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RESEARCH ARTICLE

Nano-Encapsulated Phytogenic Compounds Modulate Gut-Liver Axis Integrity, Oxidative Balance, And Immunological Activity in Broilers Exposed to Cyclic Heat Stress

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ABSTRACT

Heat stress significantly hinders broiler output by compromising gut integrity, oxidative balance, and immune function. This study evaluated nano-encapsulated phytogenic compounds (NEP) as a nutritional strategy to alleviate heat stress. Three hundred sixty males of Cobb500 broilers were allocated to a 2×3 factorial design with two ambient temperatures and three dietary treatments (6 replicate pens × 10 birds). Birds received a basal diet supplemented with a conventional phytogenic extract (CPE, 500mg/kg), NEP (250mg/kg), or no supplementation (CON). The phytogenic blend contained curcumin (40%), resveratrol (30%), and quercetin (30%) and the NEP consisted of this blend encapsulated in PEGylated liposomes (80–120nM particle size, >85% encapsulation efficiency). From 22−42 days, birds were exposed to a cyclic heat stress treatment (HS; 38°C, 8h/day) or maintained under thermoneutral conditions (TN; 24°C). In CON birds, HS reduced body weight gain (BWG) and feed intake by 24 and 11%, respectively, and increased the feed-to-gain (F:G) ratio by 16% ($P<0.05$). Compared with HS-CON, NEP increased BWG (+21%) and improved the F:G ratio (−13%). Under HS, NEP reduced serum interleukin-6 (IL-6; −55%), hepatic malondialdehyde (−50%) and jejunal NF-κB mRNA expression (−2.5-fold), and increased serum IL-10 (+89%), IgA (+114%), IgG (+47%), hepatic superoxide dismutase activity (+46%) and glutathione peroxidase (+90%) activities, and jejunal occludin (+2.4-fold), claudin-1 (+2.3-fold) and Nrf2 (+2.2-fold) mRNA expression ($P<0.05$). NEP also increased the villus height/crypt depth ratio (+2-fold) and cecal *Lactobacillus* counts (+1.43 $\log_{10}$ copies/g) and reduced cecal *Escherichia coli* counts (−1.20 $\log_{10}$ copies/g). In direct comparisons under HS, NEP produced greater responses than CPE for most variables. These findings indicate that NEP, supplemented at half the CPE inclusion rate, attenuated several heat-stress-induced impairments in broilers. Because each formulation was tested at a single, non-equivalent inclusion level without a carrier-only control and bioavailability was not measured, dose–response, carrier-controlled and pharmacokinetic studies are needed to confirm a dose-sparing effect and the underlying mechanisms.

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INTRODUCTION

Global poultry production faces increasing challenges from climate change, with heat stress imposing substantial economic losses. Particularly, broiler breeding is vulnerable under elevated ambient temperatures due to their condensed growth, excessive metabolic rate and exclusive thermoregulation (Aryal *et al.*, 2025). Cyclic heat stress, characterized by diurnal temperature fluctuations that simulate natural field conditions, triggers a cascade of physiological disruptions, such as diminished feed consumption, impaired nutrient absorption, compromised intestinal barrier function, and induced inflammation and oxidative damage (Du *et al.*, 2022).

Thermal stress provokes the cellular synthesis of reactive oxygen species (ROS), which results in lipid

peroxidation and antioxidant enzyme depletion (Sumanu *et al.*, 2023; Aryal *et al.*, 2025). The principal regulator of the cellular antioxidant defense, nuclear factor erythroid 2-related factor 2 (Nrf2), is often suppressed under chronic heat stress (Ding *et al.*, 2023), while the pro-inflammatory inducer, nuclear factor kappa B (NF-κB), becomes hyperactivated; both factors perpetuate oxidative damage and inflammatory signaling pathways (Liu *et al.*, 2022a). In addition, heat stress compromises intestinal barrier function through multiple mechanisms, such as oxidative damage to membrane lipids and proteins, downregulation of tight junction gene expression, villus atrophy, crypt hyperplasia, and dysbiosis of the cecal microbiota (Liu *et al.*, 2022b). The increased intestinal permeability allows bacterial endotoxins to enter portal circulation, triggering hepatic inflammation by reducing anti-inflammatory mediators (IL-10) and elevating pro-inflammatory cytokines, such as TNF-α and IL-6 (Li *et al.*, 2020; Reisinger *et al.*, 2020). Simultaneously, exposure to HS shifts cecal microbiota composition toward pathogenic species (*Escherichia coli*, *Clostridium perfringens*) at the expense of beneficial commensals (Lactobacillus, Bifidobacterium), further compromising gut health and immune function (Ncho, 2025).

Diet supplementation with phytogenic substances, such as curcumin, resveratrol, and quercetin, has emerged as a promising nutritional intervention to mitigate HS effects in poultry (Abd El-Hack *et al.*, 2026). These bioactive compounds exhibit pleiotropic mechanisms, including direct ROS scavenging, upregulation of endogenous antioxidant enzymes via Nrf2 activation, inhibition of NF-κB-mediated inflammation, maintenance of tight junction integrity, and modulation of intestinal microbiota (Dong *et al*., 2020; Ding *et al.*, 2023; Gharib *et al.*, 2025; Khan *et al.*, 2025). However, conventional phytogenic extracts often have poor solubility and bioavailability and may be degraded in the gastric environment, which limits *in-vivo* efficacy and motivates the development of protected delivery systems (Nantapo and Marume, 2025).

Nano-encapsulation technology offers a nanoscale delivery system for bioactive compounds, enhancing stability, bioavailability, and intestinal absorption (Kamble *et al.*, 2025). Liposomal nanocarriers, composed of phospholipid bilayers that mimic biological membranes, are particularly suitable for encapsulating lipophilic phytochemicals (Misra and Pathak, 2022). PEGylation (surface modification with polyethylene glycol) further enhances nanoparticle stability and circulation time by reducing recognition by the reticuloendothelial system (Chelliah *et al.*, 2025). Recent research have demonstrated that nano-encapsulated phytogenics achieve superior biological effects compared to conventional forms, including enhanced antioxidant enzyme activities, improved tight junction protein expression, stronger immunoglobulin responses, and more favorable microbiota profiles, often at reduced dosages (Meligy *et al.*, 2023; Abdel-Raheem *et al.*, 2024; Fathi *et al.*, 2025).

Despite growing evidence supporting the use of nano-encapsulated phytogenic (NEP) compounds in poultry, their efficacy under cyclic heat stress, which more closely reflects commercial production conditions, remains poorly understood. In particular, the integrated effects of multi-component nano-formulations on the gut–liver axis, including intestinal barrier integrity, oxidative and inflammatory status, immune responses, and cecal microbiota, have not been comprehensively evaluated, and direct comparisons with conventional phytogenic extracts (CPE) are limited. Therefore, this study investigated the protective efficacy of supplementing broiler diets with an NEP product containing curcumin, resveratrol, and quercetin, compared with that of a CPE, in alleviating growth and physiological deterioration under heat-stress conditions. We hypothesized that NEP would enhance the bioavailability and biological activity of the phytogenic blend, providing superior protection against heat-stress-induced physiological disturbances at a lower dietary inclusion level than the CPE.

MATERIALS AND METHODS

Ethical approval statement: All experimental procedures were approved by the Ethical Research Council of King Faisal University, Saudi Arabia (Approval No. KFU-REC-2025-NOV-ETHICS3821).

Birds, housing and management: Three hundred sixty males of Cobb500 broiler chicks (1-d-old, 42.03±0.05g) were randomly allocated to floor pens (10 birds/pen) in environmentally controlled chambers. Birds were reared under standard commercial conditions with *ad-libitum* feed and water. Three-phase feeding diets (starter, grower, and finisher) were formulated to meet or exceed the nutritional recommendations of Cobb500 broiler (Table 1). Standard lighting, vaccination, and biosecurity programs were applied throughout the 42-day experiment.

Table 1: Composition and nutritional analysis of the basal diets administered during the phase feeding program to Cobb500 broiler chickens

Ingredient (per kg as fed)	Starter (1-10d)	Grower (11-24d)	Finisher (25-42d)
Soybean meal (g)	320	320	300
Wheat bran (g)	23	30	30
Corn yellow grain (g)	560	550	585
Corn gluten meal (g)	52	25	0
Vegetable oil (g)	6	35	45
Di-calcium phosphate (g)	15	15	15
Limestone (g)	10	10	10
Salt (g)	4.5	4.5	4.5
Premix[1]	5.5	5.5	5.5
DL-Methionine (g)	1.5	2.5	2.5
L-Lysine (g)	1	1	1
L-Threonine (g)	1	1	1
L-Tryptophane (g)	0.5	0.5	0.5
Nutrients calculated per kg			
Metabolizable energy (MJ)	12.1	12.7	13.0
Calcium (g)	8.0	8.0	8.0
Available phosphorus (g)	4.3	4.2	4.2
Met + Cyst (total)	9.1	8.8	8.6
Nutrients determined per kg			
Moisture (g)	95.7	98.6	100.5
Ash (g)	112.2	111.5	110.8
CP (g)	220.4	205.0	183.8
CF (g)	36.6	36.5	35.1
Total lipids (g)	29.1	57.2	67.7

[1]Diet contents per kg: 120mg manganese oxide, 100mg zinc sulfate; 20mg ferrous sulfate, 16mg cupric sulfate, 1.2mg potassium iodide, 0.32mg sodium selenite, 36mg α-tocopherol acetate, 250mg choline chloride, 29.65mg pantothenic acid,18mg retinol, 9.8mg niacin, 6.6mg vitamin B2, 4mg cholecalciferol, 2.94mg vitamin B6, 2mg vitamin K3, 1.75mg vitamin B1, 1mg folic acid, 0.1mg biotin, 0.015mg vitamin B12.

Treatments and experimental design: Two ambient temperature conditions and three dietary treatments were used in a completely randomized 2×3 factorial design (6 replicate pens per treatment group). Birds in dietary treatments were fed a basal diet supplemented with a conventional phytogenic extract (CPE; 500mg/kg feed), nano-encapsulated phytogenic compounds (NEP; 250mg/kg feed), or without supplementation (control; CON) from day 1 to 42. From days 22−42, birds in each dietary treatment were either maintained at thermoneutral conditions (24±1°C; TN groups) or subjected to cyclic heat stress treatment (HS groups; 38±1°C for 8 h/day, returning to 24°C during the remaining period). All treatment groups were maintained at 60±5% relative humidity. The HS protocol (38±1°C for 8 h/day) was selected to simulate the daytime peak temperatures that commonly exceed 35°C in open and semi-closed poultry houses during the summer season in Saudi Arabia, while the nightly return to 24°C reproduces the diurnal recovery phase of cyclic HS models used in broilers (Li *et al*., 2020; Du *et al*., 2022). The 22–42 d window was chosen because broilers are most susceptible to HS during the grower–finisher phase, when metabolic heat production increases with rapid growth and complete feathering (Brugaletta *et al*., 2022). Ambient temperature and relative humidity were monitored continuously in each chamber and recorded at 10-min intervals using calibrated data loggers (DL2000 data logger, Vaisala, Vantaa, Finland); the mean recorded temperatures were 38.2±0.6°C during the daily heat exposure and 24.1±0.5°C during the remaining period. Rectal temperature and respiratory rate were not recorded; the severity of HS was therefore assessed from its effects on feed intake, growth performance and serum and hepatic stress-related indicators.

CPE and NEP preparation: Both CPE and NEP were prepared from a phytogenic blend, containing curcumin, resveratrol and quercetin (HPLC analytical grade, purity ≥94, ≥99 and ≥95%, respectively; Sigma-Aldrich, MO, USA), according to Abdallah *et al*. (2024). For the CPE, the three compounds were weighed at the 40:30:30 (w/w) ratio and homogenized in a laboratory blender for 15min to obtain a uniform, non-encapsulated powder without any carrier or excipient. The CPE inclusion rate (500mg/kg) was selected to match a previously used broiler supplementation level for these polyphenols (Gümüş *et al.*, 2024). Accordingly, the CPE diet supplied 200, 150 and 150mg/kg feed of curcumin, resveratrol and quercetin, respectively. The NEP level was deliberately set at 50% of CPE rate (250mg/kg) to test whether nano-encapsulation could preserve efficacy at a reduced inclusion rate, as previously reported by Fathi *et al.* (2025). Thereby, the NEP diet supplied approximately 100, 75 and 75mg/kg feed of curcumin, resveratrol and quercetin, respectively. The NEP was prepared by encapsulating the phytogenic blend in PEGylated liposomes (NanoFabTx™, Sigma-Aldrich) following the manufacturer's protocol (Kishawy *et al.*, 2023), with minor modifications. Briefly, the phytogenic blend and the PEGylated lipid mixture supplied with the kit (hydrogenated soy phosphatidylcholine, cholesterol and DSPE-mPEG2000 at a molar ratio of 56.5:38:5.5) were co-dissolved in ethanol at a blend-to-lipid mass ratio of 1:4. The organic phase was then mixed with PBS (10mM, pH7.4) using the NanoFabTx™ microfluidic device at an aqueous-to-organic flow rate ratio of 3:1 and a total flow rate of 12mL/min at room temperature. The resulting liposomal suspension was dialyzed against PBS (MWCO 10kDa, 4°C, 24h, with three buffer changes) to remove ethanol and non-encapsulated compounds, lyophilized for 48h with Alpha 1-2 LD Plus (Martin Christ, Germany), and stored at 4°C in the dark until use. To prepare the experimental diets, NEP and CPE were first premixed with 1kg of the basal diet and then blended with the remaining feed in a horizontal mixer for 10min. Supplemented diets were prepared weekly and stored in sealed, light-protected bags. The identity and quantity of all carrier materials supplied by each diet, together with the calculated concentrations of each active compound in the final feed (Table 2).

Table 2: Supplement composition, carrier materials and concentrations of active compounds in the experimental diets

Item	CON	CPE	NEP
Supplement added to feed (mg/kg)	0	500	1,250[1]
Phytogenic blend (mg/kg)	0	500	250
Active compounds (calculated)[2], mg/kg			
Curcumin	0	200	100
Resveratrol	0	150	75
Quercetin	0	150	75
Carrier materials, mg/kg[3]			
Hydrogenated soy phosphatidylcholine	0	0	597
Cholesterol	0	0	197
DSPE-mPEG2000	0	0	206
Total liposomal lipid	0	0	1,000

CON, control basal diet without supplementation; CPE, conventional phytogenic extract (non-encapsulated); NEP, nano-encapsulated phytogenic compounds (PEGylated liposomes); DSPE-mPEG2000, 1,2-distearoyl-sn-glycero-3-phosphoethanolamine-N-[methoxy(polyethylene glycol)-2000]. [1]Amount of lyophilized liposomal product needed to supply 250mg of the phytogenic blend per kg of feed (250mg blend + 1,000mg lipids). [2]Calculated from the inclusion rate and the 40:30:30 (w/w) blend composition. [3]Lipid amounts were calculated from the blend-to-lipid mass ratio (1:4) and the phosphatidylcholine: cholesterol: DSPE-mPEG2000 molar ratio (56.5:38:5.5), using molecular weights of 790, 387 and 2,806g/M, respectively.

Characterization of the NEP: Particle size and size distribution: The mean hydrodynamic diameter (Z-average) and polydispersity index (PDI) of the liposomes were determined by dynamic light scattering (DLS) using a Zetasizer Nano ZS (Malvern Instruments, Malvern, UK) equipped with a 633nm He–Ne laser, with detection at a backscatter angle of 173°. Samples were diluted 1:100 (v/v) in deionized water filtered through a 0.22μM membrane to avoid multiple scattering, equilibrated at 25°C for 120s, and measured in disposable polystyrene cuvettes. Intensity-weighted size distributions were obtained by cumulants analysis. Three independently prepared batches were analyzed, each in triplicate (12 sub-runs per measurement), and the results are presented as the size range (80–120nm) and mean PDI (PDI=0.202±0.016). Analytical recovery ranged from 96.8 to 103.1%, and intra-assay coefficients of variation were <3.5%.

Zeta potential: Surface charge was determined by electrophoretic light scattering on the same instrument using disposable folded capillary cells (DTS1070,

Malvern Instruments) at 25°C. Samples were diluted 1:100 (v/v) in 10mM NaCl to maintain a constant ionic strength, and electrophoretic mobility was converted to zeta potential using the Smoluchowski approximation (Henry's function, f(κa)=1.5). Each batch was measured in triplicate with at least 10 runs per measurement, and values are reported as mean±SD (−32±1.9mV).

Encapsulation efficiency (EE): EE was determined indirectly by separating the non-encapsulated (free) phytochemicals from the liposomes by ultrafiltration using Amicon Ultra-4 centrifugal filter units (MWCO 10kDa; Merck Millipore, Darmstadt, Germany) at 4,000×g for 20min at 4°C. To determine the total phytochemical content, an aliquot of the unfiltered suspension was disrupted with methanol (1:9, v/v) and sonicated for 10min to release the encapsulated compounds. The phytochemical content of the filtrate (free fraction) and of the disrupted suspension (total content) was determined spectrophotometrically using a UV–visible spectrophotometer (CE1010, Cecil Instruments Limited, Cambridge, UK) at 425nM against a methanol blank. Concentrations were calculated from an external calibration curve prepared from the curcumin analytical standard (Sigma-Aldrich) and were expressed as curcumin equivalents. EE was calculated as follows: EE(%)=[(total content−free content)/total content]×100. Determinations were performed in triplicate for each of three independently prepared batches, and the EE was 88.2±1.1%.

Growth performance and sample collection: Feed consumption and BW were recorded by pen at 1, 10, 24 and 42 day of age. Body weight gain (BWG), feed intake (FI) and feed-to-gain (F:G) ratio were calculated for each feeding period (1–10, 11–24 and 25–42 d) and for the whole trial (1–42 d). Mortality was recorded daily, and the F:G ratio was corrected for mortality by adding the body weight of dead birds to the total weight gain of the corresponding pen. At day 42, two birds per replication (n=12/group) underwent an 8-hour fast, after which blood samples were obtained from the brachial vein. Serum was isolated by centrifugation (3,000×g at 4°C for 15 min) and kept at −80°C until analysis. After that, birds were killed by cervical dislocation. Liver and jejunum specimens and cecal contents were collected and then snap-frozen or fixed in formalin as appropriate for subsequent analyses.

Cytokine and immunoglobulin assay: Serum interleukins (IL-6 and IL-10) and immunoglobulins (IgA and IgG) were determined in duplicate in accordance with the manufacturer's guidelines of chicken-specific ELISA kits (Cusabio Biotech, Wuhan, China). The measurements were obtained by a microplate reader (BioTek Instruments ELx808^{TM}, Santa Clara, CA, USA). The intra- and inter-assay CVs for these assays were less than 10 and 15%, respectively.

Liver oxidative status assay: Liver samples (0.5 g) were homogenized (1:9, w/v) in ice-cold physiological saline and centrifuged (3,000×g, 10min, 4°C), and the supernatant was used for the assays. Malondialdehyde (MDA) concentration (thiobarbituric acid method; kit A003-1), superoxide dismutase (SOD) activity (WST-1 method; kit A001-3) and glutathione peroxidase (GPx) activity (kit A005-1) were determined using commercial kits (Nanjing Jiancheng Bioengineering Institute, Nanjing, China) according to the manufacturer's instructions, and absorbance was read on a microplate reader (BioTek ELx808™, Santa Clara, CA, USA). One unit of SOD activity was defined as the amount of enzyme producing 50% inhibition of the WST-1 reduction reaction, and one unit of GPx activity as the amount of enzyme that consumed 1μmol/L of reduced glutathione per min at 37°C after subtraction of the non-enzymatic reaction. The protein concentration of the supernatant was quantified by the Bradford method using bovine serum albumin as the standard, and MDA, SOD and GPx were normalized to protein concentration and expressed as nmol/mg protein, U/mg protein and U/mg protein, respectively. All samples were analyzed in duplicate, and the intra- and inter-assay coefficients of variation were <8 and <12%, respectively.

Gut integrity assay: Total RNA was extracted from jejunal tissue with TRIzol reagent (Invitrogen, Carlsbad, CA, USA), cDNA was established by reverse transcription with a PrimeScript RT Reagent Kit (Takara Bio, Shiga, Japan) and then quantitative real-time PCR was performed with SYBR Green chemistry on a StepOnePlus Real-Time PCR System (Applied Biosystems, Foster City, CA, USA). Negative control (no template) was included in each run, and all samples were analyzed in triplicate. Gene-specific primers for occludin, claudin-1, nuclear factor erythroid 2-related factor 2 (Nrf2) and nuclear factor kappa B (NF-κB) were designed using Primer3 software and BLAST analysis (Table 3). Relative expression of target genes was calculated using the $2^{-\Delta\Delta Ct}$ technique with β-actin as the reference gene.

Jejunal samples were processed routinely (Alaqil *et al.*, 2026), stained with hematoxylin and eosin and microscopically examined. The VH/CD ratio was derived using histomorphological assessments of villus height (VH) and crypt depth (CD) utilizing a light microscope fitted with a digital camera and ImageJ (National Institutes of Health, Bethesda, MD, USA). Jejunal segments (approximately 2 cm) were collected from the midpoint between the end of the duodenal loop and Meckel's diverticulum, fixed in 10% neutral-buffered formalin, embedded in paraffin and sectioned at 5 μm. Three non-consecutive cross-sections per bird were examined at 40× magnification, and 10 well-oriented villi with their associated crypts were measured per section (30 villi and 30 crypts per bird). Only villi with an intact epithelium and lamina propria, sectioned longitudinally along their full length, were measured. VH was measured from the villus tip to the villus–crypt junction, and CD from the villus–crypt junction to the base of the crypt. All measurements were performed by an investigator blinded to the treatment groups. Measurements were averaged per bird, and the bird means (2 birds/pen; n=12 per treatment) were used for statistical analysis.

A QIAamp DNA Stool Mini Kit (Qiagen, Hilden, Germany) was used to isolate total DNA from cecal contents. Quantitative PCR was conducted with group-specific primers targeting the 16S rRNA genes of

Lactobacillus spp. (LACT) and *Escherichia coli* (EC). Bacterial abundance was quantified as $\log_{10}$ gene copies per gram of cecal content. The primer sequences are listed in Table 3. Absolute quantification was performed using standard curves generated from 10-fold serial dilutions (102–108 copies) of genomic DNA from reference strains (Lactobacillus acidophilus ATCC 4356 and E. coli ATCC 25922). Primer specificity was confirmed by BLAST analysis, a single peak in the melting-curve analysis and a single amplicon of the expected size on agarose gel electrophoresis. Amplification efficiencies were 95.2% (R^2 = 0.998) for Lactobacillus and 97.4% (R^2 = 0.997) for E. coli. Gene copy numbers were calculated from the standard curves, corrected for the dilution factor and DNA elution volume, and expressed as $\log_{10}$ copies per gram of wet cecal content; differences between treatments are therefore reported as differences in $\log_{10}$ copies/g.

Statistical analysis: The MIXED technique in SAS (version 9.4, SAS Institute Inc., Cary, NC, USA) was performed to analyze the data using a 2×3 factorial ANOVA. The model established the effects of temperatures, dietary treatments and their interactions, as follow: $Y_{ijk} = \mu + T_i + D_j + (T\times D)_{ij} + e_{ijk}$, where Y_{ijk} is the observed value, μ is the overall mean, T_i is the fixed effect of ambient temperature (i = TN, HS), D_j is the fixed effect of dietary treatment (j = CON, CPE, NEP), $(T\times D)_{ij}$ is the temperature × diet interaction, and e_{ijk} is the random residual error. The pen (n = 6 per treatment) was the experimental unit for growth performance, resulting in 30 error degrees of freedom. For serum, hepatic, jejunal and cecal variables, two birds per pen were sampled and the individual bird was used as the observational unit (n=12 per treatment; 66 error degrees of freedom). The homogeneity of variances was assessed using Levene's test, and the normality of residuals was assessed using the Shapiro–Wilk test and inspection of residual plots. The main effects of temperature and diet and their interaction are reported separately with exact P-values. Means of the six treatment combinations and the main effect means of diet were compared using Tukey's HSD test. Effect estimates for dietary treatments under heat stress are presented as differences between means with Tukey-adjusted 95% confidence intervals (Table 9). Results are presented as means with the pooled SEM, and differences were considered significant at P<0.05.

RESULTS

Growth performance: The effects of cyclic heat stress and dietary supplementation with CPE or NEP on growth performance from 1 to 42d of age (Table 4). Significant temperature × diet interactions were detected for final BW, FI, BWG and F:G ratio (P<0.001). Under TN conditions, BWG of TN-NEP birds was 4.4% higher than that of TN-CON birds (P<0.05), whereas TN-CPE did not differ from either group. Cyclic heat stress markedly reduced BWG and FI, with HS-CON birds showing 23.7 and 11.4% reductions, respectively, compared with TN-CON birds (P<0.05). Phytogenic supplementation partially mitigated this decline, with BWG improvements of 7.2 and 21.0% over HS-CON in the HS-CPE and HS-NEP groups, respectively (P<0.05), and HS-NEP birds also gained more than HS-CPE birds (P<0.05; Table 9). A similar pattern was observed for the F:G ratio. Under TN conditions, the F:G ratio was improved by 5.2% in TN-NEP relative to TN-CON (P<0.05), whereas the 2.3% improvement in TN-CPE was not significant. Heat stress substantially impaired feed efficiency, resulting in a 16.2% higher F:G ratio in HS-CON than in TN-CON birds (P<0.05). Both CPE and NEP improved feed efficiency under HS conditions, decreasing the F:G ratio by 5.4 and 13.2%, respectively, relative to HS-CON (P<0.05), and the F:G ratio of HS-NEP birds did not differ from that of TN-CON birds.

Body weight at 10, 24 and 42d and FI and F:G ratio for each feeding period are also presented in Table 4. Before heat exposure (1–10 d), BW at 10d, FI and F:G ratio did not differ among treatments (P>0.05 for temperature, diet and their interaction). At 24d, after the first 3 days of heat exposure, HS reduced BW by 10.0% in CON birds compared with TN-CON birds (P<0.05), whereas BW of HS-NEP birds was 10.6% higher than that of HS-CON birds and did not differ from that of TN-CON birds. Over the 11–24 d period, HS increased the F:G ratio by 11.0% in CON birds, whereas the F:G ratio of HS-NEP birds was 11.9% lower than that of HS-CON birds and did not differ from that of TN-CON birds (P<0.05). During the finisher period (25–42 d), HS reduced FI by 6.7% and increased the F:G ratio by 40.2% in CON birds compared with TN-CON birds (P<0.05). Under HS, NEP increased FI by 3.7 and 2.7% compared with HS-CON and HS-CPE, respectively, and reduced the F:G ratio by 20.6% compared with HS-CON (P<0.05). Overall mortality was 3.89±0.82% and was not affected by temperature, diet or their interaction (P>0.05).

Table 3: List of the primer sequences used for the qPCR analysis of jejunal gene expression and cecal bacteria in broiler chickens.

Target Gene	Accession No.	Primer Sequence (5' → 3')	Product Size (bp)
Nrf2	NM_001006655	F: GCTCCAGTTTCCTCACTCCG	125
		R: TGGCTCTTGTAGTCGTTGTCG	
NF-κB	NM_205134.1	F: GAGGATGGCTTCTATGAGGCTG	156
		R: CGCACTGTCACCTGGAAGC	
Occludin	XM_046904540.1	F: GGCCACCATGTTCAGCAAGAA	99
		R: GACCCGTAGCCGTAATCAGCC	
Claudin-1	NM_001013611.2	F: AGAGGCATCAGGTATCTGGGT	214
		R: CCCATCGAGAAGTAGGAGCCA	
β-actin	NM_205518.1	F: CAACACAGTGCTGTCTGGTGGTA	139
		R: ATCGTACTCCTGCTTGCTGATCC	
Lactobacillus spp. (16S rRNA)	—	F: AGCAGTAGGGAATCTTCCA	341
		R: CACCGCTACACATGGAG	
E. coli (16S rRNA)	—	F:CATGCCGCGTGTATGAAGAA	96
		R: CGGGTAACGTCAATGAGCAAA	

Abbreviations: Nrf2, nuclear factor erythroid 2-related factor 2; NF-κB, nuclear factor kappa B.

Table 4: Effects of cyclic heat stress and dietary phytogenic supplementation on body weight, feed intake and feed-to-gain ratio of broiler chickens during different feeding periods

(A) Body weight (g) and body weight gain (g)				
Item	10 d	24 d	42 d	BWG, 1–42 d
Temperature × Diet				
TN-CON	356.0	1,287.4^{b}	3,006.8^{b}	2,964.8^{b}
TN-CPE	355.9	1,311.6^{ab}	3,072.1^{ab}	3,030.1^{ab}
TN-NEP	355.6	1,335.3^{a}	3,136.1^{a}	3,094.0^{a}
HS-CON	355.9	1,158.9^{d}	2,302.6^{e}	2,260.7^{e}
HS-CPE	355.9	1,228.0^{c}	2,465.6^{d}	2,423.3^{d}
HS-NEP	355.7	1,281.9^{b}	2,778.2^{c}	2,736.2^{c}
SEM	0.22	7.31	16.13	16.13
Main effect of temperature				
TN	355.9	1,311.4^{a}	3,071.6^{a}	3,029.6^{a}
HS	355.8	1,223.0^{b}	2,515.5^{b}	2,473.4^{b}
Main effect of diet				
CON	356.0	1,223.2^{c}	2,654.7^{c}	2,612.8^{c}
CPE	355.9	1,269.8^{b}	2,768.8^{b}	2,726.7^{b}
NEP	355.7	1,308.6^{a}	2,957.2^{a}	2,915.1^{a}
P-value				
Temperature (T)	0.900	<0.001	<0.001	<0.001
Diet (D)	0.340	<0.001	<0.001	<0.001
T × D	0.902	<0.001	<0.001	<0.001
(B) Feed intake (g/bird)				
Item	1–10 d	11–24 d	25–42 d	1–42 d
Temperature × Diet				
TN-CON	367.7	1,291.6^{a}	3,216.3^{a}	4,980.0^{a}
TN-CPE	367.1	1,289.7^{a}	3,213.4^{a}	4,974.0^{a}
TN-NEP	364.2	1,275.5^{a}	3,191.9^{a}	4,929.2^{a}
HS-CON	364.0	1,236.0^{b}	3,000.6^{c}	4,412.6^{c}
HS-CPE	365.7	1,255.0^{b}	3,031.4^{c}	4,474.5^{c}
HS-NEP	363.9	1,254.6^{b}	3,112.0^{b}	4,636.1^{b}
SEM	1.58	4.58	7.29	14.79
Main effect of temperature				
TN	366.3	1,285.6^{a}	3,207.2^{a}	4,961.1^{a}
HS	364.5	1,248.5^{b}	3,048.0^{b}	4,507.7^{b}
Main effect of diet				
CON	365.8	1,263.8	3,108.5^{b}	4,696.3^{b}
CPE	366.4	1,272.3	3,122.4^{b}	4,724.3^{b}
NEP	364.1	1,265.1	3,151.9^{a}	4,782.6^{a}
P-value				
Temperature (T)	0.166	<0.001	<0.001	<0.001
Diet (D)	0.317	0.149	<0.001	<0.001
T × D	0.552	0.003	<0.001	<0.001
(C) Feed-to-gain ratio (g:g)				
Item	1–10 d	11–24 d	25–42 d	1–42 d
Temperature × Diet				
TN-CON	1.17	1.39^{c}	1.87^{d}	1.68^{cd}
TN-CPE	1.17	1.35^{cd}	1.83^{de}	1.64^{d}
TN-NEP	1.16	1.30^{d}	1.77^{e}	1.59^{e}
HS-CON	1.16	1.54^{a}	2.62^{a}	1.95^{a}
HS-CPE	1.17	1.44^{b}	2.45^{b}	1.85^{b}
HS-NEP	1.16	1.36^{c}	2.08^{c}	1.70^{c}
SEM	0.005	0.012	0.021	0.010
Main effect of temperature				
TN	1.17	1.35^{b}	1.82^{b}	1.64^{b}
HS	1.16	1.45^{a}	2.39^{a}	1.83^{a}
Main effect of diet				
CON	1.17	1.46^{a}	2.25^{a}	1.82^{a}
CPE	1.17	1.39^{b}	2.14^{b}	1.74^{b}
NEP	1.16	1.33^{c}	1.93^{c}	1.64^{c}
P-value				
Temperature (T)	0.204	<0.001	<0.001	<0.001
Diet (D)	0.428	<0.001	<0.001	<0.001
T × D	0.579	<0.001	<0.001	<0.001

BW, body weight; BWG, body weight gain. Initial BW at 1 d did not differ among treatments (42.03 ± 0.05 g; P>0.05). Heat stress was applied from 22 to 42d; therefore, the 1–10 d period and most of the 11–24d period preceded heat exposure. TN, thermoneutral (24±1°C); HS, cyclic heat stress (38±1°C for 8h/day from 22 to 42d of age); CON, basal diet without supplementation; CPE, basal diet + conventional phytogenic extract (500mg/kg); NEP, basal diet + nano-encapsulated phytogenic compounds (250mg/kg); SEM, pooled standard error of the mean. $^{a–e}$Means within a column and within the same section (treatment combinations or main effects) without a common superscript differ significantly (Tukey's HSD test, P<0.05). Values are means of n=6 replicate pens (10 birds/pen) per treatment, with the pen as the experimental unit; degrees of freedom: T, F (1, 30); D and T × D, F (2, 30).

Cytokine and immunoglobulin levels: The effects of cyclic heat stress and dietary supplementation with CPE or NEP on serum cytokine and immunoglobulin levels are presented in Table 5. Serum IL-6 concentration increased dramatically under HS conditions, representing a 3.0-fold elevation in HS-CON relative to TN-CON (P<0.05). Conversely, serum IL-10 decreased by 46% in the HS-CON birds compared to the TN-CON (P<0.05). Phytogenic supplementation in the CPE and NEP groups attenuated this inflammatory response, with IL-6 suppression of 29 and 55% in heat-stressed birds, respectively, compared with HS-CON (P<0.05). NEP treatment effectively triggered IL-10 release by 57.7% compared to the CON group under TN conditions (P<0.05). Under HS conditions, NEP treatment preserved IL-10 levels comparable to those in TN-CON birds. On the other hand, serum IgA concentration of CON birds decreased by 46.5% in the HS *vs.* the TN group (P<0.05). In contrast, NEP treatment increased IgA concentration under TN and HS conditions, resulting in 38 and 113.6% increases over their respective controls (P<0.05). Serum IgG had the same pattern, with a 32.3% reduction in the HS-CON versus the TN-CON group (P<0.05). NEP treatment elevated IgG by 25.9 and 47.2% in TN-NEP and HS-NEP, respectively, compared with their controls (P<0.05). Table 9 shows that NEP was more effective than CPE, reducing IL-6 and increasing IL-10, IgA and IgG under heat stress conditions (P<0.05).

Table 5: Effects of cyclic heat stress and dietary phytogenic supplementation on serum cytokines and immunoglobulins of broiler chickens at 42d of age

Item	IL-6 (pg/mL)	IL-10 (pg/mL)	IgA (mg/dL)	IgG (mg/dL)
Temperature × Diet				
TN-CON	50.15^{d}	23.27^{b}	58.28^{c}	411.60^{c}
TN-CPE	41.32^{e}	27.57^{b}	66.59^{b}	467.90^{b}
TN-NEP	34.43^{e}	36.70^{a}	80.57^{a}	518.11^{a}
HS-CON	148.03^{a}	12.58^{d}	31.16^{e}	278.60^{e}
HS-CPE	104.96^{b}	17.88^{c}	46.09^{d}	349.98^{d}
HS-NEP	65.92^{c}	23.80^{b}	66.57^{b}	410.08^{c}
SEM	2.100	1.131	1.091	5.963
Main effect of temperature				
TN	41.97^{b}	29.18^{a}	68.48^{a}	465.87^{a}
HS	106.30^{a}	18.08^{b}	47.94^{b}	346.22^{b}
Main effect of diet				
CON	99.09^{a}	17.92^{c}	44.72^{c}	345.10^{c}
CPE	73.14^{b}	22.72^{b}	56.34^{b}	408.94^{b}
NEP	50.18^{c}	30.25^{a}	73.57^{a}	464.10^{a}
P-value				
Temperature (T)	<0.001	<0.001	<0.001	<0.001
Diet (D)	<0.001	<0.001	<0.001	<0.001
T × D	<0.001	0.354	<0.001	0.116

IL, interleukin; Ig, immunoglobulin. TN, thermoneutral (24±1°C); HS, cyclic heat stress (38±1°C for 8h/day from 22 to 42d of age); CON, basal diet without supplementation; CPE, basal diet + conventional phytogenic extract (500mg/kg); NEP, basal diet + nano-encapsulated phytogenic compounds (250mg/kg); SEM, pooled standard error of the mean. $^{a–e}$Means within a column and within the same section (treatment combinations or main effects) without a common superscript differ significantly (Tukey's HSD test, P<0.05). Values are means of n=12 birds (2 birds/pen) per treatment; degrees of freedom: T, F(1, 66); D and T × D, F(2, 66).

Liver oxidative status: The effects of phytogenic supplementation with CPE or NEP on hepatic oxidative balance in broilers exposed to cyclic heat stress are presented in Table 6. Hepatic MDA concentration was considerably (P<0.05) elevated by 2.6-fold in the HS-CON birds relative to TN-CON birds. Phytogenic

supplementation in CPE or NEP form under HS conditions reduced (P<0.05) the liver MDA concentration by 24.2 and 50.2%, respectively, compared to the HS-CON group. Additionally, NEP supplementation under TN conditions maintained the hepatic MDA at the lowest level among all groups. In contrast, SOD activity decreased by 30.5% in HS-CON relative to TN-CON (P<0.05). Phytogenic treatment in both CPE and NEP forms increased progressively SOD activity under TN and HS conditions (P<0.05). Notably, NEP enhanced SOD activity by 29.6% compared with CON under TN conditions and restored it completely under HS conditions to TN-CON levels (P<0.05), resulting in 46% higher SOD activity in HS-NEP compared with HS-CON. Similarly, hepatic GPx activity was reduced by 42.4% in HS-CON compared with TN-CON (P<0.05), whereas CPE and NEP increased GPx activity under HS by 35.1 and 89.7%, respectively, compared with HS-CON (P<0.05). Compared with CPE under HS conditions, NEP further reduced hepatic MDA and increased SOD and GPx activities (P<0.05; Table 9).

Table 6: Effects of cyclic heat stress and dietary phytogenic supplementation on hepatic oxidative status of broiler chickens at 42d of age

Item	MDA (nmol/mg)[1]	SOD (U/mg)[1]	GPx (U/mg)[1]
Temperature × Diet			
TN-CON	2.42^{d}	141.91^{c}	31.63^{c}
TN-CPE	2.17^{de}	162.25^{b}	35.90^{b}
TN-NEP	2.04^{e}	183.93^{a}	41.49^{a}
HS-CON	6.31^{a}	98.57^{e}	18.22^{e}
HS-CPE	4.78^{b}	123.54^{d}	24.63^{d}
HS-NEP	3.14^{c}	143.94^{c}	34.58^{b}
SEM	0.073	2.660	0.530
Main effect of temperature			
TN	2.21^{b}	162.70^{a}	36.34^{a}
HS	4.74^{a}	122.02^{b}	25.81^{b}
Main effect of diet			
CON	4.37^{a}	120.24^{c}	24.93^{c}
CPE	3.47^{b}	142.89^{b}	30.26^{b}
NEP	2.59^{c}	163.93^{a}	38.03^{a}
P-value			
Temperature (T)	<0.001	<0.001	<0.001
Diet (D)	<0.001	<0.001	<0.001
T × D	<0.001	0.671	<0.001

MDA, malondialdehyde; SOD, superoxide dismutase; GPx, glutathione peroxidase. [1]Normalized per mg protein. TN, thermoneutral (24±1°C); HS, cyclic heat stress (38±1°C for 8h/day from 22 to 42d of age); CON, basal diet without supplementation; CPE, basal diet + conventional phytogenic extract (500mg/kg); NEP, basal diet + nano-encapsulated phytogenic compounds (250mg/kg); SEM, pooled standard error of the mean. $^{a-e}$Means within a column and within the same section (treatment combinations or main effects) without a common superscript differ significantly (Tukey's HSD test, P<0.05). Values are means of n=12 birds (2 birds/pen) per treatment; degrees of freedom: T, F (1, 66); D and T × D, F (2, 66).

Jejunal gene expression: The effect of phytogenic supplementation in CPE or NEP forms on jejunal Occludin, claudin-1, NF-κB and Nrf2 gene expression in broilers exposed to cyclic HS are presented in Table 7. Occludin was downregulated by 2.33-fold in HS-CON compared with TN-CON (P<0.05). CPE demonstrated intermediate effects, achieving 1.23-fold in TN-CPE and 0.68-fold in HS-CPE, compared with TN-CON (P<0.05). NEP supplementation upregulated occludin expression to 1.52-fold under in TN-NEP and restored expression to 1.06-fold in HS-NEP, representing a 2.4-fold increase in comparison with HS-CON (P<0.05). Claudin-1 mRNA expression followed a similar pattern, being downregulated by 2.32-fold in HS-CON compared with TN-CON and restored to TN-CON levels in HS-NEP (P<0.05). Nrf2 expression was suppressed by 1.56-fold in HS-CON compared to TN-CON (P<0.05), whereas phytogenic supplementation increased Nrf2 mRNA expression by 1.33 and 1.81-fold in TN-CPE and TN-NEP groups, respectively, compared with the TN-CON group. NEP supplementation increased Nrf2 mRNA expression under heat stress, representing approximately a 2.2-fold increase over HS-CON (P<0.05). Conversely, NF-κB was markedly upregulated by heat stress, increasing to 2.84-fold in HS-CON relative to TN-CON (P<0.05). Phytogenic supplementation of CPE and NEP decreased (P<0.05) the NF-κB expression, in comparison to the CON group, under TN or HS conditions. Notably, NF-κB expression was suppressed by 2.53-fold in the HS-NEP group compared with the HS-CON group (P<0.05), returning it to normal levels. Under HS conditions, NEP exceeded CPE in upregulating occludin (+0.38), claudin-1 (+0.38) and Nrf2 (+0.44) and in suppressing NF-κB (−0.83) expression (all P<0.001; Table 9).

Table 7: Effects of cyclic heat stress and dietary phytogenic supplementation on jejunal gene expression of broiler chickens at 42d of age

Item	Occludin[1]	Claudin-1[1]	Nrf2[1]	NF-κB[1]
Temperature × Diet				
TN-CON	1.01^{c}	1.08^{c}	0.95^{c}	0.99^{c}
TN-CPE	1.23^{b}	1.24^{b}	1.33^{b}	0.82^{d}
TN-NEP	1.51^{a}	1.37^{a}	1.81^{a}	0.66^{e}
HS-CON	0.44^{e}	0.46^{e}	0.61^{d}	2.84^{a}
HS-CPE	0.68^{d}	0.68^{d}	0.88^{c}	1.95^{b}
HS-NEP	1.06^{c}	1.06^{c}	1.32^{b}	1.12^{c}
SEM	0.019	0.021	0.028	0.032
Main effect of temperature				
TN	1.25^{a}	1.23^{a}	1.36^{a}	0.82^{b}
HS	0.73^{b}	0.74^{b}	0.93^{b}	1.97^{a}
Main effect of diet				
CON	0.72^{c}	0.77^{c}	0.78^{c}	1.91^{a}
CPE	0.95^{b}	0.96^{b}	1.10^{b}	1.39^{b}
NEP	1.29^{a}	1.22^{a}	1.57^{a}	0.89^{c}
P-value				
Temperature (T)	<0.001	<0.001	<0.001	<0.001
Diet (D)	<0.001	<0.001	<0.001	<0.001
T × D	0.005	<0.001	0.027	<0.001

Nrf2, nuclear factor erythroid 2-related factor 2; NF-κB, nuclear factor kappa B. [1]Relative mRNA expression ($2^{-\Delta\Delta Ct}$) normalized to β-actin and expressed relative to the TN-CON group. TN, thermoneutral (24±1°C); HS, cyclic heat stress (38±1°C for 8h/day from 22 to 42d of age); CON, basal diet without supplementation; CPE, basal diet + conventional phytogenic extract (500mg/kg); NEP, basal diet + nano-encapsulated phytogenic compounds (250mg/kg); SEM, pooled standard error of the mean. $^{a-e}$Means within a column and within the same section (treatment combinations or main effects) without a common superscript differ significantly (Tukey's HSD test, P<0.05). Values are means of n=12 birds (2 birds/pen) per treatment; degrees of freedom: T, F(1, 66); D and T × D, F(2, 66).

Jejunal histomorphology and cecal microbiota: The effect of phytogenic supplementation in CPE or NEP forms on jejunal histomorphology and cecal bacterial counts in broilers exposed to cyclic HS are presented in Table 8. Under TN conditions, CPE and NEP treatments increased the VH/CD ratio relative to the CON group (P<0.05). HS reduced the VH/CD ratio by 48% in HS-CON compared to TN-CON (P<0.05). NEP supplementation increased (P<0.05) the VH/CD ratio in HS-NEP by 2-fold relative to HS-CON, to a level comparable to that of TN-CON. Cecal LACT counts

were decreased by HS, by 1.06 log_{10} copies/g in HS-CON compared with TN-CON (P<0.05). Compared with their respective controls, LACT counts were increased by 0.42 and 0.59 log_{10} copies/g in TN-CPE and HS-CPE, respectively, and by 0.98 and 1.43 log_{10} copies/g in TN-NEP and HS-NEP, respectively (P<0.05). Conversely, cecal EC counts were increased by HS, by 0.93 log_{10} copies/g in HS-CON compared with TN-CON (P<0.05), and were reduced by 0.37 and 0.52 log_{10} copies/g in TN-CPE and HS-CPE, respectively, and by 0.87 and 1.20 log_{10} copies/g in TN-NEP and HS-NEP, respectively, compared with their respective controls (P<0.05). Compared with HS-CPE, HS-NEP birds had a higher VH/CD ratio (difference 2.10) and Lactobacillus count (0.84 log_{10} copies/g) and a lower E. coli count (−0.68 log_{10} copies/g) (all P<0.001; Table 9).

DISCUSSION

The reduced BWG and impaired F:G ratio observed in HS-CON birds agree with previous reports of the negative effects of cyclic HS on broiler productivity (Li *et al.*, 2020; Du *et al.*, 2022). This impairment is generally attributed to reduced feed intake, impaired digestion and nutrient absorption, increased energy expenditure for thermoregulation, and the diversion of metabolic resources from growth toward stress responses (Afzal, 2026). In the present study, CPE and NEP partially mitigated the HS-induced decline in BWG and F:G ratio; however, BWG of HS-NEP birds remained lower than that of TN-CON birds, indicating that neither supplement fully offset the effects of HS. These findings are consistent with previous studies on phytogenic and nano-formulated feed additives in heat-stressed broilers (Madkour *et al.*, 2024; Elbaz *et al.*, 2025; Kusmayadi *et al.*, 2025). The larger improvement observed with NEP than with CPE (Table 9), despite its lower inclusion rate, might be related to improved intestinal absorption and bioavailability of the encapsulated phytochemicals. However, bioavailability was not measured in the present study, so this explanation remains a hypothesis. It is supported indirectly by reports of improved intestinal absorption and up to 3.6-fold higher systemic exposure with liposomal formulations (Kishawy *et al.*, 2023; Sun *et al.*, 2025). Notably, HS-NEP birds also consumed more feed than HS-CON and HS-CPE birds during the heat-exposure period (Table 4). Therefore, part of the improvement in growth performance, and possibly in other variables, may reflect the higher nutrient intake of these birds rather than a direct effect of the encapsulated phytochemicals (Ghasemi *et al.*, 2021).

Table 8: Effects of cyclic heat stress and dietary phytogenic supplementation on jejunal histomorphology and cecal bacterial counts of broiler chickens at 42d of age

Item	VH (μm)	CD (μm)	VH/CD ratio	*Lactobacillus*[2]	*E. coli*[2]
Temperature × Diet					
TN-CON	1,254.0^{c}	219.8^{c}	5.73^{c}	7.21^{c}	6.53^{c}
TN-CPE	1,395.7^{b}	205.7^{cd}	6.82^{b}	7.62^{b}	6.17^{d}
TN-NEP	1,520.0^{a}	195.6^{d}	7.80^{a}	8.18^{a}	5.66^{e}
HS-CON	848.1^{e}	285.7^{a}	2.98^{e}	6.15^{e}	7.47^{a}
HS-CPE	1,029.5^{d}	260.5^{b}	3.96^{d}	6.74^{d}	6.95^{b}
HS-NEP	1,303.2^{c}	216.0^{c}	6.06^{c}	7.58^{b}	6.26^{d}
SEM	14.41	3.92	0.132	0.057	0.052
Main effect of temperature					
TN	1,389.9^{a}	207.1^{b}	6.78^{a}	7.67^{a}	6.12^{b}
HS	1,060.3^{b}	254.1^{a}	4.33^{b}	6.82^{b}	6.89^{a}
Main effect of diet					
CON	1,051.0^{c}	252.8^{a}	4.35^{c}	6.68^{c}	7.00^{a}
CPE	1,212.6^{b}	233.1^{b}	5.39^{b}	7.18^{b}	6.56^{b}
NEP	1,411.6^{a}	205.8^{c}	6.93^{a}	7.88^{a}	5.96^{c}
P-value					
Temperature (T)	<0.001	<0.001	<0.001	<0.001	<0.001
Diet (D)	<0.001	<0.001	<0.001	<0.001	<0.001
T × D	<0.001	<0.001	<0.001	<0.001	0.008

VH, villus height; CD, crypt depth. [2]log_{10} gene copies/g of cecal content. TN, thermoneutral (24±1°C); HS, cyclic heat stress (38±1°C for 8h/day from 22 to 42d of age); CON, basal diet without supplementation; CPE, basal diet + conventional phytogenic extract (500mg/kg); NEP, basal diet + nano-encapsulated phytogenic compounds (250mg/kg); SEM, pooled standard error of the mean. $^{a-e}$Means within a column and within the same section (treatment combinations or main effects) without a common superscript differ significantly (Tukey's HSD test, P<0.05). Values are means of n=12 birds (2 birds/pen) per treatment; degrees of freedom: T, F(1, 66); D and T × D, F(2, 66).

Table 9: Effect estimates of dietary treatments under cyclic heat stress, expressed as differences between treatment means with Tukey-adjusted 95% confidence intervals

Variable	CPE − CON	NEP − CON	NEP − CPE
Final BW	162.9 (93.5, 232.3) P<0.001	475.6 (406.2, 545.0) P<0.001	312.7 (243.3, 382.1) P<0.001
FI	61.9 (−1.8, 125.5) P = 0.061	223.4 (159.8, 287.1) P<0.001	161.6 (98.0, 225.2) P<0.001
BWG	162.6 (93.2, 232.0) P<0.001	475.5 (406.1, 544.9) P<0.001	312.9 (243.5, 382.3) P<0.001
F:G ratio	−0.105 (−0.148, −0.063) P<0.001	−0.257 (−0.300, −0.214) P<0.001	−0.152 (−0.195, −0.109) P<0.001
IL-6	−43.07 (−51.79, −34.35) P<0.001	−82.10 (−90.82, −73.39) P<0.001	−39.03 (−47.75, −30.32) P<0.001
IL-10	5.30 (0.61, 9.99) P = 0.018	11.22 (6.53, 15.92) P<0.001	5.92 (1.23, 10.62) P = 0.006
IgA	14.94 (10.41, 19.46) P<0.001	35.41 (30.88, 39.94) P<0.001	20.48 (15.95, 25.00) P<0.001
IgG	71.38 (46.63, 96.13) P<0.001	131.48 (106.73, 156.23) P<0.001	60.10 (35.35, 84.85) P<0.001
MDA	−1.54 (−1.84, −1.23) P<0.001	−3.17 (−3.48, −2.87) P<0.001	−1.64 (−1.94, −1.33) P<0.001
SOD	24.96 (13.92, 36.00) P<0.001	45.36 (34.32, 56.40) P<0.001	20.40 (9.36, 31.44) P<0.001
GPx	6.40 (4.21, 8.60) P<0.001	16.35 (14.15, 18.55) P<0.001	9.95 (7.75, 12.15) P<0.001
Occludin	0.25 (0.17, 0.33) P<0.001	0.63 (0.55, 0.71) P<0.001	0.38 (0.30, 0.46) P<0.001
Claudin-1	0.22 (0.13, 0.31) P<0.001	0.60 (0.51, 0.69) P<0.001	0.38 (0.29, 0.47) P<0.001
Nrf2	0.27 (0.16, 0.39) P<0.001	0.71 (0.60, 0.83) P<0.001	0.44 (0.32, 0.56) P<0.001
NF-κB	−0.89 (−1.02, −0.76) P<0.001	−1.72 (−1.85, −1.59) P<0.001	−0.83 (−0.96, −0.70) P<0.001
VH	181.5 (121.7, 241.3) P<0.001	455.2 (395.4, 515.0) P<0.001	273.7 (213.9, 333.5) P<0.001
CD	−25.2 (−41.5, −8.9) P<0.001	−69.6 (−85.9, −53.4) P<0.001	−44.5 (−60.8, −28.2) P<0.001
VH/CD ratio	0.98 (0.43, 1.53) P<0.001	3.08 (2.54, 3.63) P<0.001	2.10 (1.56, 2.65) P<0.001
Lactobacillus	0.59 (0.35, 0.83) P<0.001	1.43 (1.19, 1.67) P<0.001	0.84 (0.60, 1.08) P<0.001
E. coli	−0.52 (−0.73, −0.31) P<0.001	−1.20 (−1.42, −0.99) P<0.001	−0.68 (−0.90, −0.47) P<0.001

Values are mean differences (95% confidence interval) between the HS-diet combinations, with Tukey-adjusted P-values from the comparison of the six treatment combinations. Abbreviations and units are as defined in Tables 4–8.

The dramatic elevation in serum IL-6 and reduction in IL-10 under HS conditions suggest a shift toward a pro-inflammatory state (Fathi *et al.*, 2024). These changes may be associated with intestinal endotoxin translocation and impaired barrier function (Li *et al.*, 2020), although neither endotoxin nor intestinal permeability was measured in the present study. Conversely, NEP supplementation shifted the circulating cytokine profile toward lower IL-6 and higher IL-10 concentrations. The reduction in IL-6 achieved by NEP in heat-stressed birds is particularly notable and might contribute to mitigating muscle protein catabolism, reduced feed intake, and impaired growth performance induced by elevated IL-6 and other proinflammatory cytokines (Kikusato *et al.*, 2021). These effects might be mediated by several mechanisms that were not directly assessed here, including direct inhibition of NF-κB signaling by polyphenolic compounds (Mamun *et al.*, 2024) and reduced intestinal permeability, which limits endotoxin translocation (Kikusato *et al.*, 2021). Furthermore, IgA serves as the primary immunoglobulin at mucosal surfaces, preventing pathogen adherence and neutralizing toxins, while IgG provides systemic immunity and pathogen resistance (Baker *et al*., 2015). The parallel increases in serum IgA and IgG concentrations with NEP supplementation, especially in HS-NEP versus HS-CON birds, are consistent with previous reports of increased circulating immunoglobulins in broilers receiving antioxidant phytogenic supplements (Al-Nasser *et al.*, 2024; Elbaz *et al.*, 2025). However, because no pathogen challenge, vaccine-response assessment or immune-cell function assay was performed, these changes in circulating biomarkers indicate an altered systemic immune status rather than improved disease resistance or protective immunity.

The elevation in hepatic MDA and reduction in SOD activity under HS conditions indicate increased lipid peroxidation and reduced antioxidant enzyme activity, consistent with HS-induced oxidative stress (Yuan *et al.*, 2025). Lipid peroxidation compromises membrane integrity, enzyme function, and cellular signaling, contributing to hepatocellular damage and metabolic dysfunction (Wu *et al*., 2008; Guo and Wang, 2016). The lower MDA concentration and higher SOD and GPx activities in heat-stressed birds receiving CPE or NEP suggest an improved hepatic antioxidant status, which may result from direct ROS scavenging by the polyphenols, upregulation of antioxidant enzymes, or preservation of mitochondrial function (Fathi *et al.*, 2024). The greater effect of NEP than CPE on these variables (Table 9) may reflect enhanced cellular uptake and intracellular delivery of nano-encapsulated compounds, enabling more effective neutralization of ROS at their sites of generation (Kishawy *et al.*, 2023). This hypothesis, however, requires confirmation by measuring phytochemical concentrations in tissues. Moreover, because only three hepatic markers were determined, these results do not provide a comprehensive assessment of hepatic redox status; additional indicators, such as catalase activity, the reduced-to-oxidized glutathione ratio and protein oxidation products, should be evaluated in future studies.

The jejunal gene expression data provide some insight into the possible mechanisms underlying the effects of NEP. Together with other tight junction proteins like claudins and zonula occludens, occludin provides the intestinal structure foundation that controls intercellular permeability and prevents the luminal translocation of antigens and pathogens (Cuddihey *et al*., 2022). Heat stress downregulates tight junction protein expression through oxidative damage, inflammatory signaling, and cytoskeletal disruption (Choi *et al.*, 2024; Wang *et al.*, 2025). In the current study, the upregulation of jejunal occludin and claudin-1 mRNA in HS-NEP versus HS-CON birds was accompanied by increased Nrf2 and decreased NF-κB mRNA expression. Nrf2 is considered a master regulator of several cytoprotective gene networks in broilers, including antioxidant enzymes (SOD, catalase, GPx), phase II detoxification enzymes (HO-1, NQO1), and anti-inflammatory mediators (Bai *et al.*, 2024; Hu *et al.*, 2024). Conversely, NF-κB activation increases oxidative stress while driving the production of pro-inflammatory cytokines, such as TNF-α and IL-1β, in broiler tissues. This is because NF-κB signaling encourages the production of ROS, which in turn activates NF-κB, creating a self-reinforcing inflammatory–oxidative pathway in broilers (Jiang *et al.*, 2025). These coordinated transcriptional changes are consistent with a cellular environment favoring antioxidant defense and reduced inflammation, and with the observed improvements in oxidative biomarkers, cytokine profiles, and tissue integrity. These results agree with earlier research showing that dietary antioxidants can activate the Keap1/Nrf2 axis and inhibit NF-κB activation in the intestine of broiler chickens (Tong *et al.*, 2020). However, these findings are based on mRNA abundance only, which does not necessarily reflect protein abundance or pathway activation correlated with antioxidant defense, inflammatory suppression and/or intestinal integrity. Other indicative measurements such as Nrf2 nuclear translocation, NF-κB p65 phosphorylation or IκBα degradation, the expression of downstream target genes (e.g., HO-1 and NQO1), tight-junction protein abundance, zonula occludens-1 expression and functional intestinal permeability (e.g., FITC-dextran flux or serum diamine oxidase and D-lactate) were not assessed. Therefore, the present results indicate transcriptional changes consistent with, but not demonstrative of, Nrf2 activation, NF-κB inhibition and improved intestinal barrier function.

Chicken exposure to HS usually causes crypt hyperplasia and villus atrophy, which decreases nutritional absorption and raises maintenance energy needs (Brugaletta *et al.*, 2022). The higher jejunal VH/CD ratio in NEP-fed heat-stressed birds indicates improved intestinal morphology, which may favor absorptive capacity, although nutrient absorption was not measured. The antioxidant and anti-inflammatory protection by phytogenic compounds in the NEP might contribute to the improvement of the VH/CD ratio in HS birds through enhanced epithelial cell proliferation and differentiation, reduced apoptosis, and improved tight junction integrity (Adil *et al.*, 2024). Additionally, NEP-supplemented birds under HS had higher cecal *Lactobacillus* and lower *E. coli* counts. These results are consistent with earlier research demonstrating that in heat-stressed or challenged broilers, resveratrol, quercetin, and nano-formulated phytogenic enhance LACT and Bifidobacterium while decreasing *E.*

coli and *Clostridium perfringens* (Adil *et al.*, 2024; Al-Nasser *et al.*, 2024; Elbaz *et al.*, 2025). Liu *et al.* (2021) reported that short-chain fatty acids, especially butyrate, are produced by LACT species and function as colonocytes' main energy source. They also improve tight junction integrity, regulate immunological responses, and competitively exclude pathogens. Conversely, elevated EC populations indicate dysbiosis and increased risk of opportunistic infection, endotoxin production, and inflammatory responses (Elnagar *et al*., 2021). It should be noted, however, that only two bacterial groups were quantified by targeted qPCR; therefore, these results do not describe overall microbial diversity, community structure or function, which would require 16S rRNA gene amplicon or metagenomic sequencing.

The synergistic combination of curcumin, resveratrol, and quercetin in the phytogenic blend employed in this study may contribute to the observed efficacy through complementary mechanisms while maintaining stability and bioavailability in the nano-formulation. Curcumin has potential anti-inflammatory and antioxidant activities by suppressing the NF-κB and promoting the Nrf2 pathway (Jin *et al.*, 2021). Resveratrol triggers SIRT1 signaling and the AMPK pathways, thereby enhancing mitochondrial and metabolic function, reducing oxidative stress, and modulating inflammatory responses. Quercetin demonstrates strong antioxidant capacity, stabilizes mast cells, reduces histamine release, and modulates immune cell function (Lv *et al.*, 2025). The greater efficacy of NEP than CPE, despite NEP being supplied at half the inclusion rate, supports the potential advantages of nano-encapsulation technology and aligns with a previous study demonstrating approximately a 2-fold increase in absorption efficiency (Thimmannagari *et al.*, 2026). Liposomal nanocarriers have been reported to protect phytochemicals from gastrointestinal degradation and to improve their permeability, absorption and bioavailability (Kishawy *et al.*, 2023; Patel *et al.*, 2025). PEGylation further enhances nanoparticle stability and circulation time by reducing recognition by the reticuloendothelial system (Chelliah *et al.*, 2025). The particle size range of 80-120nm in this study is within the range considered favorable for intestinal uptake, as particles below 200 nm can be taken up by enterocytes through endocytosis and transported via lymphatic vessels, reducing first-pass hepatic metabolism (Lemasson *et al.*, 2025). Moreover, the high encapsulation efficiency (>85%) and negative zeta potential (−32mV) indicate a stable dispersion with minimal aggregation (Andishmand *et al.*, 2024). However, no pharmacokinetic analysis, tissue phytochemical measurement or assessment of gastrointestinal release was performed in the present study. Therefore, enhanced bioavailability and targeted delivery of the encapsulated compounds remain hypotheses that require experimental confirmation.

In the current study, each NEP and CPE formulation was evaluated at a single inclusion level, and the two levels were not equivalent in active phytochemical content (250 vs 500mg/kg). Consequently, the effects of formulation and dose cannot be fully separated, and the greater responses observed with NEP cannot be attributed exclusively to nano-encapsulation. In addition, the NEP supplementation added only 1g liposomal lipids per kg of feed, which is small relative to the 35–45g/kg of vegetable oil in the grower and finisher diets (Table 1). However, dietary phospholipids, cholesterol and PEG can themselves influence lipid digestion, intestinal barrier function and microbial composition. Although NEP at half the inclusion rate produced greater improvements than CPE in most of the measured variables, the present data do not establish dose-sparing efficacy in a strict dose–response sense. Therefore, graded inclusion levels of both formulations (e.g., 125, 250 and 500 mg/kg feed) at equivalent active-phytochemical doses, together with inclusion of an empty-liposome treatment, ideally at the lipid dose supplied by NEP, are recommended in future studies to define the dose–response relationships and confirm the dose-sparing potential of NEP. In addition, the higher feed intake of HS-NEP birds may have contributed to some of the observed differences, and the severity of heat stress was inferred from performance and biochemical responses because physiological indicators such as rectal temperature and respiratory rate were not recorded. Finally, no economic evaluation was performed; the cost of the liposomal formulation, the feed cost per unit of weight gain and the scalability of the preparation process under commercial conditions remain to be determined. Therefore, the practical implications of the present findings should be regarded as prospective.

Conclusions: Under the conditions of this study, dietary supplementation with nano-encapsulated phytogenic compounds at 250 mg/kg (half the CPE inclusion rate) attenuated the adverse effects of cyclic heat stress on growth performance and was associated with favorable changes in selected indicators, including lower serum IL-6 and higher IL-10, IgA and IgG concentrations, lower hepatic MDA and higher SOD and GPx activities, higher jejunal occludin, claudin-1 and Nrf2 and lower NF-κB mRNA expression, a higher jejunal VH/CD ratio, and higher cecal Lactobacillus and lower E. coli counts. For most of these variables, the responses to NEP were greater than those to CPE. However, because the two formulations were compared at non-equivalent doses without a carrier-only control, and because bioavailability, protein-level pathway activity, intestinal permeability and economic outcomes were not assessed, these findings do not establish the mechanisms, dose-sparing efficacy, long-term safety or commercial feasibility of NEP. Future studies should include graded doses of both formulations at equivalent active-compound levels, empty-liposome controls, pharmacokinetic and functional measurements, and economic evaluation under commercial production conditions.

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