and thus explain the development of the cellular oxidative stress observed independently of any sexual determinism.

According to Medzhitov (2008), inflammation should not be considered solely as a reaction to infection or injury but should be expanded to take into account inflammatory processes induced by other types of adverse conditions. The rapid development of steatosis in the liver, as well as the associated oxidative stress, could therefore induce this type of chronic inflammatory state. TNFα (tumor necrosis factor alpha) and IL18 (interleukin-18) are cytokines which have been shown to be increased in nutritionally induced hepatic steatosis in mice or humans (Henao-Meija et al., 2012; Yamanishi et al., 2016; Tiegs and Horst, 2022; Knorr et al., 2023; Vachliotis and Polyzos, 2023). An increase in the transcription levels of the genes coding for these molecules must therefore be considered a sign of the development of an inflammatory process in the liver. In the present experiment, between the beginning (meal 0) and the end (meal 21) of the force-feeding period, we reported an increase in the transcription level of the TNFα gene in females but not in males and increases in that of IL18 for both sexes (Table 2). This indicates that the inflammatory process was present at the end of the force-feeding period in both sexes, assessed by the increase in the level or transcription of the IL18 gene, but potentially with a higher intensity in females, as illustrated by increases in both levels of transcription IL18 and TNFα genes. This differential level of inflammation in male and female mule ducks during the development of liver steatosis induced by force-feeding has been previously approximated with measurements of OH-Pro levels. On the contrary, Spruss et al. (2012) reported that TNFα levels were altered in male and female mice developing NAFLD.

BCL6 is a transcription factor that plays a key role in determining the active genetic program in male *versus* female

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TABLE 2 Fold-changes (references are mean values for 0 meal) of relative expression of selected genes involved in liver metabolisms of male and female mule ducks during the force-feeding period. Values are the means ± SD.

		Gender	0 meal	8 meals	16 meals	21 meals		
	n	Male	9	12	11	10		
		Female	12	9	11	12		
Metabolism	Gene						GIFF*	P[1]<
Carbohydrates	Glut2	Male	1.00 ± 0.08^{b}	0.94 ± 0.08^{b}	1.40 ± 0.11^{a}	1.22 ± 0.15^{ab}	→	0.0129
		Female	1.00 ± 0.10^{a}	0.67 ± 0.04^{b}	1.23 ± 0.13^{a}	1.19 ± 0.12^{a}	→	0.0005
	Eno1	Male	1.00 ± 0.04^{b}	1.31 ± 0.05^{a}	1.25 ± 0.07^{a}	1.19 ± 0.13^{ab}	↑	0.0261
		Female	1.00 ± 0.07^{b}	1.26 ± 0.12^{ab}	1.39 ± 0.12^{a}	1.42 ± 0.12^{a}	↑	0.0219
	Hk1	Male	1.00 ± 0.24^{a}	1.2 ± 0.40^{a}	1.57 ± 0.40^{a}	0.34 ± 0.01^{b}	↓	0.0060
		Female	1.00 ± 0.34^{a}	0.23 ± 0.10^{b}	0.83 ± 0.25^{a}	0.33 ± 0.08^{ab}	↓	0.0109
Lipids	Cpt1a	Male	1.00 ± 0.08^{b}	0.67 ± 0.05^{c}	1.06 ± 0.06^{b}	2.51 ± 0.31^{a}	↑	0.0001
		Female	1.00 ± 0.21^{c}	1.86 ± 0.49^{b}	1.34 ± 0.10^{b}	2.73 ± 0.25^{a}	↑	0.0001
	Acad11	Male	1.00 ± 0.08^{a}	0.39 ± 0.04^{b}	0.61 ± 0.03^{c}	0.58 ± 0.06^{b}	↓	0.0001
		Female	1.00 ± 0.07^{a}	0.58 ± 0.07^{b}	0.60 ± 0.04^{b}	0.58 ± 0.04^{b}	↓	0.0001
	Acox1	Male	1.00 ± 0.06^{b}	0.65 ± 0.06^{c}	1.05 ± 0.06^{b}	1.42 ± 0.17^{a}	↑	0.0001
		Female	1.00 ± 0.09^{b}	0.72 ± 0.08^{c}	0.97 ± 0.06^{b}	1.45 ± 0.12^{a}	↑	0.0001
	Soat	Male	1.00 ± 0.11^{b}	0.60 ± 0.05^{c}	0.77 ± 0.08^{b}	1.3 ± 0.17^{a}	↑	0.0001
		Female	1.00 ± 0.10^{b}	0.79 ± 0.07^{b}	1.00 ± 0.07^{b}	1.53 ± 0.14^{a}	↑	0.0002
	Dgat2	Male	1.00 ± 0.37	0.63 ± 0.22	1.37 ± 0.43	0.74 ± 0.30	→	0.6624
		Female	1.00 ± 0.30	0.58 ± 0.21	0.75 ± 0.25	0.70 ± 0.20	→	0.4254
	FasN	Male	1.00 ± 0.06	1.11 ± 0.12	1.02 ± 0.06	0.78 ± 0.08	→	0.0653
		Female	1.00 ± 0.13	0.93 ± 0.09	1.24 ± 0.14	1.08 ± 0.11	→	0.3841
	Scd1	Male	1.00 ± 0.04^{c}	1.60 ± 0.12^{b}	1.99 ± 0.11^{a}	1.53 ± 0.11^{b}	↑	0.0001
		Female	1.00 ± 0.15^{b}	1.88 ± 0.16^{a}	2.43 ± 0.13^{a}	2.27 ± 0.25^{a}	↑	0.0001
	Plin2	Male	1.00 ± 0.11^{c}	2.39 ± 0.23^{b}	3.87 ± 0.44^{a}	2.64 ± 0.14^{b}	↑	0.0001
		Female	1.00 ± 0.06^{b}	0.77 ± 0.11^{b}	1.94 ± 0.29^{a}	2.18 ± 0.33^{a}	↑	0.0003
	Fabp4	Male	1.00 ± 0.09^{d}	5.00 ± 1.00^{c}	49 .00 ± 10.00^{b}	121.00 ± 11.00^{a}	↑	0.0001
		Female	1.00 ± 0.12^{d}	5.92 ± 2.11^{c}	19.36 ± 2.92^{b}	87.30 ± 8.95^{a}	↑	0.0001
	Apob	Male	1.00 ± 0.40	2.08 ± 0.71	1.03 ± 0.34	1.57 ± 0.45	→	0.6743
		Female	1.00 ± 0.20	0.56 ± 0.19	1.00 ± 0.37	0.73 ± 0.28	→	0.5012
Inflammation	Il18	Male	1.00 ± 0.17^{b}	0.61 ± 0.06^{c}	0.90 ± 0.10^{b}	1.42 ± 0.14^{a}	↑	0.0001
		Female	1.00 ± 0.13^{c}	1.18 ± 0.11^{c}	1.70 ± 0.13^{b}	2.68 ± 0.18^{a}	↑	0.0001
	TNFα	Male	1.00 ± 0.08^{a}	0.56 ± 0.04^{b}	0.90 ± 0.06^{a}	0.96 ± 0.06^{a}	→	0.0001
		Female	1.00 ± 0.07^{b}	1.00 ± 0.18^{b}	1.16 ± 0.09^{ab}	1.46 ± 0.13^{a}	↑	0.0171

(Continued on following page)

TABLE 2 (*Continued*) Fold-changes (references are mean values for 0 meal) of relative expression of selected genes involved in liver metabolisms of male and female mule ducks during the force-feeding period. Values are the means ± SD.

		Gender	0 meal	8 meals	16 meals	21 meals		
Regulators	Bcl6	Male	1.00 ± 0.16	0.91 ± 0.11	1.30 ± 0.12	1.14 ± 0.11	→	0.0760
		Female	1.00 ± 0.16^{b}	0.76 ± 0.09^{b}	1.21 ± 0.16^{ab}	1.76 ± 0.23^{a}	↑	0.0014
	Pparα	Male	1.00 ± 0.04^{a}	0.54 ± 0.04^{c}	0.76 ± 0.04^{b}	0.70 ± 0.06^{b}	↓	0.0001
		Female	1.00 ± 0.07	0.88 ± 0.13	1.01 ± 0.07	1.15 ± 0.08	→	0.1214
	Srebp-1c	Male	1.00 ± 0.08^{a}	0.61 ± 0.05^{b}	0.73 ± 0.09^{b}	0.57 ± 0.05^{b}	↓	0.0006
		Female	1.00 ± 0.10	0.78 ± 0.17	0.89 ± 0.08	0.75 ± 0.10	→	0.2051
	Chrebp	Male	1.00 ± 0.03^{a}	0.60 ± 0.05^{b}	0.67 ± 0.04^{b}	0.36 ± 0.02^{c}	↓	0.0001
		Female	1.00 ± 0.07^{a}	0.78 ± 0.08^{a}	0.81 ± 0.06^{a}	0.38 ± 0.04^{b}	↓	0.0001
	Pparγ	Male	1.00 ± 0.05^{a}	0.56 ± 0.04^{c}	0.73 ± 0.04^{b}	0.71 ± 0.06^{b}	↓	0.0001
		Female	1.00 ± 0.06	0.88 ± 0.11	0.95 ± 0.04	1.01 ± 0.06	→	0.3008

Within a line, values with different superscripts are different ($p < 0.05$).
GIFF*: global impact of force-feeding between 0 and 21 meals.
[1]: *p*-values are related to the effect of the number of meals.

mice and, therefore, their survival under different conditions (Nikkanen et al., 2022). BCL6 is a master transcription factor for the regulation of T follicular helper cells but also acts as a potent antagonist of PPARα-directed gene regulation (Sommars et al., 2019). A high level of hepatic BCL6 in male mice leads to hepatic steatosis and glucose intolerance during dietary excess, while its low level in females may explain better hepatic lipid handling (Salisbury et al., 2021). In the present study, the level of transcription of the BCL6 gene increased only in female ducks during the force-feeding period (Table 2) while it remained constant in males. Since low levels of BCL6 gene transcription in female mice are associated with their increased resistance to the development of hepatic steatosis, we can hypothesize that the observed increase in BCL6 gene transcription in female ducks during the force-feeding period illustrates a decrease in their resistance to hepatic steatosis, which could possibly be linked to their higher inflammatory response. The levels of other transcription factors evaluated (Pparα, Srebp-1c, and Pparγ) decreased in male ducks, while they remained constant in female ducks, during the force-feeding period (Table 2). On the contrary, the transcription level of Chrebp decreased in both sexes. Chrebp (Regnier et al., 2023) and Srebp-1c (Ferré et al., 2021) are involved in lipogenesis pathways when large amounts of glucose are available. In force-fed male ducks, Han et al. (2015) reported that their transcript levels were increased, while we reported them to be downregulated in the present experiment, probably because our birds were fasted for 12 hours before slaughter and, therefore, the level of circulating glucose must have been very low. However, given the enormous amount of lipids accumulated in hepatocytes during force-feeding, this fasting period was not sufficient to increase the level of transcriptional factors such as Pparα or Pparγ, which are generally activated to absorb circulating fatty acids during prolonged periods of fasting (Ruppert and Kersten, 2024).

Other transcript levels regarding carbohydrate or lipid metabolisms in the liver presented changes in male and female ducks during the force-feeding period (Table 2). They are close to previous results (Pioche et al., 2020; Tavernier et al., 2020; Massimino et al., 2021; Andrieux et al., 2023) describing the development of hepatic steatosis induced by force-feeding in male mule ducks. Here, we reported, in male and female mule ducks, that force-feeding influences the transcription of genes (Scd1, Soat, FasN, Dgat2, and Plin2) involved in the neo-synthesis of different types of lipids (fatty acids, polar or neutral lipids, and cholesterol-derived lipids) in response to increasing amounts of carbohydrates delivered. Among the transcripts analyzed and related to lipogenesis, only ACAD11 was significantly decreased during the force-feeding period, as observed by Massimino et al. (2021) in force-fed male mule ducks. For genes related to lipid transport, the relative expression of APOB remained constant, while that of FABP4 was increased significantly during the force-feeding period in both sexes. This has already been observed by Massimino et al. (2021), who proposed two ways to explain these opposing regulations: first, depending on the direction of lipid transport through the liver cells (in or out), their expression can be either increased or decreased by force-feeding; second, the timing of RNA collection after the last meal can have a significant impact on the expression level, as demonstrated by a kinetic study of these same genes by Annabelle et al. (2018). The increase in the level of Fabp4 gene transcripts in the liver is spectacular (121 and 87 times higher in males and females during the entire force-feeding period, respectively), as previously reported in Tavernier et al. (2018) and Pioche et al. (2020). In birds, unlike what is observed in mammals, the liver is the main site of lipid biosynthesis (Cui et al., 2018), and the increase in Fabp4 levels observed during the development of liver steatosis reflects simply the increase in hepatic lipogenesis and transport, as occurring in mammalian adipocytes developing NAFLD (Moreno-Vedia et al., 2022).

Conclusion

Our experience does not reveal large differences in the independent development of hepatic steatosis in response to specific force-feeding programs adapted for male and female mule ducks. The overall evolution of the different parameters measured was visible in both sexes despite the existence of a proven sexual body

dimorphism in lean animals prior to force-feeding. However, female birds could have a higher level of liver inflammation at the end of the force-feeding period. In mammals, we know that females are more resistant than males to developing hepatic steatosis in response to unbalanced diets (Spruss et al., 2012; Gasparin et al., 2018; Smati et al., 2022) but which are incommensurate with those caused by force-feeding of palmipeds. It would therefore be bold to conclude that male and female birds differ from mammals on this point as the modifications induced by force-feeding are particular with regard to the nature of the food, the quantities delivered, and the duration of the force-feeding period.

Our study demonstrates that the natural propensity of palmipeds to rapidly develop hepatic steatosis in response to an energy-rich diet, delivered in large quantities for a very short time, is not specific to males, although only those are traditionally used for the production of foie gras in France. It is better to conclude that despite their domestication, mule ducks, whatever their genus, have retained a particular capacity to produce and store lipids in large quantities in different body compartments. This allows them to support the large energy expenditure initially associated with long migratory flights.

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 in the article/Supplementary Material.

Ethics statement

Ethical review and approval were not required for the animal study because we collected samples from a commercial slaughterhouse after the regular operations of anesthesia, bleeding, scalding, plucking, and evisceration. We do not manipulate live animals, and no specific experimental manipulations were performed during the times of rearing. All the samples we harvested were commercial ones, ready to be purchased by consumers.

Author contributions

EA: formal analysis, writing–original draft, methodology, and investigation. ST: writing–original draft, methodology, investigation, and formal analysis. VA-B: writing–original draft, methodology, and formal analysis. FL: writing–original draft, methodology, and formal analysis. JA: writing–review and editing and conceptualization. CM: writing–review and editing and conceptualization. HR: writing–original draft, supervision, methodology, investigation, data curation, writing–review and editing, and conceptualization.

Funding

The author(s) declare that financial support was received for the research, authorship, and/or publication of this article. N. C4697/INRAE.

Acknowledgments

The authors would like to thank all the technical staff from Euralis Gastronomie for taking care of the animals during the experiment and for facilitating access to the slaughterhouse for sample collection.

Conflict of interest

Authors JA and CM were employed by Euralis Gastronomie.

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

Supplementary material

The Supplementary Material for this article can be found online at: https://www.frontiersin.org/articles/10.3389/fphys.2024.1392968/full#supplementary-material

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frontiers | Frontiers in Physiology

TYPE Original Research
PUBLISHED 03 July 2024
DOI 10.3389/fphys.2024.1396301

OPEN ACCESS

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RECEIVED 05 March 2024
ACCEPTED 03 June 2024
PUBLISHED 03 July 2024

CITATION
Li X, Uyanga VA, Jiao H, Wang X, Zhao J, Zhou Y, Li H and Lin H (2024), Effects of low dietary calcium and lipopolysaccharide challenges on production performance, eggshell quality, and bone metabolism of laying hens.
Front. Physiol. 15:1396301.
doi: 10.3389/fphys.2024.1396301

Effects of low dietary calcium and lipopolysaccharide challenges on production performance, eggshell quality, and bone metabolism of laying hens

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Dietary calcium supply is essential for bone development and egg production in laying hens. This study investigated the effects of low dietary calcium and lipopolysaccharide (**LPS**) induced immune challenge in aged laying hens. A total of thirty-two Hy-Line Brown laying hens at 80 weeks old with an average laying rate of 62% were randomly divided into two groups and fed a normal calcium diet (3.57% Ca, **NCA**) or low calcium diet (2.08% Ca, **LCA**). At 88 weeks, the experiment was designed using a 2 × 2 factorial arrangement, and hens were intraperitoneally injected with saline (**SAL**) or LPS (0.5 mg/kg, 0.5 mg/kg, or 1.5 mg/kg body weight) once every 48 h intervals over 5 days. Production performance, egg quality, and bone physiology were evaluated. Results showed that LPS challenge decreased the hen-day egg production, egg mass, and eggshell traits ($p < 0.05$), but increased ($p < 0.05$) the calcium content of the tibia compared to SAL-injected hens. LCA diet decreased ($p < 0.05$) the hen-day egg production, and eggshell traits such as weight, percentage, strength, and thickness compared to the NCA diet. LCA diet increased the serum alkaline phosphatase (**ALP**) activity ($p < 0.01$) and tibial expression of *ALP* ($p < 0.05$) compared to NCA diet. LPS injection suppressed both the serum ALP activity ($p < 0.05$) and tibial expression of *ALP* ($p < 0.001$) compared to SAL injection. Furthermore, LPS injection increased ($p < 0.05$) the expression of both pro and anti-inflammatory cytokines in the spleen and tibia. The expression of cathepsin K (***Cts K***) and matrix metalloproteinase 9 (***MMP-9***) were downregulated by LPS injection ($p < 0.001$). Broken and shell-less egg production and calcium content of eggshell, as well as tibial mRNA expression of osteocalcin (***Ocn***), tumor necrosis factor-alpha (***TNF-α***) and tartrate-resistant acid phosphatase (***TRAP***) were affected by the interaction ($p < 0.05$) of diet and

Abbreviations: ALP, alkaline phosphatase; BMD, bone mineral density; Ca, calcium; Cts K, Cathepsin K; FGF23, fibroblast growth factor 23; GAPDH, glyceraldehyde-phosphate dehydrogenase; IL-1, Interleukin-1; IL-6, Interleukin-6; IL-10, Interleukin-10; IL-17, Interleukin-17; IFN-γ, interferon-gamma; LPS, lipopolysaccharide; MMP-9, matrix metallopeptidase 9; N, Newton; Ocn, osteocalcin; P, phosphorus; TNF-α, tumor necrosis factor-alpha; TRAP, tartrate-resistant acid phosphatase.

injection. Therefore, this study demonstrated that to certain extents, low dietary calcium and LPS challenge dysregulated bone homeostasis and metabolism, with detrimental effects on the performance and eggshell quality of aged laying hens.

KEYWORDS

calcium, lipopolysaccharide, bone homeostasis, egg quality, aged laying hen

1 Introduction

In laying hens, calcium (**Ca**) deposition and mobilization in bone occurs daily to meet the requirements for egg production. The delicate balance between calcium and phosphorus deposition and resorption (i.e., bone synthesis and bone resorption) may be disrupted by intestinal malabsorption (Bielke et al., 2017), high output at the peak laying period (Whitehead, 2004), and immune challenge (Walsh et al., 2018). Bone health and the immune system are closely related since they both share a rich set of molecules and similar regulatory mechanisms (Takayanagi, 2007). There is sufficient evidence demonstrating that bone cells are primarily affected by various immune agents under normal and pathogenic conditions (Dar et al., 2018). Studies have also shown that inflammatory cytokines, mainly produced by T cells and B cells, can directly or indirectly regulate the functions of osteoclasts and osteoblasts (Takayanagi, 2007; Walsh et al., 2018). The osteoblasts and osteoclasts mainly mediate the process of bone formation and bone resorption (Horowitz, 1998). Osteoblasts promote self-produced osteoid mineralization by secreting alkaline phosphatase (**ALP**) and matrix vesicles (Knothe Tate et al., 2004). On the other hand, osteoclasts are involved in the hydrolysis of mineral composition and osteoid by generating acids and proteolytic enzymes (Zhang et al., 2011).

The pro-inflammatory cytokine, tumor necrosis factor-alpha (**TNF-α**) is considered a potent inducer of bone resorption and plays a crucial role in bone metabolism and inflammatory bone diseases (Amarasekara et al., 2015). It was shown that interleukin-17 (**IL-17**) inhibited the osteogenesis of rat calvarial osteoblast precursors, and reduced the expression of ALP and osteocalcin (**Ocn**) (Kim et al., 2014). In addition, bone cells can also regulate the function of immune cells (Tsukasaki and Takayanagi, 2019). Osteoblasts secrete multiple cytokines, including interleukin-1 (**IL-1**), interleukin-6 (**IL-6**), and interleukin-7, which regulate hematopoietic stem cells and support the differentiation of immune cells (Mercier et al., 2011). Inflammatory response via lipopolysaccharide (**LPS**) has been reported to affect bone homeostasis in broilers (Mireles et al., 2005) and laying hens (Nie et al., 2018; Akbari MoghaddamKakhki and Kiarie, 2021; Liu et al., 2022).

Conventionally, laying hens are reared up to 70 weeks with an average of 290 eggs during the first laying cycle (Ledur et al., 2000). However, some producers have considered that extending the laying period to more than 80 weeks to achieve up to 500 eggs can accrue significant economic benefits, increase profitability, and minimize the environmental impacts for sustainable production (Molnar et al., 2017; Molnar et al., 2018). Extended lay is associated with several problems such as a decline in egg production, a decrease in egg quality, shell quality, and bone quality, and a rise in birds' health and welfare concerns (Molnar et al., 2017). To address these problems, improved genetics, along with appropriate nutrition strategy and management practices have been widely studied in aged laying hens (Xin et al., 2022). More so, it is necessary to understand the calcium metabolism of aged laying hens during the late laying stage.

Calcium supply is essential for bone development and eggshell formation in laying hens. Dietary supplementation of calcium is an important nutritional strategy for promoting the skeletal health of laying hens. Relative to hens fed normal calcium levels (3.69%), it was demonstrated that hens fed low calcium diets (1.56%) had greater bone pathological damage characterized by loss of bone mass and bone strength, lowered bone mineral content, increased micro-structural damage and a higher bone turnover (Jiang et al., 2019). The bone is an important source of calcium for eggshell formation, thus exploring bone homeostasis during the late egg-laying period will provide new ideas for prolonging the laying period. Bone metabolism is essential for laying hens because it is related to bone health and eggshell quality. However, the intestinal efficiency for calcium absorption decreases in aged hens, thus limiting the calcium supply to the blood, which results in poor eggshell quality (Molnar et al., 2018). The decrease in blood calcium does not only affect the calcium supply for eggshell formation but directly impacts the metabolism and adjustment for calcium in the bone (Gu et al., 2021). Therefore, low calcium levels can disrupt normal bone metabolism, and thus affect eggshell formation.

Since bone homeostasis is established by the metabolic balance between osteoblasts and osteoclasts, it is worth understanding the underlying changes that occur during immune challenge and low calcium conditions. Therefore, this study investigated the interaction of dietary calcium levels and LPS challenge on the production performance, egg quality, bone immune function, and metabolism-related genes in aged laying hens.

2 Material and methods

This research was performed in accordance with the "Guidelines for Experimental Animals" of the Ministry of Science and Technology (Beijing, P. R. China), and the study protocols were approved by the Institutional Animal Care and Use Committee (No. 2020002) of the Shandong Agricultural University, China.

2.1 Experimental animals and management

Hy-Line Brown laying hens at 78 weeks old (n = 99) were randomly divided into two groups and fed a normal calcium diet, with a 2-week adaptation period before the start of the experiment. Hens were raised in battery cages that housed two birds per cage (60 cm length × 45 cm width × 75 cm height). The housing temperature and relative humidity were maintained at 23°C ± 2°C and 65% ± 3%, respectively, while the photoperiod was 16 h light and

8 h dark. Each cage was equipped with 1 nipple drinker and 1 feeding trough. All hens had free access to feed and water throughout the experimental period. Thirty eggs were collected from each group at 78 weeks of age for eggshell quality measurement.

At 80 weeks of age, 32 hens with similar body weight (1.98 ± 0.02 kg) and an average laying rate of 62% were selected and housed two hens per cage. The hens were divided into two groups and fed either the normal calcium diet (3.57% Ca, **NCA**) or a low calcium diet (2.08% Ca, **LCA**) until they reached 88 weeks of age. The formulation of NCA diet adhered to the guidelines of the Feeding Standard of Chickens (NY/T33-2004, China), and the dietary Ca level of LCA diet was based on a previous report (Table 1) (Jiang et al., 2019). Eggs were collected daily in the morning and evaluated weekly on an individual basis to determine the laying rate (%). Three or four eggs were collected from each cage (30 eggs in each group) on three consecutive days at 83 and 87 weeks, respectively.

At 88 weeks of age, hens were individually reared and grouped into a 2 × 2 factorial arrangement for a five-day trial and were subjected to either normal calcium diet + saline (**NCA + SAL**), normal calcium diet + LPS (**NCA + LPS**), low calcium diet + saline (**LCA + SAL**), or low calcium diet + LPS (**LCA + LPS**). Thus, the experiment was arranged as 4 treatments, each consisting of 8 replicates. A cage with one hen was considered as an experimental unit. Laying hens were intermittently injected intraperitoneally with either sterile saline, or LPS (derived from *Escherichia coli*, L2880; Sigma-Aldrich, St. Louis, MO, USA) at doses of 0.5, 0.5, or 1.5 mg/kg body weight at 11:00 a.m. on days 1, 3, and 5 of week 88 (every 48-h interval) (Nie et al., 2018). Performance, egg production, and egg quality data were collected during the trial. All data were collected on an individual basis. Feed intake was recorded weekly, and egg weights and number of eggs laid from each chicken were recorded daily to determine the daily feed intake (g), hen-day egg production (%), and average egg weight (g). Egg mass was determined by multiplying the average egg weight by egg numbers and then dividing it by the number of days. Feed conversion ratio was calculated as grams of feed: grams of egg mass produced. The diagram depicting the experimental schedule experimental design is shown in Figure 1.

2.2 Sample collection

Five eggs from each hen and in total 40 eggs from each treatment were collected on the second and fourth days for eggshell quality assessment. On the fifth day of trial, a blood sample was collected at 14:00 p.m., 3 h after LPS or saline injection, from a wing vein of all the 32 experimental hens. Serum samples were obtained after centrifugation at 1,500 × g for 15 min at 4°C and stored at −20°C for further analysis. After blood collection, birds were sacrificed by exsanguination after cervical dislocation (Close et al., 1997; Sun et al., 2020). The spleen and right tibia were collected and immediately snap-frozen in liquid nitrogen, then stored at −80°C for further analysis. The left tibia was isolated and stored at −20°C for bone quality measurement. The distal right femur was obtained and fixed in 10% neutral buffered formalin for histological analysis.

2.3 Egg quality evaluation

The egg weight was measured using the egg multi-tester (EMT-5200, Robotmation, Japan), and eggshell strength was measured with an egg force reader (EFG-0503, Robotmation Co., Ltd., Tokyo, Japan). Eggshell thickness was measured with an eggshell thickness tester (ETG-1061, Robotmation Co., Ltd.) at three different locations (air cell, equator, and sharp end) on the egg, and the average was computed. Eggs were cracked, and the shells were cleaned, dried at 25°C for 12 h, and then weighed. The eggshell percentage was calculated using the formula: (eggshell weight/egg weight) × 100%.

2.4 Serum biochemical parameters

Serum ionized calcium and phosphorus levels were analyzed using an automatic biochemical analyzer (7170A, Hitachi, Japan) and corresponding kits provided by Maccura (Sichuan, China). Activities of alkaline phosphatase (ALP) (A059-2, Nanjing Jiancheng Bioengineering Institute, Nanjing, China) and tartrate-resistant acid phosphatase (**TRAP**) (P0332, Beyotime Biotechnology, Shanghai, China) were assessed with specific commercial assay kits, following the manufacturer's guidelines.

2.5 Tibia physical parameters

Bone mineral density (**BMD**) of the left tibia was measured using the Dual-energy X-ray absorptiometry device (InAlyzer; MEDIKORS Inc., Gyeonggi-Do, Korea). Bone breaking strength of the left tibia was measured using a 3-point bending test with a microcomputer-controlled electronic universal testing machine (Jinan Shijin Group Co., Ltd., China). The bone was positioned on two supports with a 3 cm span, and a load of 1,000 N was applied at the midpoint of each bone at a loading speed of 2 mm/min until fracture. The computerized monitor recorded the deformation curve, with the peak load indicating the breaking strength in Newton. Left tibia samples were treated with a mixture of alcohol and benzene (2:1) for 96 h to get rid of fat and were then dried at 105°C to a constant weight. The defatted bone index was calculated as defatted bone weight (g)/body weight (kg) × 100.

2.6 Calcium and phosphorus content in diet, tibia, and eggshell

Eggshell and defatted left tibia samples were ground into a powder with a pulverizer. About 0.5 g of diet, tibia, and eggshell samples were weighed into a crucible and carbonized using an induction cooker. Samples were ashed in a muffle furnace at 550°C for 6 h, then dissolved in a mixture of hydrochloric acid and concentrated nitric acid, and transferred to a 100 mL volumetric flask after boiling to prepare a sample decomposition solution. The Ca content was measured using potassium permanganate titration, and the P content was determined by the ammonium molybdate spectrophotometric method, as previously reported (Song et al., 2022).

10.3389/fphys.2024.1396301

TABLE 1 Ingredients and nutrient composition of experimental diets.

Ingredient, %	NCA[a]	LCA[a]
Corn	55.40	58.27
Soybean meal (43 CP%)	23.83	22.76
Wheat bran	6.24	7.50
Soybean oil	2.22	1.22
Limestone	9.29	2.73
Calcium hydrogen phosphate	1.22	1.20
NaCl	0.34	0.34
Zeolite powder	1.00	5.50
Lysine (99%)	-	0.01
Methionine (98%)	0.11	0.11
Threonine (98%)	-	0.01
Choline chloride (50%)	0.10	0.10
Premix[b]	0.25	0.25
Calculated nutrient levels		
Metabolizable energy, MJ/kg	11.10	11.10
Crude protein, %	16.00	16.00
Calcium, %	3.61	1.32
Lysine, %	0.750	0.750
Methionine, %	0.368	0.366
Methionine + Cystine, %	0.650	0.650
Threonine, %	0.641	0.641
Tryptophan, %	0.213	0.211
Availablephosphorus, %	0.350	0.350
Analyzed nutrient levels		
Calcium, %	3.57	2.08

[a]NCA: normal calcium diet; LCA: low calcium diet.

[b]The vitamin and mineral premix provides the following quantities per kilogram of diet: vitamin A, 12,000 IU; vitamin D3, 2,400 IU; vitamin K, 0.75 mg; vitamin E, 7.5 IU; cholecalciferol, 2,400 IU; riboflavin, 3.75 mg; niacin, 30 mg; pantothenic acid, 3.3 mg; biotin, 0.15 mg; folic acid, 0.375 mg; Thiamine, 1.2 mg; Pyridoxine, 4.5 mg; Vitamin B12, 0.006 mg; Fe, 60 mg; Se, 0.3 mg; Cu, 10 mg; Zn, 80 mg; I, 0.35 mg; Mn, 60 mg.

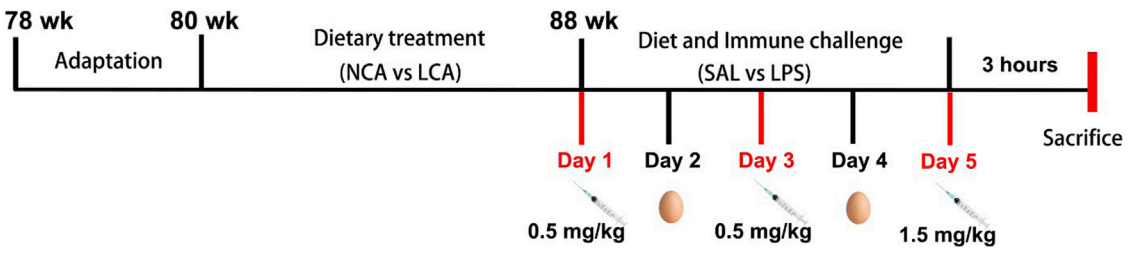

FIGURE 1
Diagram depicting the experimental schedule. NCA: Normal calcium diet (3.57% Ca); LCA: Low calcium diet (2.08% Ca). SAL: Saline; LPS: Lipopolysaccharide.

TABLE 2 Primers used for RT-qPCR.

Gene[a]	GenBank ID	Forward primers (5′-3′)	Reverse primers (5′-3′)	Product size (bp)
TNF-α	MF000729.1	GTAACGGCGTGGTGCTGAGAAG	TCCTCGGAGAAGCGGCTGAC	142
IL-1β	NM_204524.1	CAGAAGAAGCCTCGCCTGGATTC	GCCTCCGCAGCAGTTTGGTC	133
IL-6	NM_204628.1	GAGGTTGGGCTGGAGGAGGAG	TCTCGCACACGGTGAACTTCTTG	130
IL-17	NM_204460.1	CGATGAGGACCACAACCGCTTC	TGTTTGATGGGCACGGAGTTGAC	117
IL-10	NM_001004414.2	CAGCACCAGTCATCAGCAGAGC	GCAGGTGAAGAAGCGGTGACAG	94
IFN-γ	NM_205149.1	CTCGCAACCTTCACCTCACCATC	CAGGAACCAGGCACGAGCTTG	122
ALP	NM_205360.1	CACGGCGTCGATGAGCAGAAC	AGCAGAGGAGGAGGAGGAGGAG	148
Ocn	NM_205387.4	GCAGGCAGAAGCGGCACTAC	CAGCTCACACACCTCTCGTTGG	89
FGF23	XM_425663.4	AGCCAAGAGGACTGTGTGTTCAAC	ACTGGGAGTACGGTGGTGGATTC	145
TRAP	XM_015302697.2	TGCTGGCTTTGGGCGATAACTTC	GCCGTGGTGGTCGTGGTTTC	149
Cts K	NM_204971.2	GAAGGCAACGAGAAGGCTCTGAAG	AGAACTGGAAGGAGGGCAGACTG	91
MMP-9	NM_204667.1	ACCTGGACCGTGCCGTGATAG	CTGCCTCGCCGCTGTAAATCTG	102
GAPDH	NM_204305.1	CAGAACATCATCCCAGCGTCCAC	CGGCAGGTCAGGTCAACAACAG	134

[a]*TNF-α*, tumor necrosis factor alpha; *IL-1β*, interleukin 1 beta; *IL-6*, interleukin 6; *IL-17*, interleukin 17; *IL-10*, interleukin 10; *IFN-γ*, interferon gamma; *ALP*, alkaline phosphatase; *Ocn*, osteocalcin; *FGF23*, fibroblast growth factor 23; *TRAP*, tartrate-resistant acid phosphatase; *Cts K*, Cathepsin K; *MMP-9*, matrix metallopeptidase 9; *GAPDH*, glyceraldehyde-phosphate dehydrogenase.

2.7 Bone histological analysis

The fully fixed distal femurs were incubated in an EDTA decalcifying solution (E1171; Beijing Solarbio Science & Technology Co., Ltd., China) at room temperature for about 2 weeks. Decalcified femur samples were dehydrated in a graded series of ethanol and routinely embedded in paraffin. The samples were then cut into 3 μm sections for hematoxylin-eosin (**HE**) staining and Goldner Trichrome staining (G3550; Solarbio, China) according to the manufacturer's instructions. The slides were stained and visualized under an optical microscope (CK-40, Olympus, Tokyo, Japan). Images were taken of each section, and ImageJ software was used to measure the tissue area (**T.Ar**), trabecular area (**Tb.Ar**). Trabecular bone volume/tissue volume (**BV/TV**) was calculated as BV/TV = Tb.Ar/T.Ar × 100 (%).

2.8 Total RNA extraction and real-time quantitative PCR

Spleen samples were homogenized using a grinder (Genenode, China), while tibia samples were ground to powder using liquid nitrogen with a mortar. Total RNA was obtained from the spleen and tibia using Trizol reagent (Invitrogen, USA). The concentration and purity of the RNA were verified from $OD_{260/280}$ readings (Ratio ≈1.75–2.01) using spectrophotometry (Eppendorf, Germany). According to the manufacturer's instructions, reverse transcription was performed using total RNA (1,000 ng) for first-strand cDNA synthesis with the Transcriptor First Strand cDNA Synthesis Kit (Roche, Germany). The cDNA was amplified in a 20 μL reaction system containing 0.2 μM of each specific primer (Sangon, China) and the SYBR Green master mix (Roche, Germany). RT-qPCR was performed at the ABI QuantStudio 5 PCR machine (Applied Biosystems; Thermo, USA). Primers used for RT-qPCR were designed with Primer 5.0 software and synthesized by Sangon Biotech (Shanghai, China) according our previous work (Song et al., 2022), and primer sequences are shown in Table 2. The melting curves were checked to guarantee the specificity of amplification products. Data were analyzed using the method of $2^{-\Delta\Delta CT}$, and GAPDH was used to normalize the data as an internal control. The mRNA expression level of the NCA + SAL group was used as a calibrator. The expressions of genes tumor necrosis factor α (*TNF-α*), interleukin 1β (*IL-1β*), interleukin 6 (*IL-6*), interleukin 17 (*IL-17*), interleukin 10 (*IL-10*), interferon γ (IFN-γ), alkaline phosphatase (*ALP*), osteocalcin (*Ocn*), and fibroblast growth factor 23 (*FGF23*) were determined.

2.9 Statistical analysis

Statistical analysis was conducted using the PROC GLM procedure of SAS software (version 9.3, SAS Institute Inc., Cary, NC) with each hen as a replicate. Prior to LPS challenge, data was subjected to One-way ANOVA to analyze the main effect of diet (NCA vs. LCA). Following LPS challenge, two-way ANOVA was used to analyze the significant main effects of diet (NCA vs. LCA), injection (Saline vs. LPS) and their interaction effect (diet × injection). Duncan's Multiple Range Test was used for mean comparisons where the treatment effect was significant. Treatment differences were considered significant at $p < 0.05$ and the tendency towards significance was noted at $0.05 < p \leq 0.10$.

TABLE 3 Effects of dietary calcium levels on the laying rate and eggshell quality of aged laying hens prior to LPS challenge.[a].

Items	NCA	LCA	SEM	*p*-value
Laying rate (%)				
83 wk	69.64[a]	58.93[b]	3.34	0.035
84 wk	71.43[a]	59.82[b]	3.26	0.021
85 wk	77.68[a]	66.07[b]	3.84	0.045
86 wk	76.79[a]	65.18[b]	3.30	0.022
87 wk	66.67	60.42	3.40	0.201
Eggshell weight (g)				
78 wk[b]	5.86	5.84	0.12	0.919
83 wk	5.85	5.86	0.12	0.965
87 wk	6.50[a]	5.69[b]	0.08	<. 001
Eggshell percentage (%)				
78 wk[b]	9.30	9.01	0.16	0.194
83 wk	9.50	9.28	0.20	0.424
87 wk	10.08[a]	8.83[b]	0.14	<. 001
Eggshell strength (kg. f)				
78 wk[b]	3.21	3.10	0.16	0.650
83 wk	3.25[a]	2.68[b]	0.10	<. 001
87 wk	3.40[a]	2.70[b]	0.13	0.002
Eggshell thickness (mm)				
78 wk[b]	0.32	0.33	0.01	0.544
83 wk	0.30[a]	0.29[b]	0.00	0.028
87 wk	0.32[a]	0.29[b]	0.01	<. 001

NCA: Normal calcium diet (3.57% Ca); LCA: Low calcium diet (2.08% Ca); SEM: standard error of mean.
[a]with each cage as replicate (n = 8).
[b]n = 30.
[a-b]Means with different alphabetical superscripts within each row are significantly different ($p < 0.05$).

3 Results

3.1 Production performance and egg quality traits

All hens were fed a normal diet during the adaptation period, and eggshell quality did not differ ($p < 0.05$, Table 3) significantly between the two groups.

Prior to LPS challenge, laying hens were fed dietary treatments from 80 to 88 weeks of age. Table 3 shows that the laying rate was significantly reduced ($p < 0.05$) by the LCA dietary treatments during weeks 83–86. The eggshell strength and eggshell thickness were lower in the LCA compared to the NCA diet at 83 weeks. This effect was further pronounced at week 87, as LCA-fed hens had lesser eggshell weight and percentage ($p < 0.001$), decreased eggshell strength ($p < 0.01$), and reduced eggshell thickness ($p < 0.001$) than those fed NCA diets.

During LPS challenge period, LPS injection reduced hen-day egg production and egg mass ($p < 0.001$, Table 4). In contrast, LPS treatment had no effect ($p > 0.05$) on body weight, egg weight, and feed conversion ratio. The average daily feed intake, however, tended to be decreased by LPS injection ($p = 0.054$), compared to SAL-treated hens. There was a significant interaction of diet × LPS ($p < 0.05$) on the percentage of broken and shell-less egg and the LCA + LPS group produced more broken and shell-less eggs, compared to other groups. Compared to the NCA diet, LCA treatment decreased ($p < 0.05$) hen-day egg production and egg mass (Table 4), whereas had no influence ($p > 0.05$) on body weight, average daily feed intake, egg weight, and feed conversion ratio.

Compared to NCA-hens, LCA treatment decreased ($p < 0.05$) eggshell weight, eggshell percentage, eggshell strength, eggshell thickness, and P content of eggshell (Table 4). LPS challenge significantly reduced ($p < 0.05$) the eggshell thickness and P content of eggshell (Table 4). In contrast, LPS treatment had no significant ($p > 0.05$) influence on eggshell weight, percentage, and strength. There was a significant interaction of diet × LPS on the Ca content of eggshell ($p < 0.01$, Table 4). The NCA + SAL group had the highest Ca content, whereas the NCA + LPS group had the

TABLE 4 Effects of dietary calcium levels and LPS challenge on the performance and eggshell quality of aged laying hens[1].

	NCA		LCA		SEM	NCA	LCA	SAL	LPS	*p*-value		
	SAL	LPS	SAL	LPS						Diet	LPS	Diet×LPS
Performance												
Body weight (kg)	1.92	1.88	1.91	1.85	0.08	1.91	1.88	1.92	1.87	0.775	0.542	0.908
Average daily feed intake (g)	113.0	90.98	117.34	71.45	15.91	101.99	94.39	115.17	81.21	0.642	0.054	0.467
Hen-day egg production(%)	77.51	53.33	59.38	30.00	8.10	65.42^{m}	44.69^{n}	68.44^{x}	41.67^{y}	0.025	0.006	0.753
Broken and shell-less egg production (%)	-	8.33^{b}	4.71^{b}	35.42^{a}	4.12	0.04	0.20	0.02	0.22	0.003	<.001	0.017
Average egg weight (g)	64.22	61.50	61.85	62.37	1.37	62.86	62.11	63.04	61.94	0.596	0.438	0.260
Egg mass (g)	52.13	33.01	36.75	18.83	5.24	42.57^{m}	27.79^{n}	44.44^{x}	25.92^{y}	0.020	0.006	0.916
Feed conversion ratio (feed, g/egg mass, g)	2.14	3.61	3.47	4.38	1.10	2.91	3.92	2.84	3.40	0.378	0.316	0.826
Eggshell Quality												
Eggshell weight (g)	6.07	5.98	5.69	5.13	0.26	6.03^{m}	5.41^{n}	5.88	5.56	0.023	0.217	0.369
Eggshell percentage (%)	9.63	9.34	9.15	8.32	0.36	9.48^{m}	8.74^{n}	9.39	8.83	0.047	0.132	0.460
Eggshell strength (kg. f)	3.53	3.47	2.81	2.81	0.27	3.50^{m}	2.81^{n}	3.17	3.14	0.017	0.909	0.926
Eggshell thickness (mm)	0.33	0.30	0.31	0.28	0.01	31.70^{m}	29.41^{n}	31.93^{x}	29.18^{y}	0.040	0.015	0.650
Ca content of eggshell (%)	35.21^{a}	30.29^{c}	32.58^{b}	32.79^{b}	0.70	32.75	32.69	33.89	31.54	0.932	0.002	0.001
P content of eggshell (%)	14.75	14.06	14.12	11.59	0.66	14.40^{m}	12.85^{n}	14.43^{x}	12.83^{y}	0.026	0.021	0.174

NCA: Normal calcium diet (3.57% Ca); LCA: low calcium diet (2.08% Ca); SAL: saline; LPS: lipopolysaccharide; Ca: Calcium; P: phosphorus; SEM: standard error of mean.

[a]with each cage as replicate (n = 8).

[a-c]Means with different alphabetical superscripts within the same row differ significantly ($p < 0.05$).[n, m]Means with different alphabetical superscripts within column NCA, and LCA, differ significantly ($p < 0.05$).[x, y]Means with different alphabetical superscripts within column SAL, and LPS, differ significantly ($p < 0.05$).

10.3389/fphys.2024.1396301

TABLE 5 Effects of dietary calcium levels and LPS challenge on the serum biochemical parameters of aged laying hens.

	NCA		LCA		SEM	NCA	LCA	SAL	LPS	*p*-value		
	SAL	LPS	SAL	LPS						Diet	LPS	Dietx LPS
Ionized Ca (mmol/L)	5.71	5.69	5.70	4.94	0.27	5.70	5.32	5.71	5.32	0.174	0.163	0.179
Inorganic P (mmol/L)	1.05	1.17	1.30	1.23	0.11	1.11	1.27	1.17	1.20	0.168	0.819	0.428
ALP (U/L)	146.40	62.90	230.10	119.40	21.50	104.67^{n}	174.79^{m}	188.29^{x}	91.169^{y}	0.003	<.001	0.533
TRAP (U/L)	49.10	46.60	49.30	35.50	8.50	47.84	42.42	49.19	41.07	0.527	0.346	0.509

NCA: Normal calcium diet (3.57% Ca); LCA: Low calcium diet (2.08% Ca); SAL: saline; LPS: lipopolysaccharide; ALP: alkaline phosphatase; TRAP: tartrate-resistant acid phosphatase; SEM: standard error of mean, n = 8.$^{n,\ m}$Means with different alphabetical superscripts within column NCA, and LCA, are significantly different ($p < 0.05$).$^{x,\ y}$Means with different alphabetical superscripts within column SAL, and LPS, are significantly different ($p < 0.05$).

TABLE 6 Effects of dietary calcium levels and LPS challenge on the tibia bone parameters of aged laying hens.

	NCA		LCA		SEM	NCA	LCA	SAL	LPS	*p*-value		
	SAL	LPS	SAL	LPS						Diet	LPS	DietxLPS
Defatted bone index (g/kg)	3.70	3.74	3.59	4.00	0.17	3.72	3.80	3.65	3.87	0.673	0.200	0.284
Ca content (%)	16.16	18.61	17.31	17.75	0.58	17.39	17.53	16.74^{x}	18.18^{y}	0.804	0.018	0.092
P content (%)	8.77	9.32	9.02	9.07	0.26	9.05	9.05	8.89	9.20	0.996	0.261	0.357
BMD (g/cm^2)	0.29	0.31	0.28	0.29	0.02	0.30	0.29	0.29	0.30	0.582	0.502	0.803
Bone breaking strength (N)	158.1	153.5	164.2	148.8	18.60	155.82	156.56	161.20	151.19	0.968	0.596	0.776

NCA: Normal calcium diet (3.57% Ca); LCA: Low calcium diet (2.08% Ca); SAL: saline; LPS: lipopolysaccharide; Ca: Calcium; P: phosphorus; BMD: bone mineral density; SEM: standard error of mean, n = 8. $^{x,\ y}$Means with different alphabetical superscripts within column SAL, and LPS, are significantly different ($p < 0.05$).

lowest Ca content of eggshells ($p < 0.01$). In contrast, LPS treatment had no influence ($p > 0.05$) on Ca content in LCA groups.

3.2 Serum parameters

Serum concentrations of total Ca and TP, and TRAP activity were not altered by diet, LPS, or their interaction between diet and injection ($p > 0.05$, Table 5). However, serum ALP activity was significantly increased ($p < 0.01$) by the LCA diet compared to the NCA diet. In addition, the LPS challenge significantly decreased ($p < 0.001$) serum ALP activity compared to the SAL-injected hens.

3.3 Bone parameters

Neither diet nor the diet × LPS interaction significantly affected ($p > 0.05$) the defatted bone index, Ca and P content of the tibia, BMD, or bone breaking strength (Table 6). In addition, it was observed that the LPS injection significantly increased ($p < 0.05$, Table 6) the Ca content of the tibia compared to SAL-hens.

Furthermore, Goldner's trichrome staining of the distal femur showed that the mineralized bones were stained green while the osteoid or collagen was stained red (Figure 2A). HE staining showed the trabecular bone and connective tissue cells (Figure 2B). Compared with SAL treatment, LPS treatment showed more adipocytes with large volume (Figure 2B). However, there was no significant difference in trabecular bone volume/tissue volume among treatments (Figure 2C).

3.4 mRNA expression of inflammation-related genes in the spleen and tibia

In the spleen, mRNA expression levels of *TNF-α*, *IL-1β*, and *IL-6* were not affected by the main effect of diet ($p > 0.05$) or by diet × LPS interaction ($p > 0.05$, Figure 3). However, the expression of *TNF-α* ($p < 0.01$), *IL-1β* ($p < 0.01$), and *IL-6* ($p < 0.001$) was upregulated in the LPS-challenged groups compared to the SAL-injected hens (Figures 3A–C).

The tibial mRNA expression level of *TNF-α* (Figure 4A) was significantly affected by the diet × LPS interaction ($p < 0.05$) and the LPS-induced increment of *TNF-α* expression was further increased by LCA treatment. The mRNA abundances of *IL-1β* ($p < 0.001$), *IL-6* ($p < 0.01$), *IL-17* ($p < 0.05$), *IL-10* ($p < 0.001$), and *IFN-γ* ($p < 0.05$) were upregulated in the tibia of LPS-challenged hens compared to SAL-hens (Figure 4). Alongside this, there was a tendency for *IL-17* (Figure 4D; $p = 0.083$) expression to be upregulated in the LCA dietary treatment compared to the NCA diet. The expression of *IL-17* ($p = 0.053$) and *IFN-γ* (Figure 4F; $p = 0.089$) showed a tendency to be affected by the interaction effect of diet and LPS.

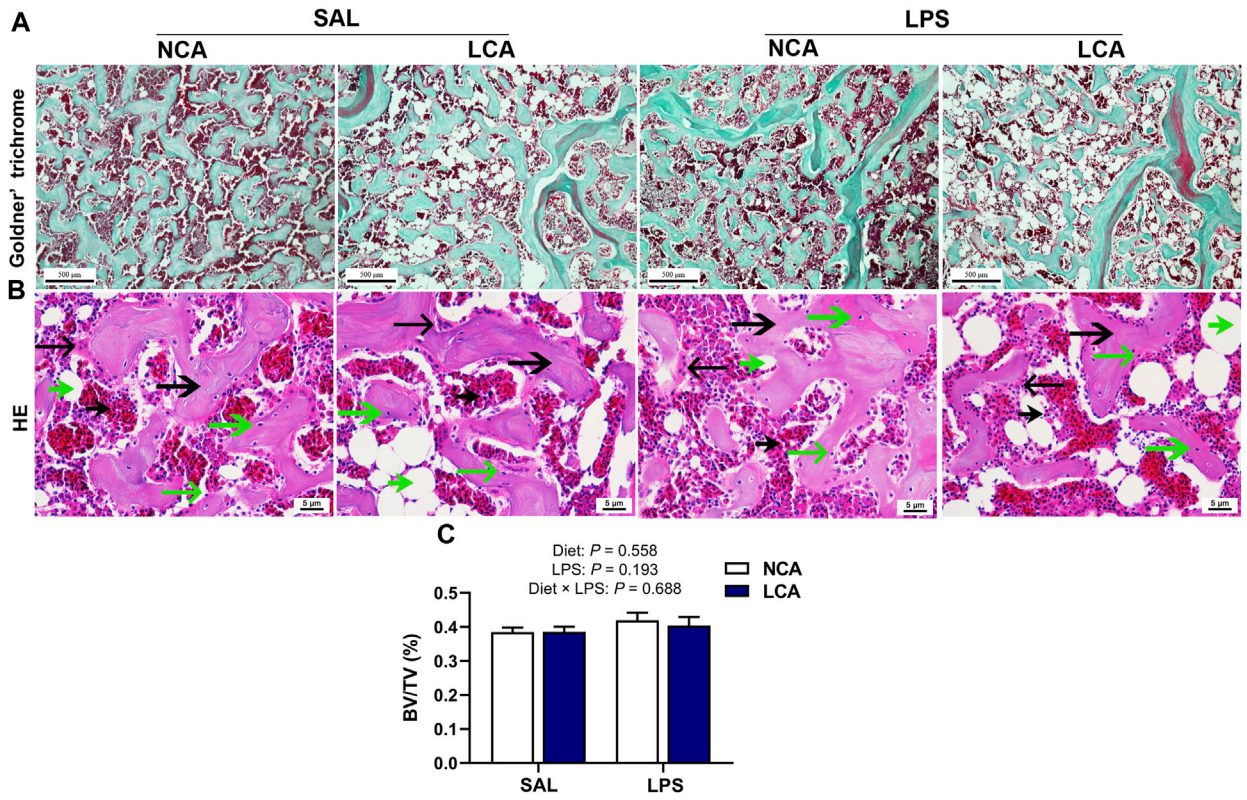

FIGURE 2
Effects of dietary calcium levels and LPS challenge on the bone microstructure of aged hens. NCA: Normal calcium diet (3.57% Ca); LCA: Low calcium diet (2.08% Ca). SAL: Saline; LPS: Lipopolysaccharide. **(A)** Goldner' Trichrome staining of the distal femur (scale bar: 500 μm); **(B)** HE staining of the distal femur (scale bar: 5 μm); **(C)** Trabecular bone volume/tissue volume (BV/TV)→trabecular bone; →connective tissue cells; →osteoclast; →osteocyte; →adipocyte→osteoblast.

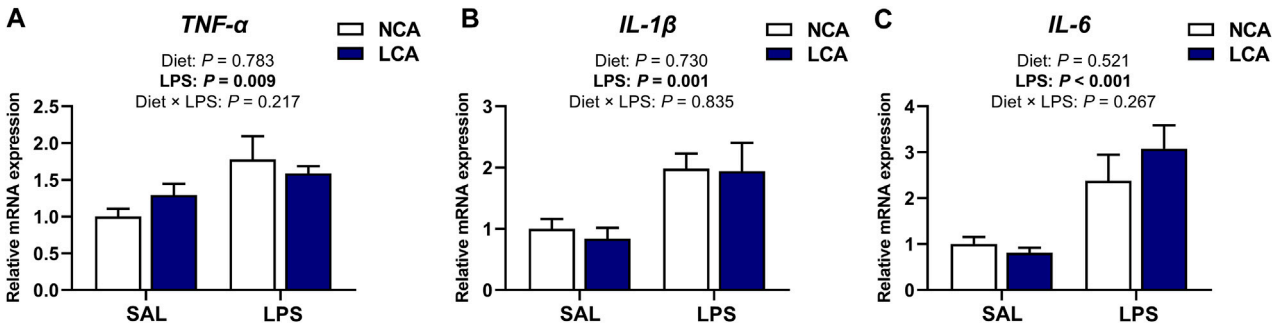

FIGURE 3
Effects of dietary calcium levels and LPS challenge on the relative mRNA expression of splenic inflammation-related genes in aged laying hens. NCA: Normal calcium diet (3.57% Ca); LCA: Low calcium diet (2.08% Ca). SAL: Saline; LPS: Lipopolysaccharide. **(A)** *TNF-α*; **(B)** *IL-1β*; **(C)** *IL-6*. Data are represented as mean ± standard error of mean (n = 8).

3.5 mRNA expression of tibial osteoblast metabolism-related genes

Figure 5 shows the mRNA expression of osteoblast metabolism-related genes in the tibia of laying hens. Feeding hens with the LCA diet upregulated (Figure 5A; $p < 0.05$) *ALP* expression compared to the NCA diet. However, hens injected with LPS showed a significant downregulation (Figure 5A; $p < 0.05$) in ALP expression levels compared to those injected with SAL.

The mRNA expression of *Ocn* was significantly influenced by the effect of diet × injection interaction (Figure 5B; $p < 0.05$). It was observed that the LCA + LPS group had a higher *Ocn* expression compared to the other treatment groups. However, the mRNA

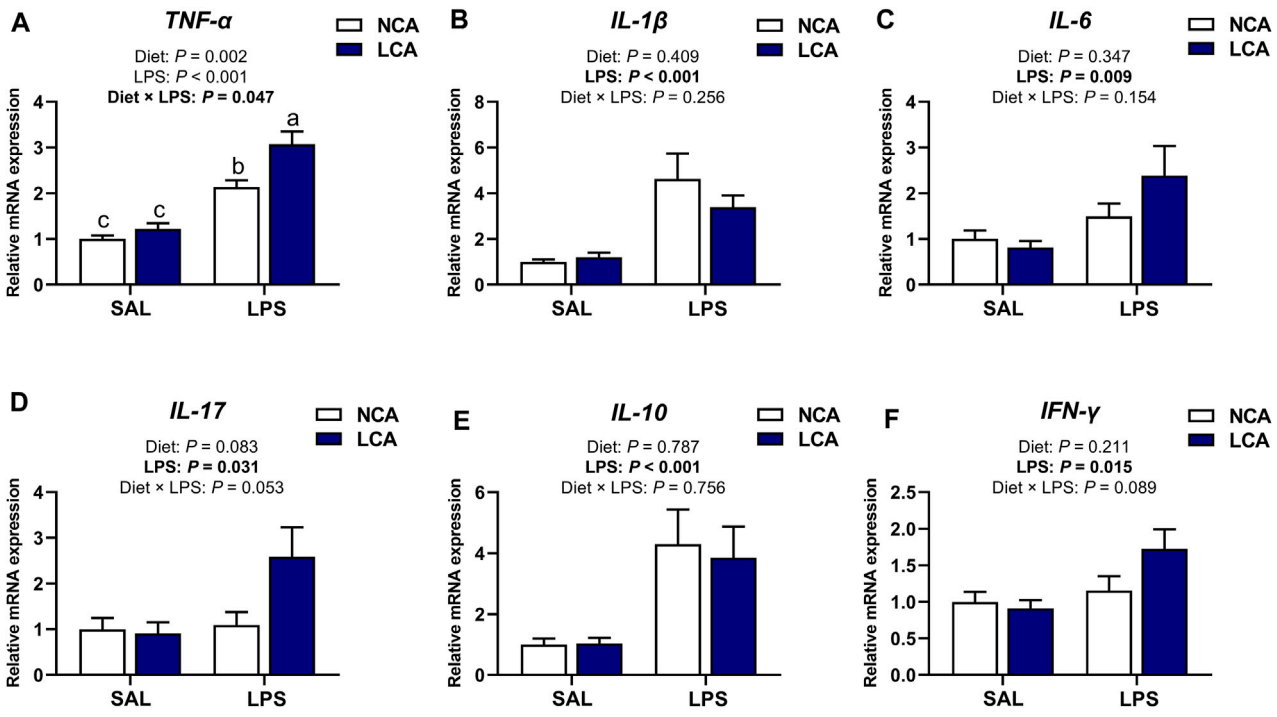

FIGURE 4
Effects of dietary calcium levels and LPS challenge on the relative mRNA expression of tibial inflammation-related genes in aged laying hens. NCA: Normal calcium diet (3.57% Ca); LCA: Low calcium diet (2.08% Ca). SAL: Saline; LPS: Lipopolysaccharide. **(A)** *TNF-α*; **(B)** *IL-1β*; **(C)** *IL-6*; **(D)** *IL-17*; **(E)** *IL-10*; **(F)** *IFN-γ*. Data are represented as mean ± standard error of mean (n = 8).

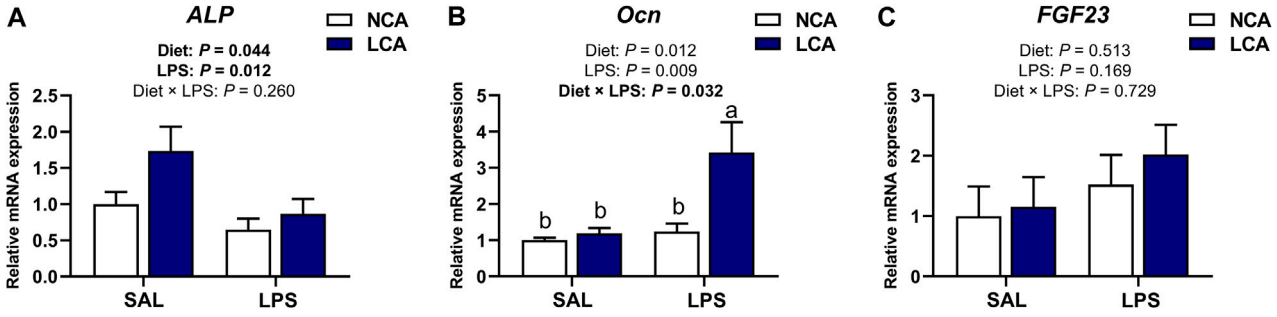

FIGURE 5
Effects of dietary calcium levels and LPS challenge on the relative mRNA expression of tibial osteoblast metabolism-related genes in aged laying hens. NCA: Normal calcium diet (3.57% Ca); LCA: Low calcium diet (2.08% Ca). SAL: Saline; LPS: Lipopolysaccharide. **(A)** *ALP*; **(B)** *Ocn*; **(C)** *FGF 23*. Data are represented as mean ± standard error of mean (n = 8). [a,b] Means with different alphabetical superscripts differ significantly, $p < 0.05$.

expression of *FGF23* was not influenced by the diet, injection or diet × injection interaction (Figure 5C; $p > 0.05$).

3.6 mRNA expression of tibial osteoclast metabolism-related genes

Figure 6 shows the mRNA expression of osteoclast metabolism-related genes in the tibia of laying hens. *TRAP* expression was changed by the interaction of diet × LPS (Figure 6A; $p < 0.05$) and the LCA + SAL group had an upregulated *TRAP* expression compared to the NCA + LPS and LCA + LPS groups.

The mRNA expression of Cathepsin K (***Cts K***) and matrix metallopeptidase 9 (***MMP-9***) were significantly affected by the main effect of injection. LPS injection downregulated the tibial expression of *Cts K* (Figure 6B; $p < 0.01$) and *MMP-9* (Figure 6C; $p < 0.001$) compared to the SAL-injected hens.

4 Discussion

The present study evaluated the effects of dietary Ca levels and immune challenge on the performance, egg quality, immune response, and bone quality of aged laying hens. Findings from this study revealed that the LPS challenge reduced hen-day egg

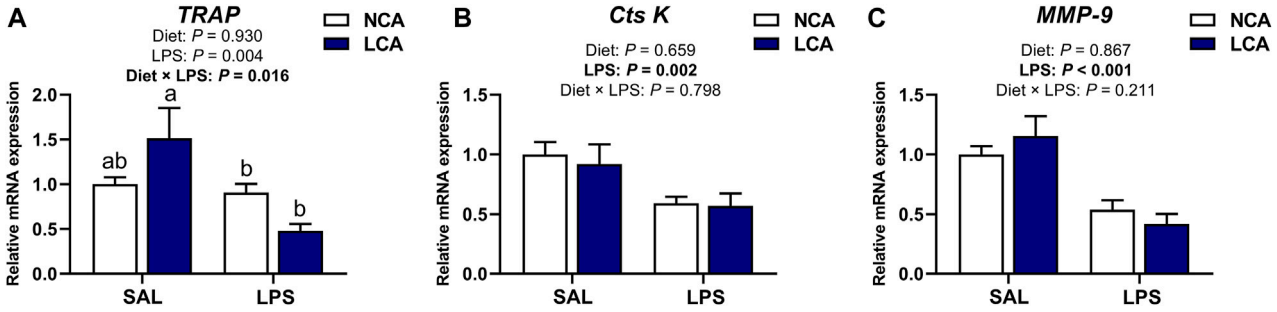

FIGURE 6
Effects of dietary calcium levels and LPS challenge on the relative mRNA expression of tibial osteoclast metabolism-related genes in aged laying hens. NCA: Normal calcium diet (3.57% Ca); LCA: Low calcium diet (2.08% Ca). SAL: Saline; LPS: Lipopolysaccharide. **(A)** *TRAP*; **(B)** *Cts K*; **(C)** *MMP-9*. Data are represented as mean ± standard error of mean (n = 8). [a,b] Means with different alphabetical superscripts differ significantly, $p < 0.05$.

production, deteriorated the eggshell quality, and induced an inflammatory response that altered bone immunity and homeostasis by suppressing osteoblasts and osteoclast activities in aged laying hens. Also, there was a significant interaction between low calcium diet and LPS challenge that induced the pro-inflammatory response of *IL-17* expression, as well as *Ocn* expression in the tibia of aged laying hens.

Several studies have shown that Ca supply is important for egg production and eggshell quality at various stages of lay. A considerable amount of Ca is required for shell formation since the eggshell constitutes ~40% Ca. However, Ca availability is largely dependent on dietary supply, intestinal absorption, and bone metabolism (Zhao et al., 2020; Hofmann et al., 2021). In a previous study, feeding low calcium diets (1.5% Ca) lowered the body weight, feed consumption, and egg production of laying hens (Zhao et al., 2020). In line with the present study, the dietary trial before LPS challenge revealed that the LCA diet depreciated the eggshell quality indices (including the eggshell weight, percentage, strength, and thickness), especially at 88 weeks of age. To corroborate this, feeding with LCA diet beyond 88 weeks of age decreased several performance and egg quality indices such as the hen-day egg production, egg mass, eggshell weight, eggshell percentage, eggshell strength, and eggshell thickness of aged hens. Thus, laying performance and eggshell quality of laying hens severely deteriorated with decreased dietary Ca supply during the extended laying phase. This may occur due to the crucial role of Ca in regulating reproductive processes and egg formation. This finding is supported by a previous report which stated that eggshell quality severely declined in hens fed a low calcium diet (Jiang et al., 2013). However, the dietary treatment in the present study did not affect the serum concentrations of ionized Ca and P, suggesting that the circulating metabolites were not strictly dependent on dietary Ca levels. Similarly, Zhao et al. (Zhao et al., 2020) reported that a low calcium diet decreased eggshell quality but without any detectable influence on serum Ca and P levels. Thus, the result suggests that circulating ionized Ca and P levels are tightly regulated by various factors in laying hens beyond dietary change. Interestingly, there was an interactive effect whereby NCA- and LCA-fed hens challenged with LPS, and LCA-fed hens injected with SAL, exhibited decreased Ca content in eggshells. This indicates that to some extent, Ca deprivation alongside exposure to immune challenge could pose detrimental effects on egg production in aged laying hens.

In the present study, the immune challenge with LPS administration also caused severe alterations to the production performance and eggshell quality of aged hens. LPS injection decreased the average daily feed intake, laying rate, and average daily egg mass. This inversely caused a disproportionate increase in the feed-to-egg ratio due to the fewer and smaller eggs. This finding corroborates the previous report that laying hens subjected to LPS-induced immune stress had poor egg quality traits and production performance including low feed intake, decreased egg production rate, and high feed-to-egg ratio (Nie et al., 2018).

In another study, maternal stimulation with LPS administration diminished the laying rate of hens and their offspring during the early and late laying stages (Liu et al., 2022). The depreciation in production performance during LPS challenge is credited to dysregulations in nutrient metabolism in order to eliminate potential threats from the body (Akbari MoghaddamKakhki and Kiarie, 2021). In the present study, LPS challenge also decreased the eggshell weight, eggshell percentage, eggshell thickness, and Ca and P content of the eggshell. Thus, exposure to immune stressors may alter the mineral metabolism and nutrient supply for eggshell formation. This can further aggravate eggshell deterioration in aged hens. Correspondingly, LPS administration was shown to decrease the eggshell percentage and eggshell weight of laying hens (Akbari MoghaddamKakhki and Kiarie, 2021). Importantly, egg production and eggshell quality are inextricably linked to Ca availability and bone metabolism in laying hens (Li et al., 2020; Zhao et al., 2020). Thus, bone homeostasis as influenced by Ca supply and immune challenge was further examined.

Bone homeostasis is achieved by a balance between bone resorption by osteoclasts and bone formation by osteoblasts (Tanaka et al., 2005). The bone mechanic parameter evaluated in the present study were unaffected while the Ca content of the tibia was even increased by LPS challenge, suggesting that the decreased bone Ca output. This observation was speculated to be related to the reduced egg production and in turn Ca deposit in eggshell. The H & E staining result indicated that LPS-challenged hens had more and larger adipocytes, suggesting that the connective tissue was dominated by adipocytes in LPS-hens. This suggested that bone health deteriorated by LPS challenge in aged laying hens. The

conclusion should be explained with caution as the quantification analysis was not conducted in the histological observation. In previous studies, low-calcium diet or age reduces bone quality (Jiang et al., 2019; Huang et al., 2020). In this study, however, the detrimental effect of low-calcium diet on bone parameters was not observed. The relative short experimental period and poor laying performance should be responsible for the different observations.

Cytokines are important in regulating bone homeostasis and immune functions (Horowitz, 1998). The differentiation and activation of the bone cells including the osteoclasts and osteoblasts are driven by cytokines and immune cells (Li et al., 2021). Thus, the role of various cytokines on bone metabolism during LPS-induced immune challenge was investigated. Administration of LPS upregulated the mRNA expression of *TNF-α, IL-1β,* and *IL-6* in the spleen thus validating the successful stimulation of the pro-inflammatory response and immune system activation in laying hens (Mireles et al., 2005; Bai et al., 2019). Similarly, the mRNA expression of *TNF-α*, *IL-17, IL-1β*, *IL-6*, *IL-10,* and *IFN-γ* was increased by LPS challenge in the tibia of aged hens. This indicates the establishment of both pro- and anti-inflammatory milieu within the bone matrix. This finding is in line with Roodman (Roodman, 1993), who explained that it was crucial to maintain an adequate pool of cytokines and immune cells for bone homeostasis. This is because the relative proportions of these factors in the bone microenvironment play a critical role in regulating the activity of osteoblasts and osteoclasts. In addition, it was found that the mRNA expression of *TNF-α*, *IL-17,* and *IFN-γ* tended to increase in the tibia of LCA-fed hens challenged with LPS injection. In a recent review, these immune cytokines (TNF-α, IL-17, and IFN-γ) were identified as particularly significant in stimulating bone resorption and suppressing bone formation, which ultimately leads to inflammatory bone loss (Li et al., 2021). Thus, it is evident that low Ca supply during immune challenge would exacerbate the incidence of bone loss and dysregulate bone homeostasis in aged laying hens.

In the present study, dietary Ca levels did not exert remarkable changes to the bone parameters and immune cytokine production, however, the serum ALP level was increased by LCA diet, whereas, decreased with LPS challenge. This was further validated with the mRNA expression analysis of osteoblast metabolism-related genes. The findings revealed that LCA diet upregulated the expression of *ALP* and *Ocn* expression in the tibia of aged laying hens. Within bone tissue, ALP is synthesized by osteoblasts and functions in osteoid formation and bone mineralization (Kumar et al., 2018). ALP is highly expressed in mineralized tissue and the serum ALP can be used as an index to examine bone formation, bone turnover, and metabolism (Zhan et al., 2019; Vimalraj, 2020). In addition, Ocn is a non-collagenous protein abundant in the bone matrix (Dirckx et al., 2019). Produced by osteoblasts, Ocn plays an important role in bone formation and mineralization (Wei et al., 2021). Therefore, coinciding with the findings of the bone microstructure, the increased ALP activity and expression of both *ALP* and *Ocn* by LCA diet indicated an increased osteoblast activity, extensive bone mineralization, and abnormal bone metabolism in aged laying hens. Additionally, *Ocn* expression was elevated in LCA-fed hens exposed to LPS challenge, revealing the role of immune stress in aggravating disorders of bone metabolism. Conversely, the administration of LPS caused an inhibitory reaction by decreasing ALP activity and gene expression. This suggests a reduction in osteoblast activity, which can potentially destabilize the bone matrix and phosphate supply for bone minerals during immune challenge (Blair et al., 2017). In primary mouse calvarial osteoblast cells, LPS stimulation suppressed osteoblast differentiation via inhibiting ALP activity and the expression of *Ocn* (Cai et al., 2019). It was also observed that the LPS challenge elicited opposing effects on the *ALP* and *Ocn* expression. These changes cannot be fully explained but may reflect metabolic adaptations during immune stress to achieve bone homeostasis. Although the osteocytes and osteoblasts are a significant source of circulatory FGF23 (Yoshiko et al., 2007), treatment with low Ca and LPS challenge did not exert profound changes to the bone *FGF23* expression in aged hens. An examination of osteoclast metabolism-related genes revealed that immune stimulation with LPS inhibited the mRNA expression of *TRAP*, *CtsK*, and *MMP-9*. TRAP is an enzyme that can degrade skeletal phosphoproteins and it is widely used as a marker of osteoclast activity (Hayman, 2008). Cts K can degrade type I collagen in osteoclastic bone resorption (Wilson et al., 2009), and MMP-9 is known to mediate bone resorption by cleaving type I and IV collagen from the bone organic matrix (Guo et al., 2019). The tibial mRNA expression of *TRAP*, *Cts K*, and *MMP-9* was downregulated with LPS challenge. These findings suggest that LPS challenge impaired bone osteoclast activity and bone resorption capacity in aged laying hens. Noteworthy, a limitation of the present study was that few hens were used per replicate to assess their physiological responses during LPS challenge. However, this was necessary to meet the experimental purpose and adapt to practical conditions with the intent of evaluating the immune responses of birds to LPS challenge. It is suggested that a large number of hens should be adopted for further studies to validate findings and meet commercial production standards. Moreover, in the present study, the experimental diet was designed to reduce Ca level with fixed P level, resulting in a changed Ca to P ratio. Hence, the result cannot be ascribed to low-calcium diet solely. The interaction of altered Ca/P ratio and LPS-challenge needs to be investigated further.

In conclusion, both dietary Ca levels and immune challenge are important factors to consider in aged laying hens, especially during the extended laying period. The findings from this study demonstrate that LPS challenge and/or LCA diet would negatively affects traits related to the production performance and eggshell quality of aged laying hens, and also significantly alter bone homeostasis. The LCA diet altered osteoblast activity, bone metabolism, and reduced eggshell quality. Importantly, LPS-induced immune responses dysregulated osteoblastic mineralization and inhibited osteoclast bone resorption capacity. LPS challenge may have altered bone metabolism via increasing bone Ca deposits, with a significant reduction in Ca output from the bone to meet the Ca needs for eggshell formation in aged hens. Therefore, further studies on the underlying mechanisms of dietary Ca on the osteo-immunity of aged laying hens are recommended to harness the production potentials of hens during extended lay.

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: https://www.ncbi.nlm.nih.gov/genbank/; NM_204524.1; NM_204628.1; NM_204460.1; NM_001004414.2; NM_205149.1; NM_205360.1; NM_205387.4; XM_425663.4; XM_015302697.2; NM_204971.2; NM_204667.1; NM_204305.1.

Ethics statement

The animal study was approved by Institutional Animal Care and Use Committee of Shandong Agricultural University. The study was conducted in accordance with the local legislation and institutional requirements.

Author contributions

XL: Data curation, Investigation, Writing–original draft. VU: Formal Analysis, Investigation, Writing–review and editing. HJ: Resources, Software, Writing–original draft. HL: Methodology, Validation, Visualization, Writing–review and editing. HL: Funding acquisition, Supervision, Writing–review and editing. XW: Methodology, Project administration, Writing–original draft. JZ: Data curation, Formal Analysis, Writing–review and editing. YZ: Methodology, Resources, Writing–review and editing.

Funding

The author(s) declare that financial support was received for the research, authorship, and/or publication of this article. This work was supported by the National Key Research Program of China (2021YFD1300405), the China Agriculture Research System (CARS-40-K09), and the Key Technology Research and Development Program of Shandong Province (2019JZZY020602).

Conflict of interest

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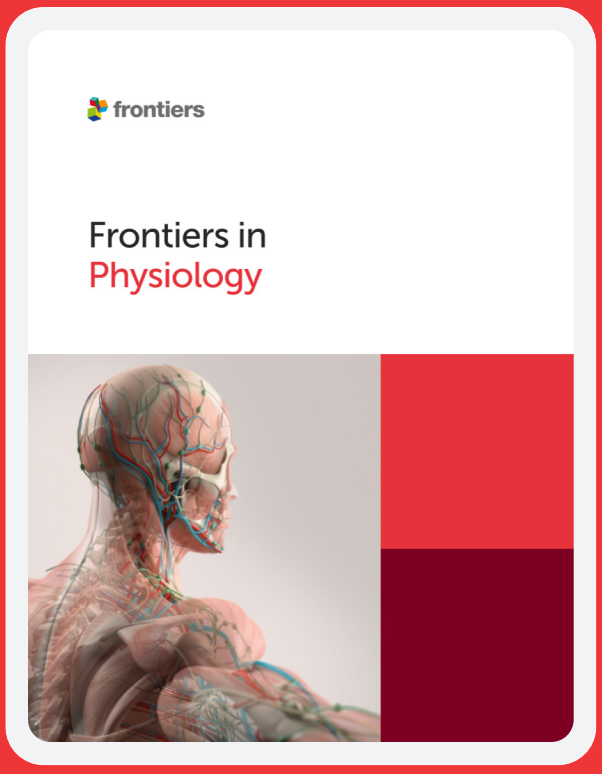

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