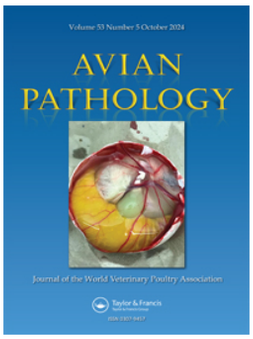

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ORIGINAL ARTICLE

Impact of liposomal hesperetin in broilers: prospects for improving performance, antioxidant potential, immunity, and resistance against *Listeria monocytogenes*

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ABSTRACT

Liposomal encapsulated phytogenics, such as liposomal hesperetin, are considered novel substitutes for antibiotics in the broiler industry owing to their improved nutritional and therapeutic properties. Therefore, our key goal was to investigate liposomal hesperetin impact on broiler growth performance, health, antioxidant status, tight junction proteins (TJP), and resistance against *Listeria monocytogenes*. Four broiler groups were fed 0, 150, 250, or 400 mg/kg of liposomal hesperetin-supplemented diets and experimentally infected with *L. monocytogenes* strain. Herein, liposomal hesperetin, especially at higher concentrations, augmented broilers FCR with upregulation of genes encoding TJP (occludin, *JAM-2*, *MUC-2*), and antioxidant attributes (*GPX-1*, *SOD-1*, *CAT*, *HO-1*, *NQO1*, *COX2*), which reflect enhancing health and welfare of broilers. Muscle antioxidant biomarkers were enhanced; meanwhile, muscle MDA, ROS, and H_2O_2 levels were reduced in response to 400 mg/kg of liposomal hesperetin. Liposomal hesperetin fortification reduced *L. monocytogenes* loads and expression levels of its virulence-related genes (*flaA, hlyA,* and *ami*). Remarkably, histopathological alterations in intestinal and brain tissues of *L. monocytogenes*-infected broilers were restored post-inclusion at higher levels of liposomal hesperetin, which reflects increase of the birds' resistance to *L. monocytogenes* infection. Transcription levels of genes encoding cytokines/chemokines (*MyD88*, *AVBD6*, *CCL20*, *IL-1β*, *IL-18*), and autophagy (*Bcl-2*, *LC3*, *AMPK*, *AKT*, *CHOP*, *Bip*, *p62*, *XBP1*) were ameliorated following dietary liposomal hesperetin fortification, which suggests enhancement of the birds' immunity and health. Collectively, our research recommends liposomal hesperetin application in broiler diets owing to its promoting impact on growth performance, antioxidant status, immunity, health, and welfare besides its antibacterial, and antivirulence characteristics to fight against *L. monocytogenes*.

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Introduction

Chicken meat is considered the most important and affordable source of birds protein, especially in developing countries, because it offers individuals high-quality protein at a reasonable cost and quick production time (Uzundumlu & Dilli, 2022). Modern intensive chicken production systems are linked to numerous stressors that impact the health, productivity, and reproductive abilities of chickens (Surai & Fisinin, 2016), as well as lowering their defence system and increasing the risk of bacterial infection (Meligy *et al.*, 2023). To overcome these stressors, enhancing the gastrointestinal barrier and altering the immune defence of birds is essential because the intestinal barrier is the initial point of dynamic contact between the host and enteric bacteria (Pastorelli *et al.*, 2013). It has been established that commensal bacteria can promote the gastrointestinal immune defence by acting as a barrier to prevent the invasion of exogenous bacteria and preventing the colonization of harmful pathogens, thereby avoiding the inflammation of the intestinal tract and

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maintaining intestinal integrity (Becattini *et al.*, 2017; Farahat *et al.*, 2021). Any unbalanced microflora can harm the birds' health by changing pH, causing dysbiosis, and promoting the growth of harmful pathogens (Michelland *et al.*, 2010; Ibrahim, Ismail, *et al.*, 2021). Additionally, numerous enteric bacteria are capable of overcoming commensal-mediated colonization resistance (Kamada *et al.*, 2013; Awad *et al.*, 2019).

Listeria monocytogenes is one of the most dangerous foodborne bacteria associated with listeriosis, and it is particularly challenging to track owing to its global distribution (Crespo *et al.*, 2013; Rothrock *et al.*, 2017; Abd El-Hamid *et al.*, 2022). Due to its high fatality rate (about 20%) and ability to cause meningitis, septicaemia, bacteraemia, gastroenteritis, perinatal infections, endocarditis, encephalitis, and abortions, listeriosis is considered a severe public health issue (Jacquet *et al.*, 2002; Elsayed *et al.*, 2022). Clinical disease in poultry is uncommon, even though many poultry species are susceptible to listeriosis (Crespo *et al.*, 2013), and the two main types of listeria infection are encephalitic and septicaemic (El-Demerdash *et al.*, 2024). Diarrhoea and emaciation are typical features of the septicaemic form, while neurologic signs like torticollis, incoordination, and depression characterize the encephalitic form (Crespo *et al.*, 2013). Of note, a previous study reported an outbreak of *L. monocytogenes* in a backyard poultry flock in the USA (Crespo *et al.*, 2013). It has been determined that *L. monocytogenes* is found in chicken, chicken products, and ready-to-eat chicken meat, which are considered a significant source of human listeriosis (Crespo *et al.*, 2013; Rothrock *et al.*, 2017). Numerous cases of human listeriosis outbreaks linked to eating contaminated food products have been documented (Upadhyay *et al.*, 2013). Due to its adaptability and persistence in various harsh conditions, its intracellular localization, the weak intracellular diffusion of certain medicines, and biofilm formation, *L. monocytogenes* is resistant to eradication, which is why it continues to be a severe concern in birds production and food processing (Upadhyay *et al.*, 2013; Stratakos *et al.*, 2020). Furthermore, during the gut stage of disease, the pathogenicity of *L. monocytogenes* originates from its capacity for adhesion, invasion, and translocation across the gastrointestinal barrier (Radoshevich & Cossart, 2017), in addition to the fact that it possesses several virulence attributes such as *hlyA* gene encoding listeriolysine O (Elsayed *et al.*, 2022), *flaA* gene encoding flagellin (Popowska, 2004), and *ami* gene encoding autolysin-adhesin amidase (Milohanic *et al.*, 2004). Thus, the best method to prevent mortality and limit the spread of the bacteria to deeper tissues is to restrict *L. monocytogenes* during the gut phase of infection. Antimicrobials can alter the gastrointestinal microbiome and reduce host immunity (Abd El-Hamid *et al.*, 2024), even though they are the preferred treatment for listeriosis. In addition, the development of multidrug-resistant pathogens and antimicrobial residues in meat were identified as major negative effects of antimicrobial overuse (Ammar, Abd El-Hamid, Hashem *et al.*, 2021; Ammar, Abd El-Hamid, *et al.*, 2021; Ammar *et al.*, 2022; Ibrahim *et al.*, 2024). Consequently, this has enabled the development of novel antimicrobial substitutes, including phytogenics (Ammar, El-Naenaeey, El-Hamid *et al.*, 2021; Abdel-Raheem *et al.*, 2023) as a preventive measure against listeriosis.

Phytogenics, which are secondary metabolites produced by plants as a result of their interactions with their environment, are naturally occurring alternatives to antibiotics (Ammar, El-Naenaeey, El-Malt, *et al.*, 2021; Ibrahim, Shahin, *et al.*, 2022). Due to their potential benefits for growth performance, antioxidant status, nutrient utilization, digestibility, quality of flesh, microbial loads, immunity, and general health status, phytogenics like flavonoid polyphenolic compounds can be used as dietary additives in poultry diets (Aljazzar *et al.*, 2022; Abd El-Hamid *et al.*, 2024). These advantageous properties stem from their ability to improve gut integrity and mucosal barriers, which boosts digestion and host immunity (Hashem *et al.*, 2022). Hesperetin, an aglycone derivative of hesperidin, is a citrus flavonoid from the flavanones subclass, which is mostly present in citrus fruits including oranges, lemons, and grapefruits (Yap *et al.*, 2021; Ortiz *et al.*, 2022). Hesperetin has a two-fold greater bioavailability than hesperidin with potential antimicrobial, antioxidant, anti-inflammatory, anti-cholesterolaemic, cardioprotective, anticancer, and antidiabetic properties (Gandhi *et al.*, 2020; Yap *et al.*, 2021; Ortiz *et al.*, 2022). Notably, hesperetin has been utilized as a dietary supplement in poultry diets due to its potential growth-enhancing, immunostimulant, antioxidant, anti-stress, and anti-inflammatory activities (Yatao *et al.*, 2018; Kamboh *et al.*, 2019), in addition to enhancing broiler meat quality (Goliomytis *et al.*, 2015; Kamboh *et al.*, 2019), improving broiler gastrointestinal health (Kamboh & Zhu, 2014), and lowering the cholesterols and triglyceride levels in chicken serum, meat, and eggs (Ting *et al.*, 2011; Kamboh *et al.*, 2019). Nevertheless, to the best of our knowledge, there has been no research investigating the impact of hesperetin on broilers experimentally challenged with *L. monocytogenes.*

Of note, the low water solubility of hesperetin may reduce its effectiveness. Hence, solubilizing agents like dimethyl sulfoxide (DMSO), which has previously been shown to exhibit toxicity, are necessary for applying this molecule (Wolfram *et al.*, 2016). Thus, to enhance the biological activity, efficiency, and stability of phytogenics including hesperetin, and get around

their limitations, liposome, a spherical colloid structure composed of an internal aqueous area and phospholipid bilayer membranes, might be utilized to encapsulate and control their release (Wolfram *et al.*, 2016; Meligy *et al.*, 2023). Liposomes are regarded as biocompatible carriers for the delivery of both hydrophilic and lipophilic bioactive substances (Emami *et al.*, 2016; Kishawy *et al.*, 2023). The liposomal encapsulation of hesperetin has been demonstrated to enhance its stability, effectiveness, and bioavailability via interacting with phospholipid membranes, most likely by incorporating it within acyl chains (Wolfram *et al.*, 2016). However, there has been no research on using hesperetin liposomes in broiler diets as far as we are aware. In light of the above, our key target was to explore, for the first time, the *in vivo* impact of liposomal hesperetin on broiler' growth performance, health, and the antioxidant potential of breast muscle, in addition to investigating its effect on immunological, and biochemical parameters, *L. monocytogenes* count, and the transcription levels of genes encoding virulence, antioxidant, tight junction, cytokines, chemokines, and autophagy after challenge with virulent *L. monocytogenes* strain.

Materials and methods

Ethical approval

The current study was carried out per the guidelines and approved standards of the Institutional Animal Care and Use Committee (IACUC), Faculty of Veterinary Medicine, Zagazig University, Egypt with the reference number ZU-IACUC/2/F/192/2022.

Liposomal hesperetin preparation and characterization

Hesperetin and cholesterol were obtained from Sigma-Aldrich (St. Louis, MO, USA). Employing ethanol for hesperetin and chloroform for lipids, liposomes, and liposomal-loaded hesperetin were made according to the earlier reported methods (Bonechi *et al.*, 2012; Kishawy *et al.*, 2023). In brief, the hydration process involved vapourizing the solvents in a vacuum for an entire night, followed by hydration for 45 min at 60°C and pH 7.5 in phosphate-buffered saline to achieve a dry lipid layer with or without hesperetin. A cautious extrusion was made of the resulting liposomal suspension, then, a 200 nm pore-size polycarbonate membrane filter was employed to achieve uniform liposomes (Alhawas *et al.*, 2023). After diluting the prepared specimen with deionized water, a drop of the diluted specimen was placed on a carbon–copper grid and allowed to evaporate at room temperature. Using a transmission electron microscope (FE-TEM; JEM 2100 F, JEOL, Tokyo, Japan) operating at a 200 kV accelerating voltage, the morphological analysis of liposomal-loaded hesperetin was performed (Figure 1(A)). Moreover, structural characterization of liposomal-loaded hesperetin using Fourier transform infrared spectroscopy was carried out (Figure 1(B)).

Diets and experimental design

Two hundred and forty Ross 308 1-day-old male broiler chicks were procured from a local hatchery. Chicks were weighed upon arrival and split into four equal experimental groups at random with six replicates in each group, each with 10 chicks. The four experimental groups included a control group that was offered the basal diet and three other groups that received a diet supplemented with graded levels of liposomal hesperetin including 150, 250, and 400 mg/kg diet. Throughout the 38-day experimental trial, the experimental diet was uniformly distributed throughout the feed by spraying following the pelleting process, and all broilers were allowed access to water and feed *ad libitum*. As indicated in Table 1, all broilers were fed coccidiostat- and-antibiotic-free meals for the starter (1–10 days), grower (11–20 days), and finisher (21–38 days) phases as per the criteria of Ross 308 broiler nutrition specification (Aviagen, 2018). Chemical analysis was performed on all feedstuff and diets to ascertain the ether extract, crude fibre, crude protein, and moisture content following the regulations of the Association of Official Analytical Chemists (AOAC, 2012).

Growth performance parameters

At the end of the starter, grower, and finisher phases, the bodyweight (BW) and feed intake (FI) were determined to calculate the bodyweight gain (BWG) and feed conversion ratio (FCR) as earlier pronounced (Ibrahim, Eldemery, *et al.*, 2022). Throughout the entire rearing period, the cumulative BWG, FI, and FCR were computed (Aljazzar *et al.*, 2022; Hashem *et al.*, 2022).

Collection of samples

At 24 days of age (before the challenge), the breast muscles were aseptically collected from chicks (four chicks/replicate) for analyses of antioxidant enzyme activities. Moreover, at 14 days post-infection (dpi) with the *L. monocytogenes* strain, blood specimens were aseptically taken from the wing veins of chicks (four chicks/ replicate). Blood specimens were aseptically collected in a sterile centrifuge tube devoid of an anticoagulant and centrifuged for 10 min at 1509 × g rpm to separate the sera. The separated serum was then stored at −20°C for subsequent biochemical

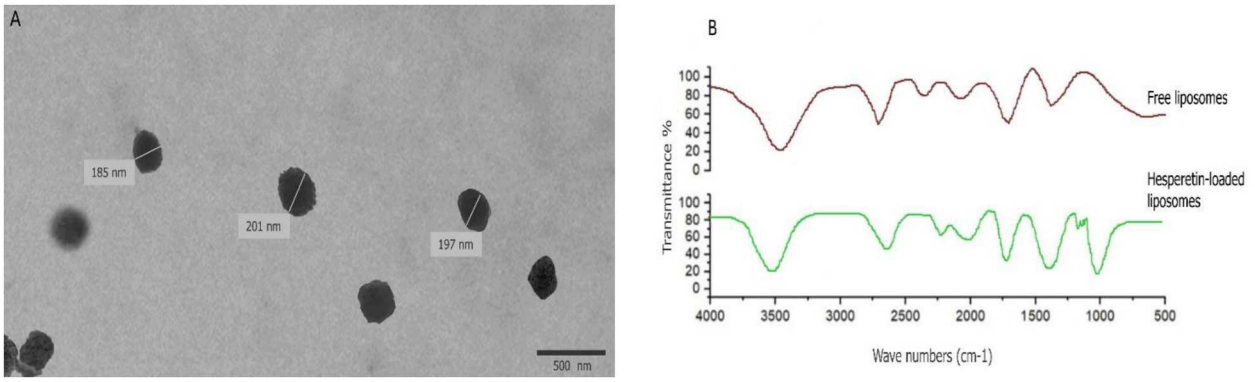

Figure 1. Transmission electron microscopy (A) and Fourier transform infrared spectrum (B) of hesperetin-loaded liposomes.

tests. Four chicks per replicate were randomly taken, and sacrificed, then, the intestinal tissues, and breast muscles were aseptically collected for analyses of immunological parameters, and the expression of genes related to inflammation, tight junction protein (TJP), antioxidant, and autophagy by reverse transcription-quantitative polymerase chain reaction (RT-qPCR) assay at 14 dpi. Additionally, the expression levels of *L. monocytogenes* virulence genes were determined in the intestinal tissues via RT-qPCR assay at 7 and 14 dpi. At 7 and 14 dpi, the caecum, liver, spleen, and brain tissues were aseptically obtained for determining the *L. monocytogenes* count via quantitative real-time PCR (RT–PCR) assay. Finally, at 14 dpi, intestinal and spleen tissues were collected and fixed in neutral buffered formalin 10% for 48 h for histopathological analysis.

Table 1. Ingredients and nutrition levels of the experimental control diet.

Ingredient, %	Starter	Grower	Finisher
	(1–10 days)	(11–20 days)	(21–38 days)
Soybean meal, 48%	34.40	30.80	25.80
Yellow corn	59.00	61.50	65.50
Soybean oil	1.80	3.00	4.00
Common salt	0.30	0.30	0.3
Calcium diphasic phosphate	1.50	1.50	1.50
Calcium carbonate	1.20	1.20	1.20
Anti-mycotoxin	0.10	0.10	0.10
Choline chloride	0.20	0.20	0.20
Premix*	0.90	0.90	0.9
DL-Methionine, 99 %	0.25	0.2	0.2
L-Lysine HCL, 78 %	0.35	0.3	0.3
Nutrient composition			
Methionine, %	0.58	0.51	0.49
Lysine, %	1.45	1.29	1.16
Available phosphorous, %	0.53	0.50	0.48
Ca, %	1.20	1.19	1.17
CF, %	2.63	2.56	2.46
EE, %	4.33	5.6	6.62
CP, %	23.01	21.5	19.50
ME (Kcal/kg)	3106	3103	3200

*Premix: each kilogram diet contained the following vitamins: Mn (oxide and sulphate), 100 mg; Zn (oxide and sulphate), 120 mg; I (iodide), 1.20 mg; Se (selenate), 0.3 mg; Cu (sulphate), 14 mg; Fe (sulphate), 30 mg; cyanocobalamin, 15 µg; biotin, 300 µg; pyridoxine, 6 mg; folate, 3 mg; niacin, 50 mg; pantothenate, 12 mg; thiamine, 4 mg; riboflavin, 7 mg; menadione, 2.5 mg; cholecalciferol, 6000 IU; tocopherol acetate, 70 mg; and retinol, 10.000 IU. Ca: calcium; CF: crude fibre; EE: ether extract; CP: crude protein; and ME: metabolizable energy.

Antioxidant evaluation

At 24 days of age (before challenge), the levels of total antioxidant capacity (T-AOC) and malondialdehyde (MDA), in addition to the concentrations of antioxidant enzymes such as catalase (CAT), superoxide glutathione peroxidase (GPX), and dismutase (SOD) in breast muscle were assessed utilizing commercial kits (Nanjing Jiancheng Bioengineering Institute, Nanjing, China) in accordance with the manufacturer's manuals. Moreover, the levels of reactive oxygen species (ROS) and hydrogen peroxide (H_2O_2) in breast muscle, at 24 days of age (before challenge), were determined as previously pronounced (Ibrahim, Abd El-Hamid *et al.*, 2022; El-Ghareeb *et al.*, 2023). The total flavonoids and phenolic contents (TFC, and TPC, respectively) in breast muscle, at 24 days of age (before challenge), were measured following the methods developed by Ibrahim, Moustafa *et al.* (2021).

Listeria monocytogenes *challenge trial*

The MDR multi-virulent *L. monocytogenes* strain employed in the present experimental trial was identified phenotypically after being isolated from clinically diseased chickens using standard bacteriological procedures as previously described (Hitchins & Whiting, 2001; Markey *et al.*, 2013). In brief, the cloacal swab samples were collected in Listeria enrichment broth (Oxoid, Cambridge, UK), and incubated under microaerophilic circumstances for 18 h at 37°C. Then, 0.1 ml of the enrichment broth was inoculated onto the surface of Listeria-selective agar base (Oxoid), and incubated at 37°C for 24 h. The obtained colonies underwent microscopic examination after being stained with Gram's stain. Several biochemical assays, including urease, oxidase, catalase, xylose, sucrose, and lactose fermentation, were used to carry out a definitive identification in accordance with the Food, and Drug Administration (FDA) bacteriological analytical manual. The strain was then confirmed by the CAMP (Christie–Atkins–Munch–Peterson) test, hemolysis onto sheep blood

agar (Oxoid), and umbrella-shaped motility techniques. According to the earlier published protocol, the obtained strain was molecularly confirmed using the *16S rRNA* gene-based PCR technique (Kumar *et al.*, 2015). Furthermore, the strain's antibiogram pattern was examined following the European Committee on Antimicrobial Susceptibility Testing (EUCAST) standard (EUCAST, 2023), and the results indicated that the strain was resistant to gentamycin, vancomycin, tetracycline, erythromycin, clindamycin, cefoxitin and chloramphenicol, being MDR. Furthermore, the strain was confirmed to be multi-virulent by PCR assay for the detection of virulence-related genes including *hlyA*, *flaA*, and *ami* genes using primers and previously published PCR cycling techniques (Jiang *et al.*, 2010; Larsen & Jespersen, 2015; Elsayed *et al.*, 2022).

In order to increase the pathogenicity of the infecting strain, it was enriched in Listeria enrichment broth (Oxoid), passed twice in healthy chicks, and then re-isolated from sacrificed chicks for the experimental trial. Before initiating the challenge trial, all chicks were examined bacteriologically using cloacal swabs to isolate and identify *L. monocytogenes* phenotypically and molecularly, as previously described. Following that, the inoculum suspension was made to achieve a concentration of 10^7 CFU/ml (Abd El-Hamid *et al.*, 2022). Each chick in the experimental groups received 1 ml of the prepared *L. monocytogenes* inoculum orally at 24 days of age. By examining the characteristic clinical signs and *post-mortem* lesions of the sacrificed chicks, in addition to re-isolating and identifying the utilized *L. monocytogenes*, the infection was confirmed.

Biochemical and immunological investigations

With the aid of analytical kits (Spinreact Co., Santa Coloma, Spain), the serum concentrations of aspartate and alanine aminotransferase (AST and ALT), total triglycerides (TG), total cholesterol (TC), and low-density lipoprotein (LDL) were determined in accordance with the manufacturer's protocols at 14 dpi.

At 14 dpi, the intestinal levels of immune-related markers such as myeloperoxidase (MPO), lysozyme (LYZ), and C-reactive protein (CRP) were measured as previously mentioned (Alhawas *et al.*, 2023). Furthermore, the level of immunoglobulin G (IgG), complement proteins C3, and C4, and proinflammatory cytokines, including tumour necrosis factor-alpha (TNF-α) and interleukin-6 (IL-6), in the intestinal tissues were detected via the enzyme-linked immunosorbent assay (ELISA) kits (Sigma Aldrich) according to the instructions of the manufacturer at 14 dpi (Alandiyjany *et al.*, 2022; Liu *et al.*, 2022).

Quantification of Listeria monocytogenes *DNA copies by quantitative real-time PCR assay*

Total DNA extraction was done utilizing the QIAamp Fast DNA Stool Mini kit (Qiagen, Hilden, Germany) from the caecal, liver, spleen, and brain samples at 7 and 14 dpi. Using a Thermo Scientific NanoDrop 2000 spectrophotometer (Thermo Fisher Scientific Inc., Waltham, MA, USA), the concentration and quality of the extracted DNA were evaluated. The purified DNA specimens were finally kept at −80°C for subsequent quantitative PCR investigation. Utilizing a Stratagene MX3005P quantitative PCR machine, RT–PCR techniques were carried out to determine the *L. monocytogenes* populations. The sequences of primers utilized in the RT–PCR targeting the *16S rRNA* gene of *L. monocytogenes* are shown in Table 2. The PCR amplification procedures were done in triplicate utilizing the QuantiTect SYBR Green PCR Master Mix (Qiagen) according to the manufacturer's guidelines. Genomic DNA obtained from pure bacterial cultures was 10-fold serially diluted to create the standard calibration curves. These standard curves were used to calculate the target genomic DNA copies and *L. monocytogenes* quantities were represented as $\log_{10}$ colony forming units (CFU)/gram of specimens.

Expression analysis of genes encoding tight junction proteins, cytokines, autophagy, and Listeria monocytogenes *virulence via reverse transcription-quantitative PCR assay*

At 14 dpi, intestinal tissues and breast muscle were utilized to measure the transcription levels of genes encoding TJP [occludin, and junctional adhesion molecule-2 (*JAM-2*)], gut barrier functions [mucin-2 (*MUC-2*)], chemokines, and cytokines [myeloid differentiation factor 88 (*MyD88*), avian β-defensin 6 (*AVBD6*), chemokine C–C motif ligand 20, also referred to as macrophage inflammatory proteins-3 (*CCL20*), interleukin-1β (*IL-1β*), *IL-10*, and *IL-18*], antioxidant attributes [glutathione peroxidase 1 (*GPX-1*), superoxide dismutase 1 (*SOD-1*), catalase (*CAT*), haemoxygenase-1 (*HO-1*), NAD(P)H dehydrogenase quinone 1 (*NQO1*), and cyclooxygenase-2 (*COX2*)], as well as autophagy [B cell lymphoma-2 (*Bcl-2*), microtubule-associated protein 1 light chain 3 (*LC3*), adenosine monophosphate-activated protein kinase (*AMPK*), serine/threonine kinase (*AKT*), transcriptional factor C/EBP homologous protein (*CHOP*), binding immunoglobulin protein (*Bip*), gene encoding a ubiquitin chain binding protein (*p62*), and x-box binding protein 1 (*XBP1*)]. Additionally, the transcription levels of the *L. monocytogenes* virulence genes (*hlyA*, *flaA*, and *ami*) were analysed using caecal samples at 7 and 14 dpi. The QIAamp RNeasy Mini kit (Qiagen) was used to extract RNA in accordance with the manufacturer's recommendations.

Table 2. Sequences of primers used in PCR assays for the examined genes.

Specificity/ Target gene	Primer sequence (5′-3′)	Accession No./Reference	Amplification efficiency (%)
Housekeeping			
GAPDH	F-GGTGGTGCTAAGCGTGTTA R-CCCTCCACAATGCCAA	NM205518	99.12
Listeria monocytogenes			
16S rRNA	F-CCTTTGACCACTCTGGAGACAGAGC R-AAGGAGGTGATCCAACCGCACCTTC	Lantz *et al.* (1994)	97.43
Virulence mediators			
hlyA	F-GCATCTGCATTCAATAAAGA R-TGTCACTGCATCTCCGTGGT	Elsayed *et al.* (2022)	98.20
flaA	F-TTACTAGATCAAACTGCTCC R-AAGAAAAGCCCCTCGTCC	Jiang *et al.* (2010)	95.41
ami	F-CGATGAATTTGCTCGTTCTATTAATAAC R-TCACTGTGCGCGCTATCAA	Larsen & Jespersen (2015)	97.48
Cytokines and chemokines			
IL-10	F: GCTGAGGGTGAAGTTTGAGG R: AGACTGGCAGCCAAAGGTC	XM_025143715.1	98.65
IL-18	F- AGGTGAAATCTGGCAGTGGAAT R- TGAAGGCGCGGTGGTTT	Kapczynski *et al.* (2014)	97.76
IL-1β	F: GCTCTACATGTCGTGTGTGATGAG R: TGTCGATGTCCCGCATGA	NM_204524	98.32
AVBD6	F-GCCCTACTTTTCCAGCCCTATT R-GGCCCAGGAATGCAGACA	NM 001001193.1	98.90
CCL20	F-AGGCAGCGAAGGAGCAC R-GCAGAGAAGCCAAAATCAAAC	NM_204438	99.61
MyD88	F-ATTCCGGTCAAGTGCAAGAC R-ATCACGGCAGCAAGAGAGAT	Karnati *et al.* (2015)	98.97
Autophagy			
Bcl-2	F- AAGCTGCTTGGGAAATGGCA R- TTTCACCGAAAAGAGCCCGC	NM_205339.3	97.79
LC3	F- GCTGCCAGTGCTGGACAAGAC R- TCCTCATCCTTCTCCTGCTCGTAG	Liu *et al.* (2021)	99.60
AMPK	F-AATTCGCAGGGAGATTCAGA R-ACAGCTCTCCTCCAGAAACG	Chen *et al.* (2022)	97.90
AKT	F-CACAGCAGTTTGGCAAGGTC R-CCTTTTGTGGACCCTTCTGC	Li *et al.* (2022)	97.54
CHOP	F- CAGGAAGAAGAGCTGGCCCCACT R- TGCTGTGCTCGCCGTGCTGT	Liu *et al.* (2021)	98.76
Bip	F-CAGACCGATGGGAATCGGAG R-GCCTTCTCTCGTTCCAGGTC	Wang *et al.* (2019)	97.13
p62	F-GCTGATGCAGTGGAGGAAGTAGAG R-GGAAGCACAGATCGGCTGGAAG	Chen *et al.* (2022)	97.76
XBP1	F-GCGAGTCTACGGATGTGAAGGA R-TGTGGAGGTTGTCAGGAATGGT	NM_001006192	96.87
Gut barrier functions and tight junction proteins			
MUC-2	F-AAACAACGGCCATGTTTCAT R- GTGTGACACTGGTGTGCTGA	NM_001318434	97.49
Occludin	F-ACGGCAAAGCCAACATCTAC R- ATCCGCCACGTTCTTCAC	XM_031604121.1	98.30
JAM-2	F-AGACAG GAACAGGCAGTGCT R- TCCAATCCCATTTGA GGCTA	XM_031556661.1	98.90
Antioxidant attributes			
GPX-1	F-AACCAATTCGGGCACCAG R-CCGTTCACCTCGCACTTCTC	HM590226	98.20
CAT	F-GGGGAGCTGTTTACTGCAAG R-GGGGAGCTGTTTACTGCAAG	NM_001031215.2	97.54
SOD-1	F-GGCAATGTGACTGCAAAGGG R-CCCCTCTACCCAGGTCATCA	NM_205064.1	97.85
COX2	F-TGTCCTTTCACTGCTTTCCAT R-TTCCATTGCTGTGTTTGAGGT	NM_001167718.1	98.53
NQO1	F-TCGCCGAGCAGAAGAAGATTGAAG R-CGGTGGTGAGTGACAGCATGG	NM_001277620.1	96.11
HO-1	F-AAGAGCCAGGAGAACGGTCA R-AAGAGCCAGGAGAACGGTCA	NM_205344	96.95

GAPDH: glyceraldehyde 3-phosphate dehydrogenase; *hlyA*: listeriolysine O gene; *flaA:* flagellin gene; *ami*: autolysin amidase gene; *JAM-2*: junctional adhesion molecule-2; *MUC-2*: mucin-2; *GPX-1:* glutathione peroxidase 1; *SOD-1:* superoxide dismutase 1; *CAT:* catalase; *HO-1:* haemoxygenase-1; *NQO1:* NAD(P)H dehydrogenase quinone 1; *COX2:* cyclooxygenase-2; *MyD88:* myeloid differentiation factor 88: *AVBD6:* avian β-defensin 6; *CCL20:* chemokine C–C motif ligand 20; *IL*: interleukin; *Bcl-2:* B cell lymphoma-2; *LC3:* microtubule-associated protein 1 light chain 3; *CHOP*: transcriptional factor C/EBP homologous protein; *XBP*1: x-box binding protein 1; *Bip:* binding immunoglobulin protein; *AMPK:* adenosine monophosphate-activated protein kinase; *p62*: gene encoding a ubiquitin chain binding protein; *AKT*: gene encoding serine/threonine kinase.

The purity and concentration of the RNA were assessed using A NanoDrop 2000 spectrophotometer. One-step RT-qPCR tests were performed in triplicate using a QuantiTect SYBR Green RT–PCR Kit (Qiagen) on the Strata-gene MX3005P real-time PCR amplification system. Melting curve analysis was used to confirm the specificity of each PCR amplification. The transcript levels of the investigated genes were compared to those of the endogenous controls, glyceraldehyde 3-phosphate dehydrogenase

(*GAPDH*), and *L. monocytogenes 16S rRNA* genes. To evaluate the relative alterations in gene expression levels, the $2^{-\Delta\Delta Ct}$ method was employed (Livak & Schmittgen, 2001). Table 2 displays the gene-specific primer sequences used in the RT-qPCR test.

Histomorphological analysis

Fixed intestinal and brain tissues were cut, rinsed with fresh water, dehydrated in increasing concentrations of ethanol (70–100%), transparentized in xylene, and finally impregnated with paraffin wax. Thin slices (5 µm in thickness) of paraffin-impregnated tissues were obtained by using an automated microtome and then stained with routine haematoxylin and eosin (H&E) and viewed under a light microscope with a computerized digital camera (Suvarna *et al.*, 2018). Lesions were identified and reported after stained slides underwent analysis.

Statistical analysis

All collected data were statistically examined utilizing the general linear model (GLM) of SPSS Inc. program version 22 (IBM Corp., Armonk, NY, USA). Shapiro-Wilk's test was used to determine the normality among the treatment groups, and Levene's test was used to determine the homogeneity. The data were represented as standard error of means (SEM) with a significance level of $P \leq 0.05$. To determine whether there were any significant variations between the mean values of the treatment groups, Tukey's test was applied. The GraphPad Prism software version 8 (San Diego, CA, USA) was used to create all graphs.

Results

Growth performance traits

Table 3 illustrates the outcomes of broiler growth performance traits after dietary fortification with graded levels of liposomal hesperetin. During the starter period, broilers supplemented with dietary liposomal hesperetin at concentrations of 400, and 250 mg/kg presented highly significant ($P < 0.001$) enhancement in BWG (326.08 g, and 317.18 g, respectively), and FCR (1.16, and 1.21), unlike the control group (303.82 g, and 1.238, respectively). Meanwhile, during the starter period, the BWG and FCR of broilers fortified with 150 mg/kg of liposomal hesperetin (1304.56 g, and 1.243, respectively) and those fed a basal control diet (303.82 g, and 1.238, respectively) presented no significant differences. The BWG and FCR levels were significantly improved ($P < 0.001$) during the grower period in all groups fortified with dietary liposomal hesperetin compared with the control group. During the grower period, broilers fortified with dietary liposomal hesperetin at levels of 400 and 250 mg/kg showed the most remarkable ($P < 0.001$) enhancement in the levels of BWG (951.83 g, and 938.35 g, respectively) and FCR (1.68, and 1.73, respectively) when compared with the control group (718.27 g, and 2.14, respectively). During the finisher and the overall rearing periods, the BWG, and FCR levels were significantly ($P < 0.001$) ameliorated in all groups fortified with liposomal hesperetin in a dose-dependent way concerning the control group. Notably, during the finisher and total growing periods, broilers offered dietary liposomal hesperetin supplementation at a concentration of 400 mg/kg exhibited the most remarkable ($P < 0.001$) enhancement in the BWG (1231.64 g and 2509.4 g, respectively), and FCR (1.74, and 1.64, respectively) compared with the control group, which reflects its enhancing impact on the performance and overall health of broilers.

Analysis of antioxidant and oxidative status in breast muscle of broilers

The effects of various levels of dietary liposomal hesperetin on oxidative and antioxidant marker activities in broiler breast muscle samples are shown in Table 4. Remarkably, compared to the control group, the breast muscle samples of broilers fortified with various concentrations of liposomal hesperetin exhibited significant ($P < 0.001$) augmentation in the activities of CAT, GPX, and SOD. Additionally, the most prominent ($P < 0.001$) enhancement in the activities of GPX (277.82, and 281.67 µmol/mg) and SOD (109.59, and 102.5 µ/ml) was presented in the breast muscle samples of broilers supplemented with liposomal hesperetin at levels of 400 and 250 mg/kg, respectively, compared with the control group (154.38 µmol/mg, and 41.85 µ/ml, respectively). Of note, there were no significant variations in the activities of CAT among all groups supplemented with liposomal hesperetin. Furthermore, the activities of T-AOC were significantly ($P < 0.001$) increased across all groups fortified with dietary liposomal hesperetin as the concentration of liposomal hesperetin supplementation elevated. The most significant elevation in the level of T-AOC was seen in broilers offered dietary liposomal hesperetin inclusion at a level of 400 mg/kg (3.37 U/mg prot), when compared with the control group (1.62 U/mg prot). Notably, the total phenolic and flavonoid contents were remarkably enhanced ($P < 0.001$) in the breast muscle samples of all groups offered dietary liposomal hesperetin, unlike the control group. Additionally, broilers offered 400 and 250 mg/kg exhibited the most significant ($P < 0.001$) augmentation in the levels of TPC (166.66, and 162.99 µg/g, respectively) and TFC (149.37, and 144.78 µg/g, respectively) concerning the control group (80.77 and 64.44 µg/g, respectively). Meanwhile,

Table 3. Effect of graded levels of liposomal hesperetin on broiler growth performance traits (starter, grower, finisher, and overall rearing periods).

	Experimental groups					
Parameter	Control	I	II	III	*P*-value	SEM
Starter period (1–10 days)						
Initial BW	43.4	43.2	43.4	43.8	0.994	0.55
BW, g/bird	347.22^{c}	347.76^{c}	360.58^{b}	369.88^{a}	<0.001	1.5
BWG, g/bird	303.82^{c}	304.56^{c}	317.18^{b}	326.08^{a}	<0.001	1.47
FI, g/bird	347.22^{c}	347.76^{c}	360.58^{b}	369.88^{a}	<0.001	1.5
FCR	1.238^{a}	1.243^{a}	1.21^{b}	1.16^{c}	<0.001	0.003
Grower period (11–20 days)						
BW, g/bird	1065.6^{c}	1211^{b}	1298.8^{a}	1322^{a}	<0.001	8.24
BWG, g/bird	718.27^{c}	863.24^{b}	938.35^{a}	915.83^{a}	<0.001	8.5
FI, g/bird	1534.4^{b}	1542.8^{b}	1620^{a}	1602.8^{a}	< 0.001	9.51
FCR	2.14^{a}	1.79^{b}	1.73^{bc}	1.69^{c}	<0.001	0.017
Finisher period (21–38 days)						
BW, g/bird	1970.6^{d}	2209.4^{c}	2436.8^{b}	2553.2^{a}	<0.001	5.03
BWG, g/bird	905.37^{d}	998.55^{c}	1138.11^{b}	1231.64^{a}	<0.001	8.54
FI, g/bird	2298.6^{a}	1972.2^{d}	2054^{c}	2140.8^{b}	<0.001	15.11
FCR	2.54^{a}	1.97^{b}	1.81^{c}	1.74^{d}	<0.001	0.01
Overall rearing period						
BW, g/bird	1970.6^{d}	2209.4^{c}	2436.8^{b}	255302^{a}	<0.001	5.03
BWG, g/bird	1927.2^{d}	2166.2^{c}	2393.4^{b}	2509.4^{a}	<0.001	5.22
FI, g/bird	4209.4^{a}	3893.8^{d}	4057.8^{c}	4122.2^{b}	<0.001	11.43
FCR	2.18^{a}	1.79^{b}	1.69^{c}	1.64^{d}	<0.001	0.009

BW: body weight, BWG: body weight gain, FI: feed intake, FCR: feed conversion ratio, control: broilers offered basal diets without any supplementations, I, II, and III: broilers fed basal diets supplemented with liposomal hesperetin at graded levels comprising 150, 250, and 400 mg/kg diets, respectively. SEM: standard error of the mean. ^{a,b,c,d} rows with different superscript letters imply statistical difference ($P < 0.05$).

broilers fortified with dietary liposomal hesperetin exhibited noticeable ($P < 0.001$) minimization in the activities of lipid peroxidation biomarker (MDA) in a dose-dependent manner, unlike the control group. Moreover, the most significant ($P < 0.001$) reductions in the levels of MDA (3.99 nmol/ml) were detected in the breast muscles of broilers fortified with dietary liposomal hesperetin at a concentration of 400 mg/kg unlike the control group (13.15 nmol/ml). Significant reduction ($P < 0.001$) in ROS and H_2O_2 generation was noted in the breast muscles of broilers offered dietary liposomal hesperetin, unlike the control group. The most prominent minimization in the level of H_2O_2 was determined in broilers supplemented with dietary liposomal hesperetin at concentrations of 400, and 250 mg/kg (1.87 and 2.07 µmol/g tissue, respectively), when compared with the control group (5.3 µmol/g tissue). Notably, there were no significant differences in the levels of ROS between all groups fortified with dietary liposomal hesperetin. These results suggested an enhancing impact of liposomal hesperetin on broiler antioxidant and oxidative status, which in turn improved their general health and welfare.

Analysis of serum biochemical, and intestinal immunological parameters post-infection with Listeria monocytogenes

Table 5 illustrates the effects of graded concentrations of dietary liposomal hesperetin on the serum biochemical and intestinal biochemical markers of the broilers at 14 dpi with *L. monocytogenes*. Broilers offered dietary liposomal hesperetin inclusion at the dose of 400 or 250 mg/kg exhibited remarkable reduction ($P < 0.001$) in the levels of serum ALT, and TG, unlike the control group at 14 dpi. On the other hand, serum ALT, AST, and TG levels of broilers fortified with 150 mg/kg of liposomal hesperetin (21.86, 53.08 U/l and 79.58 mg/ dl, respectively) and those fed a basal control diet (22.68, 53.22 U/l and 79.9 mg/ dl, respectively) showed no significant

Table 4. Impact of graded levels of liposomal hesperetin on the antioxidant and oxidative markers in the breast muscle of broilers.

		Experimental groups				
Parameters	Control	I	II	III	*P*-value	SEM
CAT (U/l)	31.9^{b}	88.05^{a}	90.42^{a}	93.11^{a}	<0.001	1.3
GPX (µmol/mg)	154.38^{c}	265.31^{b}	281.67^{a}	277.82^{ab}	<0.001	0.08
SOD (µ/ml)	41.85^{c}	93.28^{b}	102.5^{ab}	109.59^{a}	<0.001	0.36
T-AOC (U/mg prot)	1.62^{d}	2.46^{c}	2.64^{b}	3.37^{a}	<0.001	0.08
TPC (µg/g)	80.77^{c}	155.63^{b}	162.99^{ab}	166.66^{a}	<0.001	0.05
TFC (µg/g)	64.44^{c}	134.9^{b}	144.78^{a}	149.37^{a}	<0.001	0.04
MDA (nmol/ml)	13.15^{a}	9.1^{b}	7.17^{c}	3.99^{d}	<0.001	0.15
H_2O_2 (µmol/g tissue)	5.3^{a}	2.38^{b}	2.07^{bc}	1.87^{c}	<0.001	0.12
ROS (µl/g tissue)	44.66^{a}	18.35^{b}	16.98^{b}	17.12^{b}	<0.001	1.5

CAT: catalase, GPX: glutathione peroxidase, SOD: superoxide dismutase, T-AOC: total antioxidant capacity, MDA: malondialdehyde, ROS: reactive oxygen species, H_2O_2: hydrogen peroxide, TPC: total phenolic compounds, TFC: total flavonoids contents, control: broilers offered basal diets without any supplementations, I, II, and III: broilers fed basal diets supplemented with liposomal hesperetin at graded levels comprising 150, 250, and 400 mg/kg diets, respectively. SEM: standard error of the mean. ^{a,b,c,d} rows with different superscript letters imply statistical difference ($P < 0.05$).

Table 5. Effect of various levels of dietary liposomal hesperetin on the levels of serum biochemical parameters, and intestinal immunological parameters in broilers 14 post-infection with *Listeria monocytogenes*.

Parameter	Experimental groups				*P*-value	SEM
	Control	I	II	III		
Serum biochemical						
LDL (mg/ dl)	112.7^{a}	100.78^{b}	99.64^{b}	95.64^{c}	<0.001	0.76
TC (mg/ dl)	121.84^{a}	119.24^{b}	113.64^{c}	108.76^{d}	<0.001	0.65
TG (mg/ dl)	79.9^{a}	79.58^{a}	77.62^{b}	76.82^{b}	<0.001	0.38
AST (U/l)	53.22^{a}	53.08^{a}	51.04^{b}	47.76^{c}	<0.001	0.38
ALT (U/l)	22.68^{a}	21.86^{a}	20.98^{b}	20.19^{b}	<0.001	0.24
Intestinal immunological						
IgG (mg/dl)	12.94^{c}	13.74^{bc}	14.06^{b}	15.4^{a}	<0.001	0.29
MPO (µmol/l)	33.2^{a}	30.62^{b}	29.1^{c}	27.44^{d}	<0.001	0.29
CRP (mg/l)	3.66^{a}	2.92^{b}	2.58^{b}	1.82^{c}	<0.001	0.14
LYZ (µg/ml)	222.74^{a}	203.54^{b}	165.93^{c}	161.22^{c}	<0.001	1.74
TNF-α	78.8^{a}	73.06^{b}	65.76^{c}	53.4^{d}	<0.001	0.73
IL-6	57^{a}	49.68^{b}	42.58^{c}	39^{d}	<0.001	0.33
C3	2.27^{a}	2^{b}	1.86^{b}	1.28^{c}	<0.001	0.07
C4	1.8^{a}	1.62^{b}	1.38^{c}	1^{d}	<0.001	0.05

LDL: low-density lipoprotein, TC: total cholesterol, TG: total triglycerides, AST: aspartate aminotransferase, ALT: alanine aminotransferase, IgG: immunoglobulin-G, MPO: myeloperoxidase, CRP: c-reactive protein, TNF-α: tumour necrosis factor-alpha, IL-6: interleukin-6, LYZ: lysozyme, C3: complement C3, C4: complement C4, Control: broilers offered basal diets without any supplementations, I, II, and III: broilers fed basal diets supplemented with liposomal hesperetin at graded levels comprising 150, 250, and 400 mg/kg diets, respectively. SEM: standard error of the mean. ^{a,b,c,d} rows with different superscript letters imply statistical difference ($P < 0.05$).

differences at 14 dpi. Additionally, the lipid profile showed that, as dietary liposomal hesperetin concentrations increased, the serum levels of TC and LDL were minimized in the broilers, unlike the control group at 14 dpi. When compared to the control group (53.22 U/l, 121.84, and 112.7 mg/dL, respectively), the broilers supplemented with liposomal hesperetin at a concentration of 400 mg/kg showed the greatest ($P < 0.001$) reductions in the serum levels of AST, TC, and LDL (47.76 U/l, 108.76, and 95.64 mg/dl, respectively) at 14 dpi. Moreover, broilers fortified with dietary liposomal hesperetin at levels of 400, and 250 mg/kg exhibited the most remarkable minimization of the levels of ALT (20.19 and 20.98 U/l, respectively) and TG (76.82, and 77.62 mg/dl, respectively) compared with the control group (22.68 U/l, and 79.9 mg/dl, respectively) in their sera at 14 dpi.

Table 6. Quantification of *Listeria monocytogenes* loads in various tissues of broilers in response to dietary liposomal hesperetin fortification at 7-and-14 days post-infection with *L. monocytogenes* strain.

Parameter (log_{10} CFU/g)	Experimental groups				*P*-value	SEM
	Control	I	II	III		
7 days post-infection						
Caecum	4.53^{a}	4.11^{a}	3.09^{b}	2.48^{b}	<0.001	0.19
Liver	0.94^{a}	0.4^{b}	0.32^{bc}	0.24^{bc}	<0.001	0.03
Spleen	0.34^{a}	0.28^{b}	0.21^{c}	0.12^{d}	<0.001	0.02
Brain	0.22^{a}	0.14^{b}	0.11^{b}	0^{c}	<0.001	0.02
14 days post-infection						
Caecum	5.04^{a}	3.55^{b}	2.53^{c}	1.92^{c}	<0.001	0.19
Liver	1.19^{a}	0.19^{b}	0.18^{b}	0.12^{b}	<0.001	0.03
Spleen	0.49^{a}	0.13^{b}	0.09^{b}	0.09^{b}	<0.001	0.01
Brain	0.35^{a}	0.15^{b}	0.03^{c}	0^{c}	<0.001	0.02

Control: broilers offered basal diets without any supplementations, I, II, and III: broilers fed basal diets supplemented with liposomal hesperetin at graded levels comprising 150, 250, and 400 mg/kg diets, respectively. SEM: standard error of the mean. ^{a,b,c,d} rows with different superscript letters imply statistical difference ($P < 0.05$).

Regarding the level of intestinal inflammatory and immune-related markers of broilers at 14 dpi, the levels of intestinal IgG, LYZ, CRP, MPO, IL-6, TNF-α, complement C3 and C4 were remarkably ($P < 0.001$) ameliorated in broilers fortified with dietary liposomal hesperetin inclusion, unlike the control group. Of note, *L. monocytogenes* infection increased the levels of LYZ, CRP, MPO, IL-6, TNF-α, complement C3 and C4, and minimized the level of IgG in broiler intestinal samples, which lowered the birds' immunity, health and welfare. Broilers offered dietary liposomal hesperetin inclusion at levels of 400 and 250 mg/kg exhibited the most prominent reduction ($P < 0.001$) in the intestinal levels of lysozyme at 14 dpi (161.22, and 165.93 µg/ml, respectively) compared with the control group (222.74 µg/ml). Moreover, when compared with the control group (12.94 mg/dl, 3.66 mg/l, 33.2 µmol/l, 57, 78.8, 2.27, and 1.8, respectively), broilers supplemented with dietary liposomal hesperetin at a dosage of 400 mg/kg demonstrated the highest noticeable ($P < 0.001$) immune response at 14 dpi as proven by the elevated intestinal level of IgG, and reduced intestinal levels of CRP, MPO, IL-6, TNF-α, complements C3 and C4 (15.4 mg/ dl, 1.82 mg/l, 27.44 µmol/l, 39, 53.4, 1.28, and 1, respectively). Collectively, dietary liposomal hesperetin supplementation alleviated the negative effects of the *L. monocytogenes* challenge, which in turn enhanced the birds' immunity, health, and welfare.

Impact of dietary liposomal hesperetin fortification on Listeria monocytogenes *counts*

At 7 and 14 dpi with MDR multi-virulent *L. monocytogenes* strain, broilers supplemented with

dietary liposomal hesperetin inclusion demonstrated no observable clinical signs of listeriosis in contrast to control broilers, which displayed depression, lethargy, decreased feed intake, and diarrhoea, which reflect the impact of liposomal hesperetin in promoting the birds' overall health and welfare. Table 6 depicts the quantification results of *L. monocytogenes* in the caecal, spleen, liver, and brain tissues of experimentally infected broilers. At 7 and 14 dpi, *L. monocytogenes* was quantitatively and significantly ($P < 0.001$) reduced in the caecal, spleen, liver, and brain tissues of broilers offered dietary liposomal hesperetin inclusion, unlike the control group. Interestingly, our findings revealed that *L. monocytogenes* populations were at their minimum concentrations in the caecal, liver, and spleen tissues of broilers fortified with dietary liposomal hesperetin inclusion at concentrations of 400 and 250 mg/kg at both time-points following *L. monocytogenes* infection (up to 1.92, 0.12, and 0.09 $\log_{10}$ CFU/g, respectively). At 7 dpi, broilers offered dietary liposomal hesperetin inclusion at a dosage of 400 mg/kg displayed the most significant reduction in the *L. monocytogenes* load in the splenic tissues (0.12 $\log_{10}$ CFU/g) when compared with the control group (0.34 $\log_{10}$ CFU/g). On the other hand, at 7 dpi, the *L. monocytogenes* counts in the caecal tissues of broilers fortified with 150 mg/kg of liposomal hesperetin (4.11 $\log_{10}$ CFU/g) and those fed a basal control diet (4.53 $\log_{10}$ CFU/g) presented no significant differences. Of note, no CFUs were detected in brain tissues of broilers offered liposomal hesperetin at a concentration of 400 mg/kg at either time-point post-challenge with *L. monocytogenes* strain.

Gene expression analysis of virulence-related genes post-infection with Listeria monocytogenes

Figure 2 displays the mRNA expression levels of genes related to *L. monocytogenes* virulence as determined by RT-qPCR at 7-and14 dpi with MDR multi-virulent *L. monocytogenes* strain. The results showed that, in comparison to the control non-supplemented group, liposomal hesperetin fortification, especially at higher concentrations, significantly ($P < 0.001$) reduced the expression levels of *hlyA*, *flaA*, and *ami* virulence genes, which suggests its impact in promoting broiler health and immunity. Notably, dietary liposomal hesperetin fortification at a dose of 400 mg/kg significantly ($P < 0.001$) downregulated the transcription of *hlyA*, *flaA*, and *ami* genes at 14 dpi, with particular reference to the *16S rRNA* gene (0.11-, 0.15-, and 0.21-fold change, respectively). At 7 dpi, broilers offered dietary liposomal hesperetin inclusion at levels of 400, and 250 mg/kg displayed the most prominent reduction ($P < 0.001$) in the expression of *hlyA* (0.30- and 0.36-fold change, respectively), and *ami* (0.32- and 0.29-fold change, respectively) genes, unlike the control group. Moreover, dietary liposomal hesperetin supplementation at a dosage of 400 mg/kg remarkably downregulated ($P < 0.001$) the transcription level of the *flaA* gene in a dose-dependent manner at both time-points post-infection with *L. monocytogenes* strain.

*Gene expression analysis of genes related to cytokines, chemokines, and tight junction proteins post-*Listeria monocytogenes *challenge.*

Figure 3 depicts the outcomes of RT-qPCR measurements of the transcription levels of genes related to cytokines and chemokines at 14 days post-infection with the MDR multi-virulent *L. monocytogenes* strain. Our results demonstrated that *L. monocytogenes* infection upregulated the expression levels of genes encoding proinflammatory cytokines (*IL-1β*, *MyD88*, *IL-18)*, and chemokine (*CCL20),* and downregulated the transcriptional levels of genes encoding anti-inflammatory cytokine (*IL-10)*, and chemokine (*AVBD6),* which reflect lowering the birds' immunity and health. Moreover, at 14 dpi, elevating the liposomal hesperetin concentrations noticeably ($P < 0.001$) downregulated the transcription levels of *IL-1β* (Figure 3(A)), *MyD88* (Figure 3(B)), *IL-18* (Figure 3(C)), and *CCL20* (Figure 3(E)) genes compared with the control group. Furthermore, at 14 dpi, our findings showed that supplementing broilers with liposomal hesperetin remarkably ($P < 0.001$) increased the expression levels of the genes encoding *IL-10* (Figure 3(D)), and *AVBD6* (Figure 3(F)) unlike the control group. Notably, compared to the control group, broilers given 400 mg/kg of liposomal hesperetin showed the most significant ($P < 0.001$) decrease in the expression levels of *IL-1β*, *IL-18*, and *CCL20* genes (up to 0.76-, 0.29-, and 0.44-fold change, respectively), and the most significant ($P < 0.001$) upregulation in the transcription levels of *IL-10*, and *AVBD6* genes (up to 1.88-, and 1.56-fold change, respectively) at 14 dpi. Moreover, broilers fortified with dietary liposomal hesperetin inclusion at levels of 400 and 250 mg/kg had the most prominent ($P < 0.001$) downregulation in the transcription levels of the *MyD88* gene (up to 0.66, and 0.73-fold change) compared with the control group at 14 dpi. Overall, dietary liposomal hesperetin fortification alleviated the adverse impacts of the *L. monocytogenes* challenge, which in turn enhanced the broilers' immunity, health, and welfare.

The transcription levels of genes encoding TJP and gut barrier functions following fortification with liposomal hesperetin at 14 dpi with MDR multi-virulent *L. monocytogenes* strain are shown in Figure 4. Our findings showed that *L. monocytogenes* infection

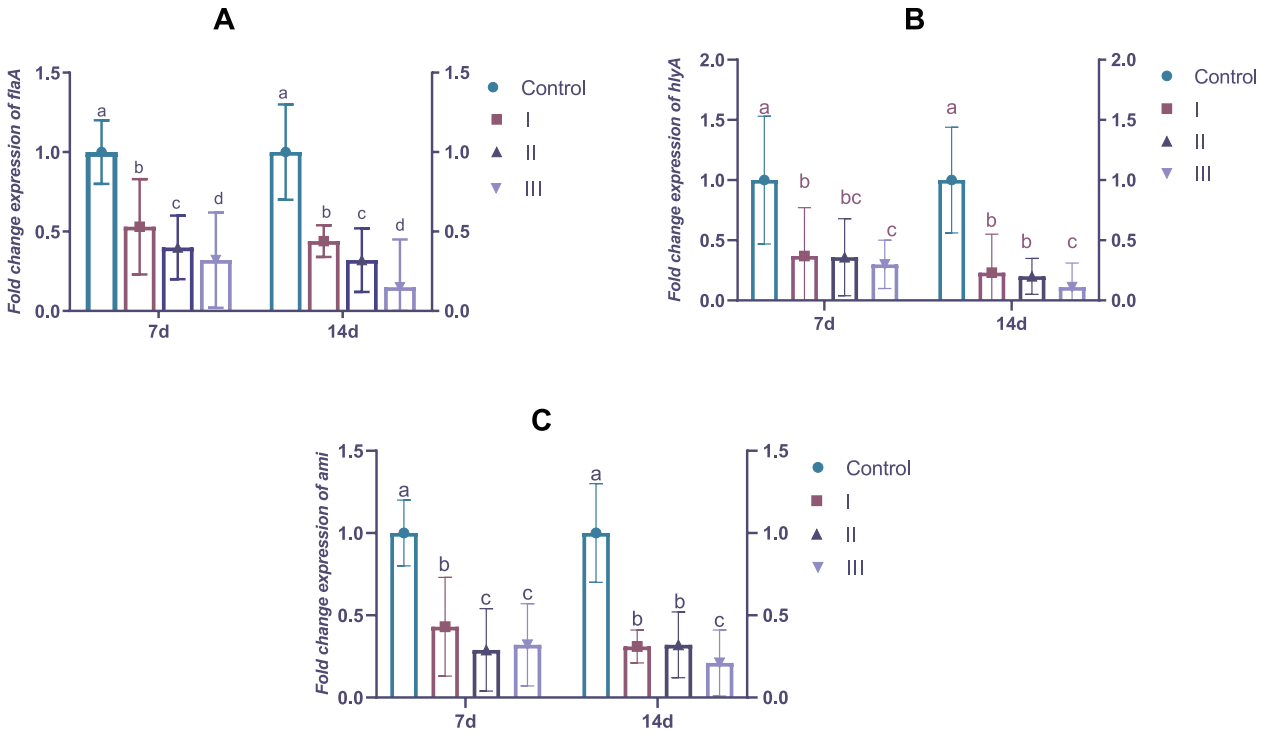

Figure 2. Expression levels of *L. monocytogenes* virulence genes; *flaA* (A), *hlyA* (B), and *ami* (C) in broiler intestinal tissues following dietary supplementation with graded levels of liposomal hesperetin at 7 and 14 dpi. Results are expressed as means ± SEM (standard error of the mean) in bars. Control: broilers offered basal diets without any supplementations, I, II, and III: broilers fed basal diets supplemented with liposomal hesperetin at graded levels comprising 150, 250, and 400 mg/kg diets, respectively. ^{a,b,c,d} bars with different superscript letters imply statistical difference ($P < 0.05$).

downregulated the expression levels of genes encoding TJP (occludin and *JAM-2),* and gut barrier functions (*MUC-2),* which suggests minimizing gut health and integrity. The mRNA expression levels of occludin (Figure 4(A)), *JAM-2* (Figure 4(B)), and *MUC-2* (Figure 4(C)) genes were remarkably ($P < 0.001$) increased across all groups fortified with dietary liposomal hesperetin supplementation as the concentration of liposomal hesperetin supplementation elevated at 14 dpi with MDR multi-virulent *L. monocytogenes* strain. Of note, broilers offered 400 mg/kg liposomal hesperetin showed the greatest upregulation ($P < 0.001$) in occludin, *JAM-2,* and *MUC-2* mRNA expression levels (up to 3.42-, 2.94-, and 2.37-fold change, respectively) when compared to the control group at 14 dpi. These results reflect the impact of liposomal hesperetin in enhancing gut health and integrity, which consequently enhanced overall health of the birds.

Gene expression analysis of antioxidant-related genes post-infection with Listeria monocytogenes

Figures 5 and 6 display the mRNA expression levels of antioxidant-related genes in the intestinal tissues and breast muscles of broilers fortified with graded levels of liposomal hesperetin at 14 dpi with MDR multi-virulent *L. monocytogenes* strain. Our findings revealed that increasing the concentrations of dietary liposomal hesperetin supplementation significantly ($P < 0.001$) upregulated the expression levels of *CAT*, *GPX-1*, *HO-1*, and *NQO1* genes in the intestinal tissues and breast muscle samples at 14 dpi with MDR multi-virulent *L. monocytogenes* strain. Additionally, at 14 dpi, our outcomes showed that fortifying broilers with liposomal hesperetin noticeably ($P < 0.001$) decreased the transcription levels of *SOD-1* and *COX2* genes in the intestinal tissues and breast muscle samples compared with the control group. Of note, supplementing broilers with dietary liposomal hesperetin inclusion at a dosage of 400 mg/kg resulted in the most notable ($P < 0.001$) upregulation of the *CAT* and *SOD-1* genes (up to 3.15- and 2.98- and 1.85 and 1.63-fold change, respectively) and the most significant downregulation in the expression level of *COX2* gene (up to 0.27- and 0.20-fold change, respectively) in the intestinal tissues and breast muscle samples in contrast to the control group at 14 dpi. Moreover, feeding broilers on liposomal hesperetin at a dosage of 400 mg/kg exhibited the most prominent ($P < 0.001$) increase in the transcription levels of intestinal *HO-1* (Figure 5(D)), and muscle *GPX-1* (Figure 6(C)) genes (up to 1.98, and 2.13-fold change, respectively) at 14 dpi compared with the control group. Furthermore, broilers supplemented with dietary liposomal hesperetin at a concentration of 400 mg/kg showed the most significant ($P < 0.001$) upregulation in the mRNA expression levels of intestinal *GPX-1* (Figure 5(C)), intestinal *NQO1* (Figure 5(E)), muscle *HO-1*

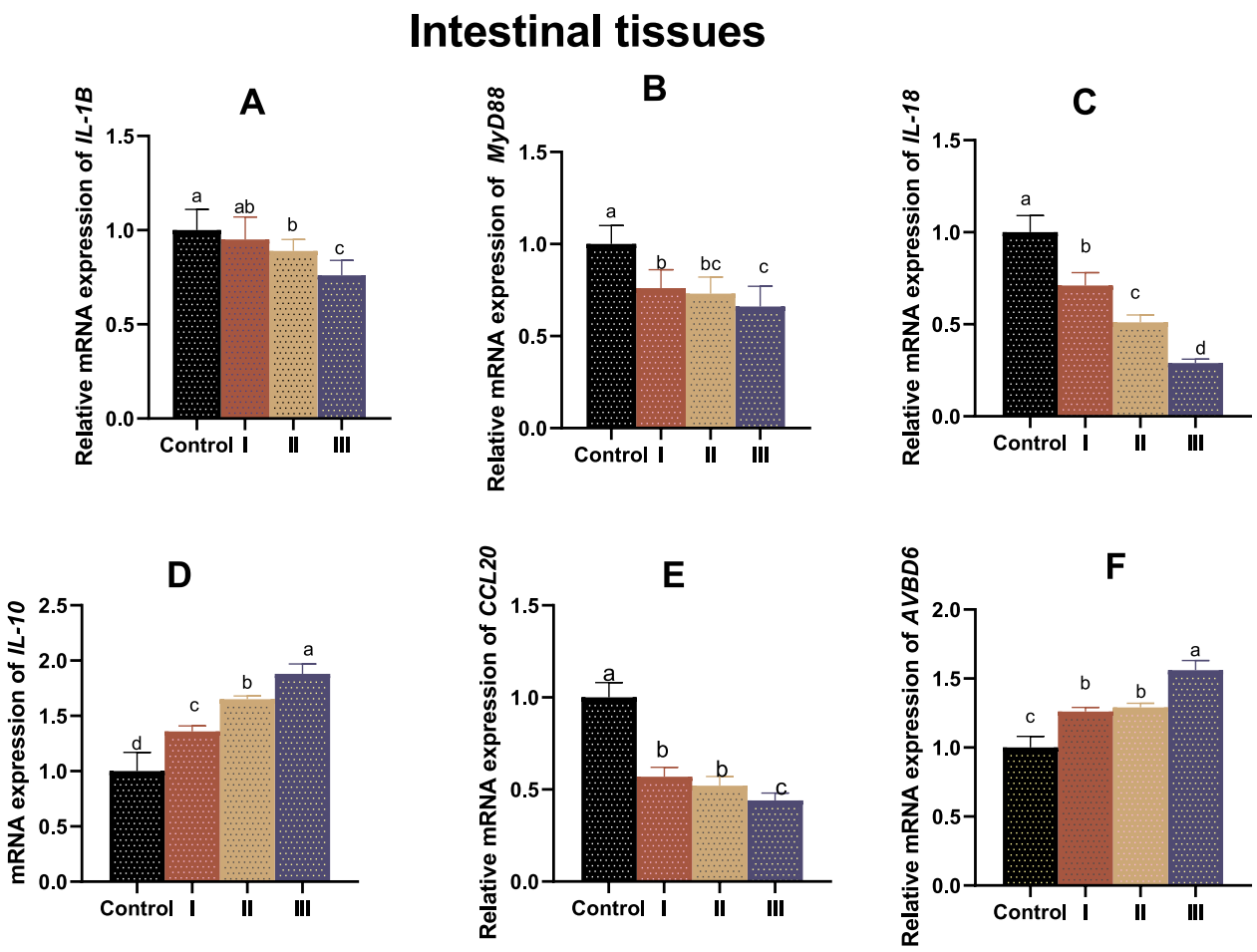

Figure 3. Expression levels of genes encoding cytokines: *IL-1β* (interleukin-1β; A), *MyD88* (myeloid differentiation factor 88; B), *IL-18* (interleukin-18; C), *IL-10* (interleukin-10; D), *CCL20* (chemokine C–C motif ligand 20; E), and *AVBD6* T (avian β-defensin 6; F) determined by RT-qPCR in the intestinal tissues of broilers fortified with various concentrations of liposomal hesperetin inclusion at 14 days post-infection with MDR multi-virulent *Listeria monocytogenes* strain. Results are expressed as means ± SEM (standard error of the mean). Control: broilers offered basal diets without any supplementations, I, II, and III: broilers fed basal diets supplemented with liposomal hesperetin at graded levels comprising 150, 250, and 400 mg/kg diets, respectively. ^{a,b,c,d} bars with different superscript letters imply statistical difference ($P < 0.05$).

(Figure 6(D)), and muscle *NQO1* (Figure 6(E)) genes (up to 1.27-, 1.23-, 2.11-, and 1.29-fold change, respectively) concerning the control group at 14 dpi.

Gene expression analysis of autophagy-related genes post-infection with Listeria monocytogenes

Figure 7 illustrates the effectiveness of graded levels of dietary liposomal hesperetin inclusion on the expression of autophagy encoding genes in the intestinal tissues of broilers at 14 dpi with MDR multi-virulent *L. monocytogenes* strain. Our results revealed that elevating the levels of dietary liposomal hesperetin inclusion substantially ($P < 0.001$) upregulated the expression levels of *Bcl-2* (Figure 7(A)), *LC3* (Figure 7(B)), *AMPK* (Figure 7(C)) and *AKT* (Figure 7(D)) genes in the intestinal tissues at 14 dpi with MDR multi-virulent *L. monocytogenes* strain, unlike the control group. Furthermore, our findings displayed that fortifying broilers with liposomal hesperetin significantly ($P < 0.001$) downregulated the expression levels of intestinal *CHOP* (Figure 7(E)), *Bip* (Figure 7(F)), *p62* (Figure 7(G)) and *XBP1* (Figure 7(H)) genes compared with the control group at 14 dpi with *L. monocytogenes* strain. Of note, fortifying the broiler diet with liposomal hesperetin at a dosage of 400 mg/kg resulted in the highest upregulation ($P < 0.001$) in the transcription levels of intestinal *Bcl-2, LC3, AMPK,* and *AKT* genes (up to 4.73-, 3.75-, 4.73-, and 3.65-fold change, respectively), and the most prominent ($P < 0.001$) downregulation in the expression levels of intestinal *CHOP*, *Bip*, and *p62* genes (up to 0.27, 0.21, and 0.41-fold change, respectively) in contrast to the control group at 14 dpi with *L. monocytogenes* strain. Moreover, dietary liposomal hesperetin supplementation at concentrations of 400 and 250 mg/kg showed the most significant ($P < 0.001$) reduction in the expression level of intestinal *XBP1* gene (up to 0.29-fold change) compared with the control group at 14 dpi with *L. monocytogenes* strain. These findings reflect the enhancing effect of

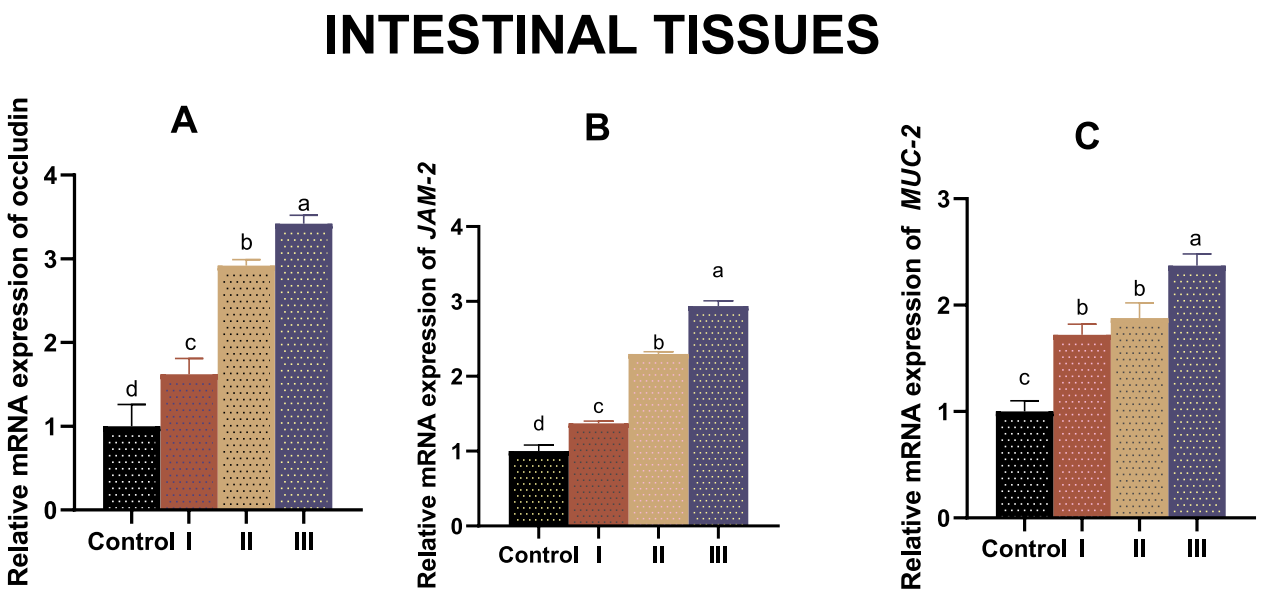

Figure 4. RT-qPCR analysis of the transcription levels of genes related to tight junction proteins and gut barrier functions; occludin (A), *JAM-2* (junctional adhesion molecule-2; B), and *MUC-2* (mucin-2; C) in the intestinal tissues of broilers offered graded levels of liposomal hesperetin at 14 days post-infection with MDR multi-virulent *Listeria monocytogenes* strain. Results are expressed as means ± SEM (standard error of the mean) Control: broilers offered basal diets without any supplementations, I, II, and III: broilers fed basal diets supplemented with liposomal hesperetin at graded levels comprising 150, 250, and 400 mg/kg diets, respectively. [a, b,c,d] bars with different superscript letters imply statistical difference ($P < 0.05$).

liposomal hesperetin on the autophagy process, which consequently enhanced broiler immunity and health.

Histopathological modifications post-infection with Listeria monocytogenes

At 14 dpi with MDR multi-virulent *L. monocytogenes* strain, the intestine of *L. monocytogenes*-challenged broilers (positive control group) showed destruction of some villus epithelium with the presence of necrotic debris (Figure 8(A)). The histopathological findings of the intestinal tissues of broilers challenged with *L. monocytogenes* and supplemented with liposomal hesperetin are shown in Figure 8(B–D). The examined sections of the intestine displayed improvement of intestinal villi, intestinal glands, submucosal layer, muscularis, and serosa in broilers offered liposomal hesperetin at a dosage of 150 mg/kg (Figure 8(B)). Moreover, the intestinal sections of broilers offered liposomal hesperetin at a dosage of 250 mg/kg showed preserved architectures of columnar epithelial lining mucosa, with elongated, broad-end intestinal villi (Figure 8(C)). However, more elongated and branched intestinal villi were seen in broilers supplemented with liposomal hesperetin at a concentration of 400 mg/kg (Figure 8(D)).

At 14 dpi with *L. monocytogenes* strain, the brain tissues of *L. monocytogenes*-challenged broilers (positive control group) showed minute scattered necrotic areas, represented by empty cavities "encephalomalacia", in addition to the presence of satellitosis, and neuronophagia was seen within some examined sections. Dilated cerebral vasculatures with perivascular exudates were also detected (Figure 9(A–B)). Additionally, the brain sections of broilers offered liposomal hesperetin at a dosage of 150 mg/kg exhibited a low number of degenerated neurons surrounded by glia cells (Figure 9(C)). Furthermore, the brain sections of broilers offered liposomal hesperetin at concentrations of 250 and 400 mg/kg revealed normal histological structures of neurons, glia cells, cerebral vasculatures, and neuropil (Figure 9(D and E)). Overall, liposomal hesperetin restored the histopathological changes in the intestinal and brain tissues of challenged broilers, which suggests its anti-inflammatory effect that consequently enhanced the birds' immunity and general health.

Discussion

Bioactive components of phytogenics have recently been extensively researched for their advantageous qualities as substitutes for chemicals and antibiotics in human and birds health (Abd El-Hamid *et al.*, 2021; Ibrahim, Shahin *et al.*, 2022). Birds productivity is enhanced when phytogenics are added to their diets because phytogenics promote growth performance, immunity, health, antioxidant defence, digestive system, and nutrient utilization (Ibrahim, Abdelfattah-Hassan *et al.*, 2021; Hashem *et al.*, 2022). Notably, nutrient utilization and FCR have significant impacts on the broiler industry's profitability (Connerton *et al.*, 2018). Despite increasing attention to the biological properties of phytogenics, their application in

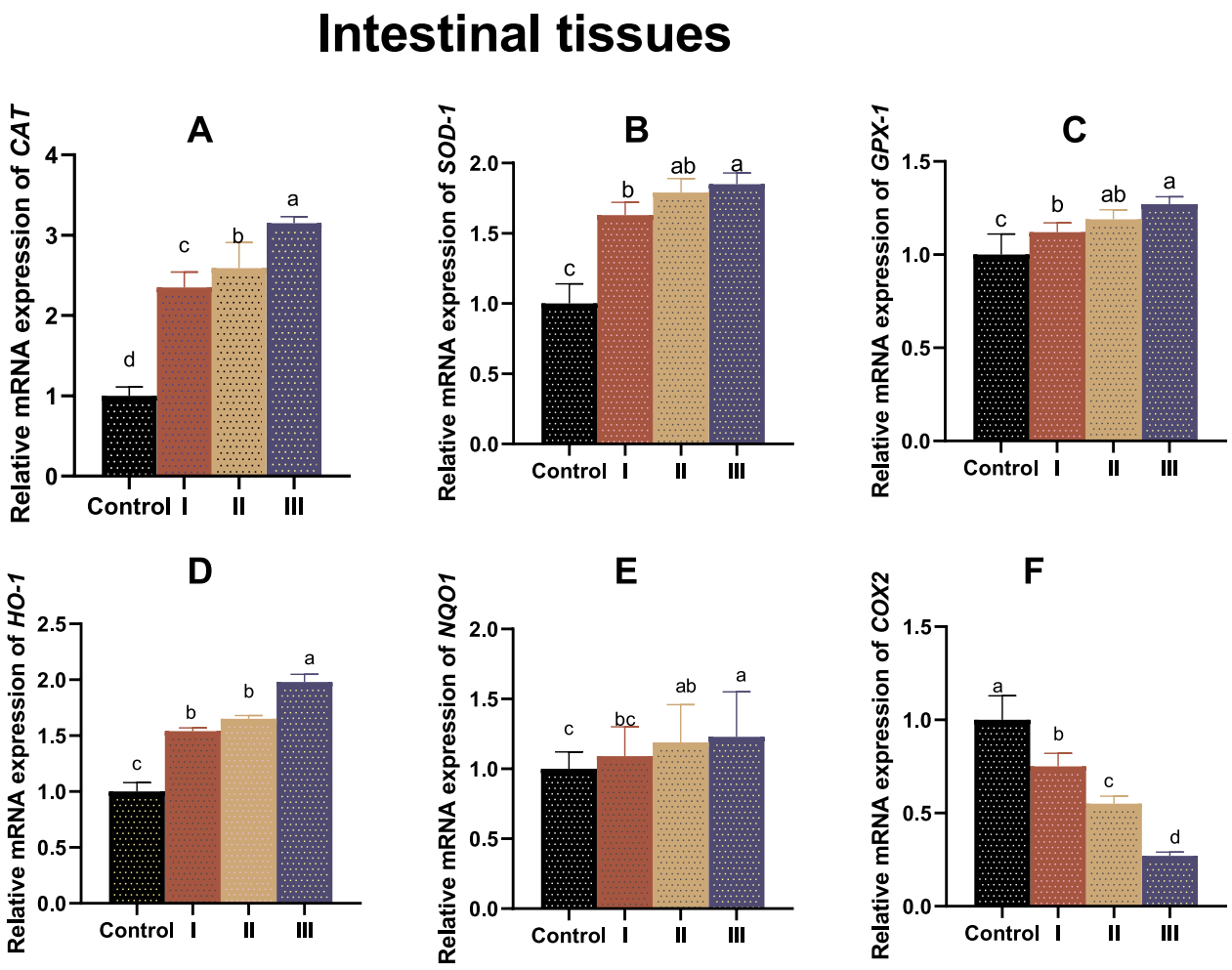

Figure 5. Expression levels of antioxidant-related genes; *CAT* (catalase; A), *SOD-1* (superoxide dismutase 1; B), *GPX-1* (glutathione peroxidase 1; C), *HO-1* (haem oxygenase-1; D), *NQO1* (NAD(P)H dehydrogenase quinone 1; E), and *COX2* (cyclooxygenase-2; F) determined by RT-qPCR in the intestinal tissues of broilers offered graded levels of dietary liposomal hesperetin inclusion at 14 days post-infection with MDR multi-virulent *Listeria monocytogenes* strain. Results are expressed as means ± SEM (standard error of the mean). Control: broilers offered basal diets without any supplementation, I, II, and III: broilers fed basal diets supplemented with liposomal hesperetin at graded levels comprising 150, 250, and 400 mg/kg diets, respectively. ^{a,b,c,d} bars with different superscript letters imply statistical difference ($P < 0.05$).

the broiler sector has been restricted by their instability in response to changes in light, temperature, and oxygen levels. Liposomes are now seen to be good options for safeguarding phytogenics, boosting their stability, and regulating their release (Sherry *et al.*, 2013). The potential impacts of liposomes on poultry production have not been thoroughly studied, and there is no information regarding the usage of liposomal hesperetin in the poultry sector as far as we are aware. The present study revealed the promoting impact of liposomal-encapsulated hesperetin on the growth performance and antioxidant capacity in broilers, in addition to reducing *L. monocytogenes* load, downregulating the expression of *L. monocytogenes* virulence-related genes, enhancing immunity, health, welfare, and ameliorating the transcription of genes encoding TJP, antioxidants, cytokines, and autophagy in broilers experimentally infected with MDR multi-virulent *L. monocytogenes* strain. Herein, during the grower, finisher, and overall rearing periods, the growth attributes were significantly ameliorated in all groups fortified with liposomal hesperetin, especially at higher levels, unlike the control group. During the starter and grower periods, broilers supplemented with dietary liposomal hesperetin at concentrations of 400 and 250 mg/kg revealed maximum BWG and superior FCR, which suggests the enhancing impact of liposomal hesperetin on the birds' growth performance and health. Moreover, during the finisher and total growing periods, broilers offered dietary liposomal hesperetin supplementation at a concentration of 400 mg/kg exhibited the most remarkable enhancement in the BWG and FCR regarding the control group. In accordance, earlier research showed that broilers offered dietary citrus flavonoid supplementation exhibited better FCR during the total growing period than the control group (Rodsatian *et al.*, 2023). Likewise, a previous study showed that hesperidin fortification significantly improved the FCR of broilers unlike the control group

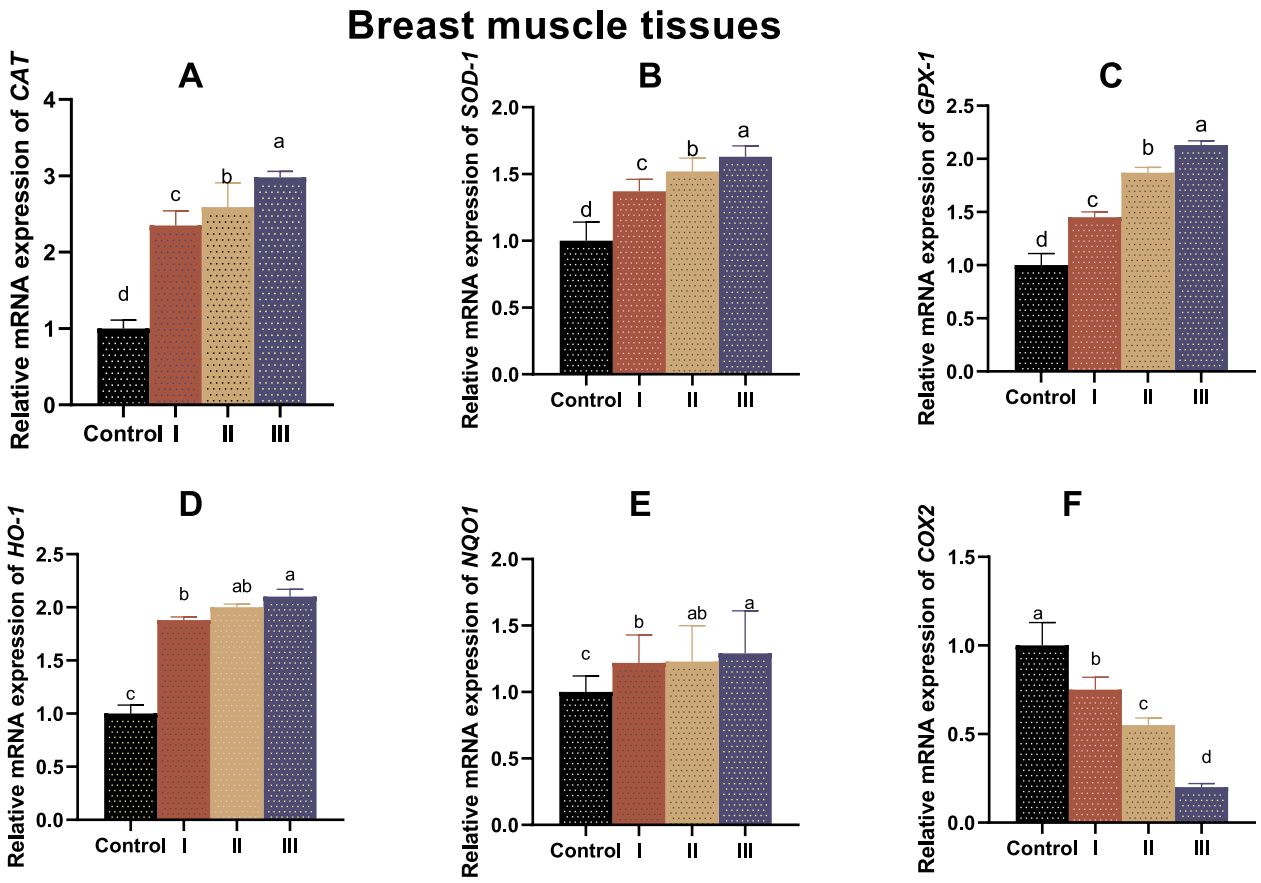

Figure 6. Expression levels of antioxidant-related genes; *CAT* (catalase; A), *SOD-1* (superoxide dismutase 1; B), *GPX-1* (glutathione peroxidase 1; C), *HO-1* (haem oxygenase-1; D), *NQO1* (NAD(P)H dehydrogenase quinone 1; E), and *COX2* (cyclooxygenase-2; F) determined by RT-qPCR in the breast muscles of broilers offered graded levels of dietary liposomal hesperetin inclusion at 14 days post-infection with MDR multi-virulent *Listeria monocytogenes* strain. Results are expressed as means ± SEM (standard error of the mean). Control: broilers offered basal diets without any supplementation, I, II, and III: broilers fed basal diets supplemented with liposomal hesperetin at graded levels comprising 150, 250, and 400 mg/kg diets, respectively. ^{a,b,c,d} bars with different superscript letters imply statistical difference ($P < 0.05$).

(Tarif, 2020). Similar to this, prior work found that dietary with liposomal-encapsulated oregano, cinnamon, and clove essential oils enhanced the growth rate and FCR in broilers throughout the rearing period, in contrast to the control group (Meligy *et al.*, 2023). Additionally, a recent study displayed that liposomal-loaded polyphenols enhanced the growth rate of broilers in comparison with the control group (Kishawy *et al.*, 2023). Nevertheless, up till now, no research has been conducted concerning the augmentation of growth attributes in broilers supplemented with dietary liposomal hesperetin. The growth-promoting effect of phytogenics may be attributed to the ability of their flavonoid polyphenolic compounds to improve the broilers' immune systems, food consumption, appetite (Aljazzar *et al.*, 2022), and digestive enzyme activities (Hashem *et al.*, 2022), in addition to having antioxidant and antimicrobial properties (Abd El-Hamid *et al.*, 2024). In contrast, previous research stated that dietary hesperidin supplementation did not affect the growth performance attributes of broiler chickens (Kamboh & Zhu, 2014; Goliomytis *et al.*, 2015), laying hens (Goliomytis *et al.*, 2019), and quail (Özbilgin *et al.*, 2021). The variations in supplementation dosages, ingredients, extraction methods, and suppliers may be the cause of the disparities in the effects of phytogenics between different studies (Ibrahim, Shahin *et al.*, 2022). Encapsulation of hesperetin by liposomes, which regulate the release of its bioactive constituents and enhance its stability, results in the favourable enhanced activities of hesperetin on growth performance attributes (Sherry *et al.*, 2013).

Notably, there is a substantial association between the immunity and general health of poultry and their antioxidant defence mechanisms. When microorganisms infect a bird, the immune system, antioxidant defences, and the production of ROS/nitrogen species are all out of balance. This leads to physiological changes that are associated with oxidative stress (Ibrahim, Shahin *et al.*, 2022). Overproduction of ROS can damage tissue, induce lipid peroxidation, and interfere with the physiological processes of cells, reducing performance, health, welfare, and survival and causing financial losses (Ibrahim, Moustafa *et al.*, 2021; Zhou *et al.*, 2022). Contrarily, increased

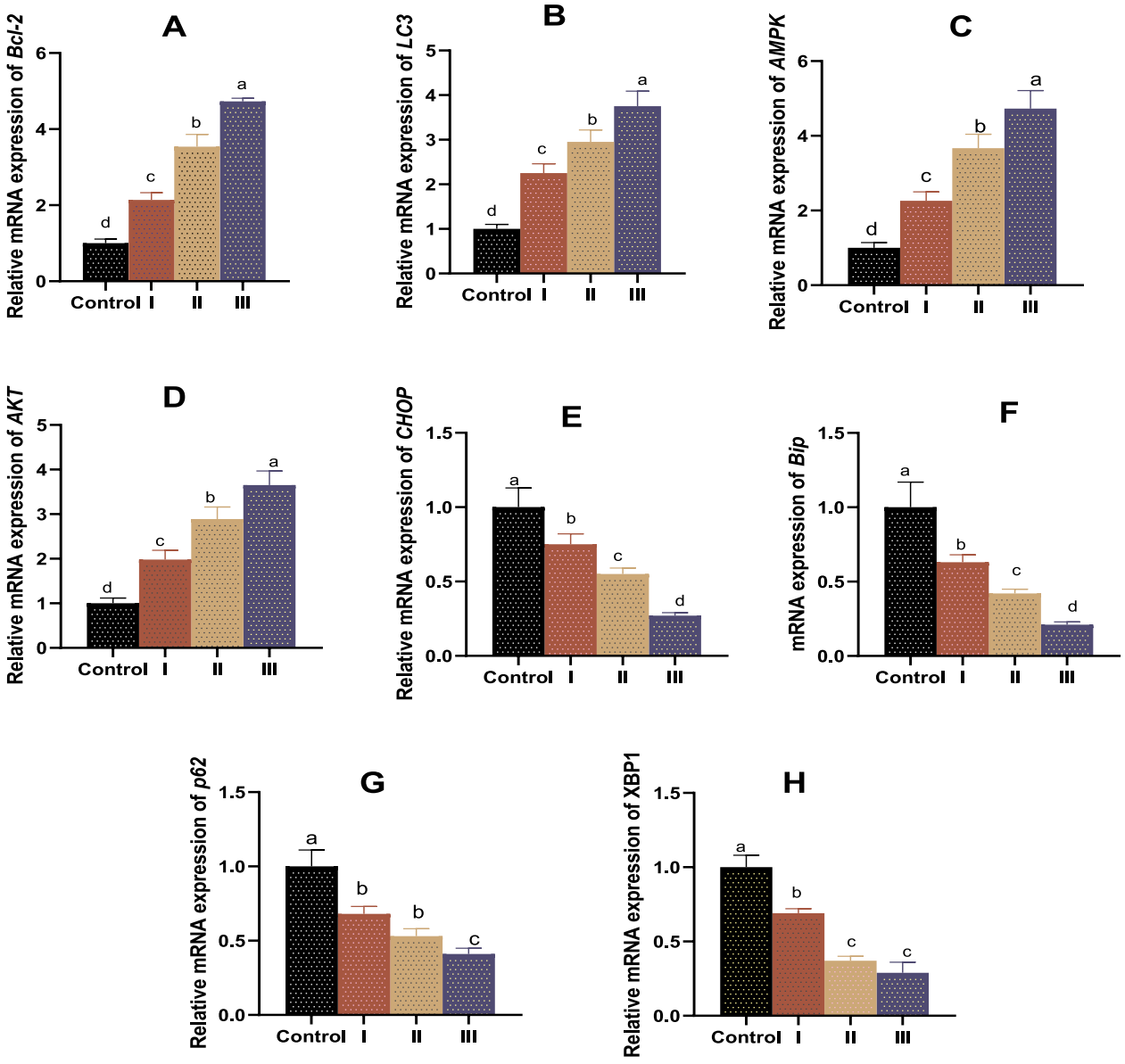

Figure 7. Analysis of RT-qPCR of autophagy-related genes; *Bcl-2* (B cell lymphoma-2; A), *LC3* (microtubule-associated protein 1 light chain 3; B), *AMPK* (adenosine monophosphate-activated protein kinase; C), *AKT* (serine/threonine kinase; D), *CHOP* (transcriptional factor C/EBP homologous protein; E), *Bip* (binding immunoglobulin protein; F), *p62* (gene encoding a ubiquitin chain binding protein; G), and *XBP1* (x-box binding protein 1; H) in the intestinal tissues of broilers offered graded levels of dietary liposomal hesperetin inclusion at 14 days post-infection with MDR multi-virulent *Listeria monocytogenes* strain. Results are expressed as means ± SEM (standard error of the mean). Control: broilers offered basal diets without any supplementation, I, II, and III: broilers fed basal diets supplemented with liposomal hesperetin at graded levels comprising 150, 250, and 400 mg/kg diets, respectively. ^{a,b,c,d} bars with different superscript letters imply statistical difference ($P < 0.05$).

levels of free radicals stimulate lipid peroxidation, resulting in oxidative stress, and elevate MDA content, which causes *post-mortem* meat deterioration (Kim *et al.*, 2012). The elimination of excessive ROS by powerful endogenous antioxidant defence mechanisms components such as CAT, SOD-1, and GPX-1 enzymes preserves the haemostasis of the cell and protects it from oxidative stress (Abd El-Hamid *et al.*, 2024). Furthermore, oxidative stress may disrupt the redox-sensitive signalling pathway and transcription factors, which may compromise the physiological functioning of the cell. Through boosting the production of phase-2 detoxifying enzymes and antioxidant proteins, the transcription factors NQO1, HO-1, and NRF2 are thought to have important regulatory roles in the cellular oxidative stress reaction (Kitakaze *et al.*, 2019; Khater *et al.*, 2022). Additionally, the T-AOC is thought to be an index that reflects the body's antioxidant state (Ibrahim, Abd El-Hamid *et al.*, 2022). Of note, previous studies found a significant relationship between free radical-scavenging capabilities and TPC and TFC concentration (Tayade *et al.*, 2013; Ibrahim, Moustafa *et al.*, 2021). Notably, the key mediators (antioxidant glutathione, ROS,

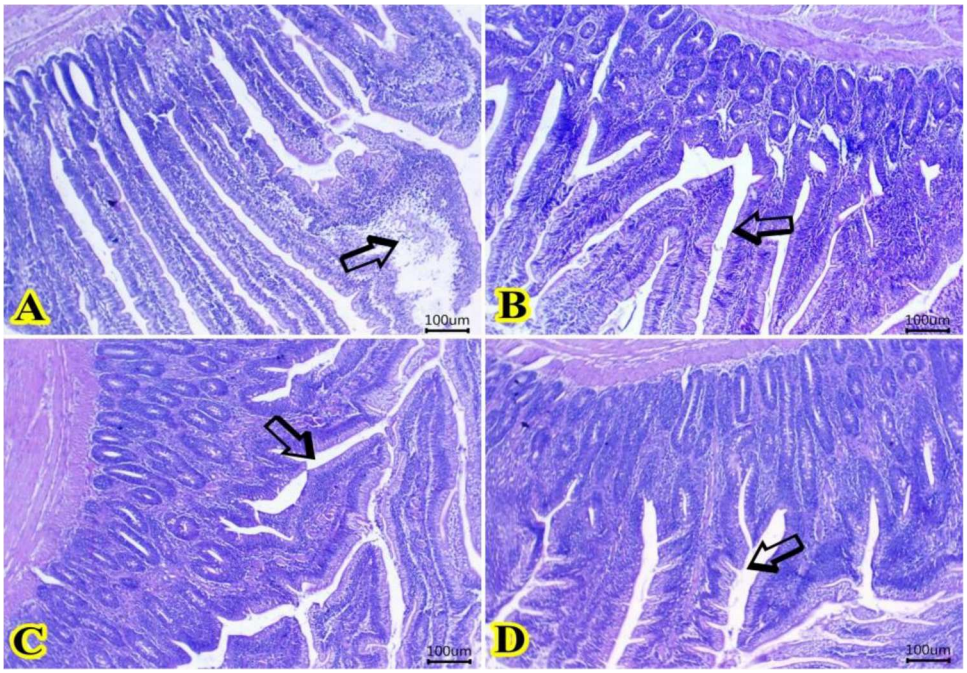

Figure 8. Histopathological modification of broiler intestine at 14 days post-infection with *Listeria monocytogenes* strain (scale bar 100 µm). (A) positive control group (broilers offered basal diets without any supplementations and challenged with *L. monocytogenes*). The intestine showed destruction of some villus epithelium with the presence of necrotic debris (arrow). (B) group I: broilers fed basal diets supplemented with liposomal hesperetin at the level of 150 mg/kg. Improvement of intestinal villi (arrow), intestinal glands, submucosal layer, muscularis, and serosa in comparison with the control group. (C) group II: broilers fed basal diets supplemented with liposomal hesperetin at the level of 250 mg/kg. Preserved architectures of columnar epithelial lining mucosa, with elongated, broad-end intestinal villi (arrow), unlike the control group. (D) group III: broilers fed basal diets supplemented with liposomal hesperetin at the level of 400 mg/kg. More elongated and branched intestinal villi (arrow), unlike the control group.

and COX-2) reciprocally regulate the inflammation that is triggered by the microbial infection. The enzyme COX-2 is responsible for mediating the biological transformation of arachidonic acid into inflammatory prostaglandins, which in turn triggers the production of cytokines (Yu *et al.*, 2012). Consequently, feeding birds a diet rich in phytogenics with immunostimulant properties may strengthen their antioxidant defence mechanism via scavenging free radicals and preventing the harmful effects of ROS, which in turn improves the meat quality and prolongs its shelf life after slaughter (Kurutas, 2016; Pereira *et al.*, 2022). In this regard, liposomal hesperetin is a naturally occurring antioxidant, but further research is needed to understand how it influences the antioxidant defence in broilers and whether using it could provide an additional benefit for improving this function. Our findings revealed a remarkable augmentation in the levels of T-AOC, TPC, TFC and SOD, CAT, and GPX enzymes and a significant reduction in the concentrations of MDA, ROS, and H_2O_2, at 24 days of age, in the breast muscles of broilers fortified with graded concentrations of dietary liposomal hesperetin, unlike the control group, indicating its activity in triggering the antioxidant defence mechanism, which in turn enhanced the birds' immunity and general health. Additionally, the most prominent enhancement in the levels of TPC, TFC, GPX, and SOD and the most significant reduction in the level of H_2O_2 were presented in the breast muscle samples of broilers supplemented with liposomal hesperetin at levels of 400 and 250 mg/kg. The most significant elevation in the level of T-AOC and the most significant reductions in the level of MDA were seen in broilers offered dietary liposomal hesperetin inclusion at a level of 400 mg/kg. In agreement with our findings, earlier studies revealed that dietary hesperidin supplementation significantly increased the levels of T-AOC, GPX, and SOD enzymes and minimized the MDA content in broilers (Kamboh & Zhu, 2014; Kamboh *et al.*, 2016) and layers (Lien *et al.*, 2008; Goliomytis *et al.*, 2014; Iskender *et al.*, 2016). Similarly, previous work showed that dietary hesperidin supplementation significantly reduced the MDA content in the broiler breast muscle, unlike the control group (Goliomytis *et al.*, 2015). Likewise, a prior study demonstrated that dietary hesperidin inclusion remarkably enhanced the tissue SOD, CAT, and GSH levels, and minimized the MDA concentration in Japanese quail (Özbilgin *et al.*, 2023). However, up till now, no research has been conducted concerning the enhancement of antioxidant potential in broilers fortified with dietary liposomal hesperetin. Herein, in parallel with enhancing the antioxidant

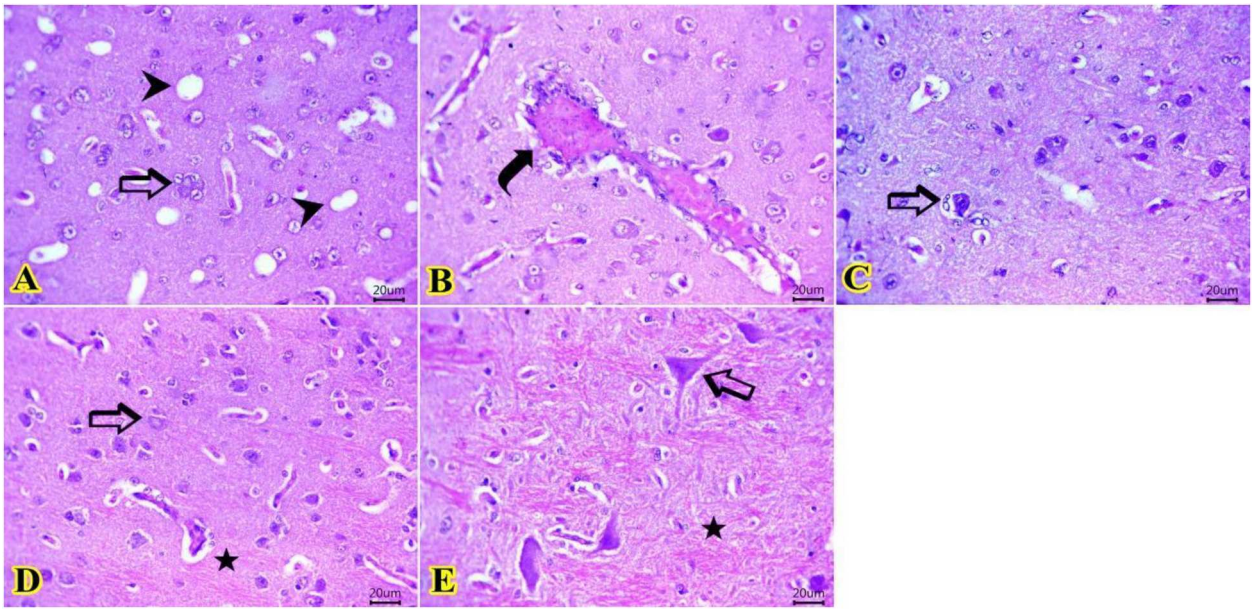

Figure 9. Histopathological modification of broiler brain tissues at 14 days post-infection with *Listeria monocytogenes* strain (scale bar 20 μm). (A, B) control (broilers offered basal diets without any supplementation and challenged with *L. monocytogenes*). The brain tissues showed minute scattered necrotic areas (arrowheads), with the presence of satellitosis (arrow), and dilated cerebral blood vessels with perivascular exudates (curved arrow). (C) I: broilers fed basal diets supplemented with liposomal hesperetin at the level of 150 mg/kg. Degenerated neurons surrounded by glia cells (arrow). (D, E) II, and III: broilers fed basal diets supplemented with liposomal hesperetin at the levels of 250, and 400 mg/kg, respectively. Normal histological structures of neurons (arrows), glia cells, cerebral vasculatures, and neuropil (stars).

and oxidative biomarkers before the challenge, increasing the concentrations of dietary liposomal hesperetin supplementation significantly upregulated the expression levels of *CAT, GPX-1, SOD-1*, *HO-1,* and *NQO1* genes and noticeably downregulated the transcription level of *COX2* genes in the intestinal tissues and breast muscle samples at 14 dpi with MDR multi-virulent *L. monocytogenes* strain. Of note, supplementing broilers with dietary liposomal hesperetin inclusion at a dosage of 400 mg/kg resulted in the most notable upregulation of intestinal and muscle *CAT,* intestinal *HO-1,* and muscle *GPX-1* and *SOD-1* genes and the most significant downregulation in the expression levels of intestinal and muscle and *COX2* genes at 14 dpi. Furthermore, broilers supplemented with dietary liposomal hesperetin at concentrations of 400 and 250 mg/kg showed the most significant upregulation in the expression levels of intestinal *GPX-1*, intestinal *NQO1*, muscle *HO-1,* and muscle *NQO1* genes at 14 dpi. Our results suggest that liposomal hesperetin inclusion effectively counteracted the adverse oxidative reaction in *L. monocytogenes*-challenged broilers indicating its potent antioxidant effect, which in turn improved the broilers' performance and general health. Similar to this, recent studies stated that dietary phytogenic supplementation significantly increased the level of T-AOC, and minimized the MDA, ROS, and H_2O_2 levels (El-Ghareeb *et al.*, 2023), in addition to upregulating the expression levels of *CAT, GPX-1, HO-1, SOD-1* and *NQO1* genes (El-Ghareeb *et al.*, 2023; Meligy *et al.*, 2023), and noticeably downregulated the transcription level of *COX2* gene in broilers (El-Ghareeb *et al.*, 2023). Likewise, prior studies showed that dietary phytogenic supplementation significantly improved the levels of TFC, TPC, and GPX enzyme, and lowered the MDA content in broilers (Ibrahim, Moustafa *et al.*, 2021; Vasilopoulou *et al.*, 2023), which might be attributed to their polyphenolic contents that have free radical-scavenging, and chelating properties (Pazos *et al.*, 2005; Ibrahim *et al.*, 2024). Nevertheless, no research has been conducted concerning the antioxidant potential of dietary liposomal hesperetin in broilers challenged with *L. monocytogenes* up till now. In the present study, incorporating liposomal hesperetin into the broilers' diet increased their antioxidant capacity. This may be linked to the effectiveness of liposomal encapsulation in increasing the bioactivity and bioavailability of hesperetin, which facilitates easier absorption by cells and more profound tissue penetration, thereby improving the antioxidant response.

The aminotransferase (ALT and AST) enzymes are indicators of liver health (Alhawas *et al.*, 2023). Our findings revealed that broilers offered dietary liposomal hesperetin inclusion at dosages of 400 and 250 mg/kg exhibited a remarkable reduction in the levels of serum ALT, and TG, unlike the control group at 14 dpi, which indicates that liposomal hesperetin may have a modulatory influence on the activity of enzymes associated with the metabolism of lipid. Additionally, broilers supplemented with

liposomal hesperetin at a concentration of 400 mg/kg showed the greatest reductions in the serum levels of AST, TC, and LDL at 14 dpi, which implies that liposomal hesperetin has a protective effect on the liver tissues of broilers. Our findings suggest that liposomal hesperetin supplementation successfully counteracted the adverse reaction on the liver function in *L. monocytogenes*-challenged broilers indicating its potent enhancement impact on the liver tissues. Similarly, an earlier study showed that dietary hesperidin supplementation significantly reduced the serum TC and TG levels in broilers (Kamboh & Zhu, 2014) and layers (Lien *et al*., 2008; Goliomytis *et al*., 2014; Iskender *et al*., 2016). In agreement with this, previous research showed that dietary hesperidin inclusion remarkably reduced the levels of serum AST, ALT, and LDH in Japanese quail, but did not affect the levels of serum TC, and TG in comparison with the control group (Özbilgin *et al*., 2023). In contrast, prior work showed that dietary hesperidin inclusion did not affect the plasma cholesterol level in layers (Goliomytis *et al*., 2019). Furthermore, by reducing key lipogenic factors that encourage the synthesis of bile acids and increase cholesterol elimination, phytogenic nanoemulsions may change the transcription of genes in the liver and inhibit the manufacture of cholesterol (Shin *et al*., 2011). Accordingly, broiler serum activities of LDL, TG, and TC, were reduced following essential oil fortification (Kishawy *et al*., 2022).

The immune system of birds is mostly responsible for maintaining their health. Due to their ability to enhance lymphocyte and immunoglobulin production activities, phytogenics have a positive effect on the birds' immunological defence, and general health (Faramarzi *et al*., 2013; Krishan & Narang, 2014). The antioxidant defence mechanism and immune response of birds are strongly connected, offering protection against dangerous bacteria that invade their environment. Enhancing the immune system of broilers by feeding them natural antioxidants may mitigate the negative effects of stressful environments of intensive farming (Meligy *et al*., 2023). Additionally, tissue and serum immunological parameters are important indicators that offer valuable insight into the overall health of poultry (Hashem *et al*., 2022). It is noteworthy that bacterial infections cause systemic inflammatory responses, which act as stressors on the immune system and lead to deterioration of the birds' overall health and performance (Xie *et al*., 2000; Ibrahim, Kishawy *et al*., 2021). The immunological response of poultry can be enhanced by phytogenics, which may contribute to better gastrointestinal health, gut microbiome, and resistance to microbial infections (Hashem *et al*., 2022). The activities of nitric oxide, LYZ, and MPO, which aid in the defence mechanisms of the immune system to get rid of the contaminating bacteria, have been linked to the bactericidal properties of neutrophils, monocytes, and macrophages, and they are considered key markers of the inflammatory response, and microbial infection may raise their concentrations (Ibrahim, Ismail *et al*., 2021; Ibrahim *et al*., 2023). A possible indicator of reduced inflammation in the body and cell injury is CRP, which is thought to be an acute inflammatory protein that reduces the degree of inflammation and is extensively generated at the site of inflammation or infection by numerous cells, including endothelial cells, lymphocytes, and macrophages (El-Ghareeb *et al*., 2023). Furthermore, CRP is essential for the generation of proinflammatory cytokines, especially TNF-α, IL-1β, and IL-6, phagocytosis, apoptosis, and nitric oxide release in response to bacterial infection (Sproston & Ashworth, 2018). TNF-α is an essential proinflammatory cytokine that controls the host immune defence against microbes by promoting immune cell differentiation and proliferation. Nevertheless, prolonged overproduction of the proinflammatory cytokines can cause gut damage (Hashem *et al*., 2022). Furthermore, by attracting macrophages and neutrophils, which are regarded as antimicrobial cells, proinflammatory cytokines, TNF-α, IL-1β, and IL-6, can induce an inflammatory reaction (Aljazzar *et al*., 2022). As crucial elements of the humoral immune response, immunoglobulins play a major role in immunological processes such as phagocytosis and the neutralization of pathogenic microbes (Magnadottir, 2010). Furthermore, IgG is one of the three primary immunoglobulin isotypes that respond to both local and systemic pathogens (Salinas *et al*., 2011). Complement is an enzyme glycoprotein that plays a role in the body's immunological control. Additionally, immunoglobulin could induce an increase in complements C3 and C4, thus, strengthening the liver's defences against infection and strengthening the immune system (Liu *et al*., 2022). Our findings revealed that, at 14 dpi with *L. monocytogenes*, broilers offered dietary liposomal hesperetin inclusion at levels of 400 and 250 mg/kg exhibited the most prominent reduction in the intestinal levels of LYZ. Moreover, when compared with the control group, broilers supplemented with dietary liposomal hesperetin at a dosage of 400 mg/kg demonstrated the highest noticeable immune response at 14 dpi as proven by elevated intestinal levels of IgG, and reduced intestinal levels of CRP, MPO, IL-6, TNF-α, complements C3 and C4. Our outcomes indicate that liposomal hesperetin fortification efficaciously counteracted the adverse effect on the immune system in *L. monocytogenes*-challenged broilers indicating its potent enhancing impact on broiler immunity, overall health, and welfare. Similar to this, a previous report stated that hesperetin reduced the levels of TNF-α and IL-6 in mice (Wang *et al*., 2019). In accordance, a recent study demonstrated

that dietary phytogenics inclusion significantly increased the level of IgG, and reduced the levels of MPO, CRP, TNF-α, IL-1β, and IL-6 in broilers (El-Ghareeb *et al.*, 2023). Likewise, a prior report showed that dietary plant extract significantly elevate the level of IgM, and minimize the levels of complements C3, and C4, IL-1β, and IL-6 in broilers (Liu *et al.*, 2022). Similarly, previous studies demonstrated that dietary phytogenics supplementations such as essential oils, resveratrol, and curcumin, have an immunostimulatory impact by elevating the IgM, IgA, and IgG levels, suppressing LYZ, MPO and lowering MPO mRNA expression in neutrophils, hence preventing the production of ROS and nitric oxide (Castro *et al.*, 2008; Chang *et al.*, 2013; Hashem *et al.*, 2022; Abd El-Hamid *et al.*, 2024). Nevertheless, up to now, the anti-inflammatory and immunostimulant properties of liposomal hesperetin in broilers challenged with *L. monocytogenes* strain remained unexplored.

Of note, the interplay between phytogenics and the superior immune system can impact the durability of integrity and barrier functions of the gastrointestinal tract by modifying the expression of mucin, TJP, and cytokines (Abd El-Hamid *et al.*, 2024). Cytokines have key regulatory functions in the inflammatory reaction of the digestive tract and they are fundamental for the host defence mechanisms against pathogenic organisms. The immune cells of the gastrointestinal tract are prompted to release cytokines when microbes infiltrate the digestive tract epithelium (Aljazzar *et al.*, 2022). The MyD88-dependent signalling cascades and MyD88-independent signalling can alter the inflammatory response by stimulating the generation of proinflammatory cytokines (Ibrahim *et al.*, 2020). Proinflammatory cytokines, including interleukin TNF-α, IL-6, IL-1β, and IL-18, play crucial roles in the acute-phase inflammatory processes, which are linked to both metabolic and general alterations. Furthermore, during an infection, these proinflammatory cytokines play critical functions in regulating the host immunological response (El-Ghareeb *et al.*, 2023). On the contrary, IL-10 is a vital anti-inflammatory cytokine with an opposing influence on inflammation via inhibiting inflammatory and immunological responses (Abd El-Hamid *et al.*, 2024). Additionally, the chemotactic cytokines CCL4 and CCL20, collectively referred to as inflammatory proteins of macrophage, are crucial for regulating the immunological defences of the host against illness. In broilers, the CCL20 chemokine is essential for the onset of chronic inflammation of the gut (Cardoso Dal Pont *et al.*, 2023). Of note, the immune system of the host relies heavily on the peptide defensin, which provides immediate protection against bacterial infection. For broiler macrophages, *AVBD6* and *AVBD12* have chemotactic and lipopolysaccharide-neutralizing impacts (Zhao *et al.*, 2016; El-Ghareeb *et al.*, 2023). Of note, when pathogens infiltrate the intestinal epithelial cells, the gastrointestinal immune cells start producing cytokines, which further enhances the immune system's defence against the pathogens (Kayamuro *et al.*, 2010). In this regard, *L. monocytogenes* infection could upregulate the transcription of *IL-1β*, *IL-6, IL-8,* and *TNF-α* genes, increasing intestinal epithelial permeability (Abd El-Hamid *et al.*, 2022). Our results demonstrated that, in parallel with enhancing the intestinal immunological markers, broilers given 400 mg/kg of liposomal hesperetin showed the most significant downregulation in the expression levels of *IL-1β*, *IL-18*, and *CCL20* genes, and the most significant upregulation in the transcription levels of *IL-10*, and *AVBD6* genes comparing with the control group at 14 dpi with *L. monocytogenes* strain. Moreover, broilers fortified with dietary liposomal hesperetin inclusion at levels of 400 and 250 mg/kg had the most prominent downregulation in the transcription level of the *MyD88* gene at 14 dpi. Our outcomes suggest that liposomal hesperetin supplementation effectively counteracted the strong inflammatory reactions in *L. monocytogenes*-challenged broilers indicating its potent immunostimulant and anti-inflammatory properties, which consequently enhanced broiler immunity, health, and welfare. In accordance, hesperidin downregulated the transcription of *IL-1β*, *IL-6,* and *TNF-α* genes in mice, suggesting its anti-inflammatory effect (Sugasawa *et al.*, 2019; Famurewa *et al.*, 2022). Similarly, previous studies stated that phytogenics exhibit immunostimulant and anti-inflammatory properties in a range of inflammatory and immunologic diseases in poultry and they have enhancing effects on anti-inflammatory cytokines like IL-10, peptide defensins such as *AVBD6* and *AVBD12,* as well as proinflammatory cytokines like IL-1β, IL-18, IL-6, and TNF-α, and chemotactic cytokines CCL4 and CCL20, which prevents the progression of gut inflammation and preserves gut haemostasis (Aljazzar *et al.*, 2022; Hashem *et al.*, 2022; El-Ghareeb *et al.*, 2023; Abd El-Hamid *et al.*, 2024). Nevertheless, liposomal hesperetin immunostimulant and anti-inflammatory properties have not yet been studied in broilers challenged with *L. monocytogenes* strain.

Autophagy is a significant process, which maintains the hemostasis of cells and physiological functions such as immunity, development, and reproduction (Kishawy *et al.*, 2022). Through the process of autophagy, destroyed macromolecules and organelles and microbes are eliminated by the cells using lysosomes; thus it is considered a defence process against dangerous stimuli (Ibrahim, Shahin *et al.*, 2022; Kishawy *et al.*, 2022). A distinct set of autophagy-related proteins, including atg5-atg12, mtoR, AKT, and LC3 is thought to contribute to the occurrence of autophagy

(Ibrahim, Arisha *et al.*, 2022). Moreover, *Bcl-1* and *LC3* are considered pro-autophagy genes, while *p62* is an anti-autophagy gene (Li *et al.*, 2022). Additionally, mtoR and AKT, serine/threonine kinases, play critical roles in the metabolism of cells and the process of autophagy. Activation of the *mtoR* gene coincides with the start of autophagy in the opposite direction (Kim & Guan, 2015). Additionally, AKT regulates mtoR, and activated AKT could directly phosphorylate mtoR (Li *et al.*, 2022). Meanwhile, a higher level of AMPK promotes the generation of energy through autophagy, lipolysis, and glycolysis, and inhibits energy-exhausting processes such as protein synthesis (Kishawy *et al.*, 2022), in addition to downregulating the expression of the *mTOR* gene (Li *et al.*, 2022). Of note, CHOP, Bip, and XBP1 are crucial factors for endoplasmic reticulum (ER) stress-induced apoptosis (Huang *et al.*, 2018; Wang *et al.*, 2019; Ma *et al.*, 2021). Moreover, the ER stress-induced pro-apoptotic effect of the *CHOP* gene is accomplished by suppressing the ER stress-induced anti-apoptotic gene *Bcl-2* and regulating caspase-3 (Ma *et al.*, 2021). Our results revealed that fortifying the broilers' diet with liposomal hesperetin at a dosage of 400 mg/kg resulted in the highest upregulation in the transcription levels of intestinal *Bcl-2, LC3,* and *AMPK, AKT* genes, and the most prominent downregulation in the expression levels of intestinal *CHOP*, *Bip,* and *p62* genes in contrast to the control group at 14 dpi with *L. monocytogenes* strain, which suggest the promoting impact of liposomal hesperetin on the autophagy process that in turn enhanced the birds' immunity and overall health. Moreover, dietary liposomal hesperetin supplementation at concentrations of 400 and 250 mg/kg showed the most significant reduction in the expression level of the intestinal *XBP1* gene at 14 dpi with *L. monocytogenes* strain. Similarly, earlier research stated that hesperidin upregulated the expression of *Bcl-2* (Hager-Theodorides *et al.*, 2021; Famurewa *et al.*, 2022; Hussain *et al.*, 2022), and *AMPK* genes (Xiong *et al.*, 2019). Additionally, previous work showed that hesperetin downregulated the *CHOP* and *XBP1* genes and upregulated the *Bcl-2* gene in mice (Hussain *et al.*, 2022; Song *et al.*, 2024). In accordance, previous studies stated that phytogenics upregulated the transcription levels of *lc3-II, atg12,* and *atg5* genes, and reduced the expression level of the *mtoR* gene in broilers (Kishawy *et al.*, 2022), and challenged fish (Ibrahim, Shahin *et al.*, 2022). However, the impact of liposomal hesperetin supplementation on the expression of autophagy-related genes in broilers challenged with *L. monocytogenes* strain has not yet been explored.

A crucial role of the intestinal epithelium is to create a natural barrier that prevents harmful bacteria and hazardous substances from penetrating the mucosa and coming into contact with the immune defence, which maintains the homeostasis of the gastrointestinal tract (Turner, 2009; Patra, 2019). The tight junction barriers serve as both physical and functional barriers (Tabler *et al.*, 2020), and the primary stimulators of their formation are intestinal tight junctions and their associated proteins such as *JAM-2, CLDN-1,* and occludin (Shen *et al.*, 2011; Ibrahim, Eldemery *et al.*, 2022). The production of TJP is frequently disrupted during the onset of many inflammatory diseases (König *et al.*, 2016), which can lead to a reduction of feed absorption, an increase in luminal antigen permeability, translocation of microorganisms, prolonged inflammation, and cell destruction (Peterson & Artis, 2014). Notably, mucin, which is controlled by the *MUC-2* gene, is thought to be the first line of defence in the gut; therefore, boosting its production might help reduce the invasion of pathogens and the production of toxins (Murai *et al.*, 2018). Inflammatory conditions can hinder the production of mucin by goblet cells, delay the regeneration of the gut mucous membranes, and increase intestinal inflammation and translocation of pathogens (Forder *et al.*, 2012; Ibrahim *et al.*, 2020). In this context, herbal extracts have been shown to raise the number of goblet cells that support the mucous layer, which strengthens the integrity of intestinal barriers (Abd El-Hamid *et al.*, 2022). Earlier studies have discussed the impact of TJP on the permeability of the gastrointestinal tract (Shen *et al.*, 2011; Slifer & Blikslager, 2020). It has been shown that gut microbial populations are effectively linked to the establishment of the intestinal immune defence, the enhancement of the epithelial barrier, and the restriction of microbial colonization (Kamada *et al.*, 2013). However, nutritional modifications, stress, antimicrobial usage, and diseases can all alter the gut microbiome in ways that can induce an unbalanced state of gut homeostasis (Karl *et al.*, 2017). Intestinal barrier integrity disruption in stressful environments may result from the downregulation of the transcription levels of genes encoding TJP (Abd El-Hamid *et al.*, 2022). Notably, phytogenics may reduce the stress associated with intensive farming by improving the performance of the intestinal barrier and maintaining gut homeostasis (Elmowalid *et al.*, 2022). An earlier report found that EOs provoked gut health and barrier integrity (Wlodarska *et al.*, 2015). Herein, in parallel with the improved growth performance attributes, broilers offered 400 mg/kg liposomal hesperetin showed the greatest upregulation of genes encoding TJP (occludin, and *JAM-2*) and gut barrier functions (*MUC-2*) at 14 dpi with *L. monocytogenes* strain. Our results suggest that liposomal hesperetin inclusion effectively counteracted the adverse reaction in *L. monocytogenes*-challenged broilers indicating its potent enhancement effect on the gut barriers and overall health. In accordance with our findings, a

previous study showed that phytogenics enhanced the gut barrier functions (Kapan *et al.*, 2012). Similarly, it was noted that the transcription levels of occludin, *JAM-2,* and *MUC-2* genes were significantly upregulated in broilers following dietary fortification with eugenol nanoemulsions (Ibrahim, Eldemery *et al.*, 2022), garlic nano-hydrogel (Ibrahim, Ismail *et al.*, 2021), essential oils mixture (Hashem *et al.*, 2022), quercetin nanoparticles (Khater *et al.*, 2022) and thymol nanoemulsion (Ibrahim, Abdelfattah-Hassan *et al.*, 2021). However, the effect of liposomal hesperetin on the transcription levels of gut barrier function and TJP-encoding genes in *L. monocytogenes*-challenged broilers has not been studied until now. Of note, the herbal extracts may affect the operation of the gut barrier and the transportation of nutrients through a variety of molecular pathways that may control the transcription of the occludin, *JAM-2*, and *CLDN-1* genes (Patra, 2019).

Concerning listeriosis, *L. monocytogenes* can colonize the gastrointestinal tract and proceed to infect the spleen, liver, brain, and immune cells (Abd El-Hamid *et al.*, 2022). Antimicrobials have been used for many years to treat microbial infections and are considered a vital tool in the fight against infectious diseases, but they have several grave negative effects such as the emergence of MDR pathogens (Aljazzar *et al.*, 2022). In this regard, possible benefits of phytogenics have been interpreted as a logical attempt to combat gut pathogens (Ammar, El-Naenaeey, El-Malt *et al.*, 2021; Hashem *et al.*, 2022; Ibrahim, Shahin *et al.*, 2022). There is little information available about the ability of phytogenics to prevent or treat *L. monocytogenes* infection in broilers. Our findings revealed that *L. monocytogenes* populations were at their minimum concentrations in the caecal, liver, and spleen tissues of broilers fortified with dietary liposomal hesperetin inclusion at concentrations of 400 and 250 mg/kg at 7 and 14 dpi following *L. monocytogenes* infection. At 7 dpi, broilers offered dietary liposomal hesperetin inclusion at a dosage of 400 mg/kg displayed the most significant reduction in the *L. monocytogenes* load in the splenic tissues when compared with the control group. Of note, no CFUs were determined in brain tissues of broilers offered liposomal hesperetin at a concentration of 400 mg/kg at either time-point post-challenge with *L. monocytogenes* strain. Our findings suggested the antibacterial efficacy of liposomal hesperetin, which in turn improved the broilers' immunity, overall health, and welfare. Similarly, previous studies reported *in vitro* antibacterial activities of plant extract against *L. monocytogenes* (Over *et al.*, 2009; Pirbalouti *et al.*, 2010; McMurray *et al.*, 2020; Cacciatore *et al.*, 2022). In accordance, phytogenics were shown to have *in vivo* antibacterial properties, as prior research found that they significantly reduced the *Staphylococcus aureus* and *Pasteurella multocida* loads in challenged broilers (Radi *et al.*, 2020; Hosseini-Vashan *et al.*, 2021) and rabbits (Elmowalid *et al.*, 2022; Abd El-Hamid *et al.*, 2024); however, the effect of liposomal hesperetin on *L. monocytogenes* count in the caecal, spleen, brain, and liver tissues of challenged broilers has not been investigated to date. Additionally, significant histological alterations resembling septicaemia and encephalomalacia were found in the intestine and brain tissues of the broilers challenged with *L. monocytogenes* (positive control group). Comparable observations were seen earlier (Abd El-Hamid *et al.*, 2022) in the organs of rabbits challenged with *L. monocytogenes*. Liposomal hesperetin fortification inhibited *L. monocytogenes* translocation to other organs, as seen by restoring the normal histopathological architecture of the intestine and brain of broilers, which suggests its promoting impact on bird immunity, health, and welfare. Likewise, following administration of phytogenics to challenged broilers, notable improvements were observed in the histological architecture of their tissues (Ibrahim, Abdelfattah-Hassan *et al.*, 2021). This may be related to the beneficial effects of phytogenics, as demonstrated by our findings, on boosting broiler immunity against intestinal pathogen infection and fortifying intestinal barriers, which subsequently prevents infections from spreading to other organs. Furthermore, concerning *L. monocytogenes* resistance in experimentally infected broilers following liposomal hesperetin supplementation, previous research revealed that herbal plant extract can modify the function of the innate immune response by reducing bacterial survival, increasing the production of nitric oxide, and enhancing macrophage phagocytic capacity (Elmowalid *et al.*, 2022; Abd El-Hamid *et al.*, 2024).

A novel strategy for preventing illnesses in the poultry industry is nutritional immunology, which circumvents the restrictions of immunization programmes by using dietary fortifications (Abd El-Hamid *et al.*, 2022; Elmowalid *et al.*, 2022). Furthermore, raising broilers will be more cost-effective and productively efficient if their diet and veterinary treatment are improved to minimize infections. Remarkably, many therapeutic approaches now focus on bacterial pathogenicity instead of bacterial survival (Abd El-Hamid *et al.*, 2024). Therefore, we evaluated the expression levels of *hlyA*, *flaA*, and *ami* virulence genes in response to liposomal hesperetin fortification at 7 and 14 dpi with the *L. monocytogenes* strain to study the anti-virulence characteristics of liposomal hesperetin. Our results showed that liposomal hesperetin fortification, especially at higher concentrations, significantly reduced the expression levels of *hlyA*, *flaA*, and *ami* virulence genes, suggesting its anti-virulence characteristics, which consequently improved the birds' immunity, health, and welfare. In

accordance, recent work showed the *in vivo* anti-virulence properties of dietary thymoquinone nanoemulsion fortification against *P. multocida* in challenged rabbits (Abd El-Hamid *et al.*, 2024). Moreover, prior research reported the *in vivo* anti-virulence characteristics of thymol nanoemulsion against *Salmonella* Enteritidis in experimentally infected broiler chickens (Bendary *et al.*, 2021). Of note, previous studies showed the *in vitro* anti-virulence characteristics of phytogenics against *L. monocytogenes* (Upadhyay *et al.*, 2012; Xu *et al.*, 2015; Pieta *et al.*, 2017; J. Li *et al.*, 2021); nevertheless, the *in vivo* anti-virulence impact of liposomal hesperetin in broilers challenged with *L. monocytogenes* has not yet been investigated. The bacterial gene regulation system known as quorum sensing (QS), which regulates the gene expression of several virulence indicators, may have been inhibited by liposomal hesperetin, giving rise to its anti-virulence properties (Ibrahim, Shahin *et al.*, 2022). According to a recent study, phytogenics have a dose-dependent influence on a variety of QS indicators and inhibit QS at sub-inhibitory concentrations (Miller *et al.*, 2015). The potential cause of phytogenic anti-QS activities could be their direct influence on the synthesis of signalling molecules of QS and deactivation of cognate receptors. This, therefore, prevented transcriptional activation of the virulence genes that control cooperative behaviours (Ibrahim, Shahin *et al.*, 2022; Abd El-Hamid *et al.*, 2024). According to our perspective, liposomal hesperetin possesses growth-promoting, antioxidant, antimicrobial, immunostimulant, and anti-inflammatory qualities that improve serum cellular and humoral immunity, hence improving the overall health and welfare, besides diminishing the growth of pathogenic microbes and inflammation in broilers.

Overall, our interesting findings showed that dietary liposomal hesperetin fortification enhanced the broilers' growth performance, health, and antioxidant status by promoting the levels of oxidative and antioxidant markers. Additionally, dietary liposomal hesperetin supplementation for broilers experimentally infected with MDR multi-virulent *L. monocytogenes* strain decreased the severity of clinical signs and the microbial localization or translocation via lowering the *L. monocytogenes* loads in the caecal, liver, spleen and brain tissues of broilers, reducing the transcription levels of genes linked to *L. monocytogenes* virulence, and improving the expression levels of genes encoding cytokines, antioxidant, TJP, and autophagy, which reflects its enhancing impact on broilers' immunity, overall health and welfare. Consequently, our results point to the potential application of liposomal hesperetin as an innovative dietary supplement that is claimed to be essential for controlling *L. monocytogenes* infection in broilers.

Disclosure statement

No potential conflict of interest was reported by the authors.

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Data availability statement

The data presented in this study are available upon request from the corresponding author.

Author contributions

Conceptualization, M.I.A., S.I.K., D.I.; methodology, M.I.A., R.M.S.E., D.I.; software, M.I.A., A.A.A., D.I.; validation, M.I.A., T.K., D.I.; formal analysis, M.I.A., R.A.A., D.I.; investigation, M.I.A., R.M.S.E., D.I.; resources, M.I.A., E.M.Y., D.I.; data curation, M.I.A., M.A.A., D.I.; writing – original draft preparation, M.I.A., R.M.S.E., D.I.; writing – review and editing, M.I.A., R.M.S.E., D.I.; visualization, M.I.A., D.I.M., D.I.; supervision, M.I.A., S.S.L., D.I.; project administration, M.I.A., S.J.D., D.I.; funding acquisition, M.I.A., R.M.S.E., D.I.

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