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Enhancing impact of dietary nano formulated quercetin on laying performance: egg quality, oxidative stability of stored eggs, intestinal immune and antioxidants related genes expression

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Abstract

Background Nutritional interventions with natural antioxidants can provide a pragmatic solution for modifying hens' performance and maintaining oxidative stability of eggs during storage. Quercetin is the most abundant flavonoids with potent antioxidant and immune stimulant activities. The concept of incorporating of quercetin, as potent antioxidant and immunostimulant, into effective nano-carriers (QNPs) has promoted their bioavailability and stability thus, their effectiveness for the first time were assessed on laying hens' performance and immunity, eggs quality during storage. Four hundred 12-weeks-old Hy-line brown laying hens were distributed to four experimental groups: control group fed basal diets, and other 3 groups fed basal diets fortified with 100, 200 and 300 mg/kg QNPs for 60 weeks.

Results Laying performance and quality of laid eggs were improved as expressed by elevated laying rate, egg mass %, eggs weight and yolk weight in QNPs200 and 300. Fortification of QNPs300 remarkably decreased layers serum total cholesterol concurrently with decreased egg yolk saturated fatty acids and cholesterol while increased polyunsaturated fatty acids. Over- 45 days storage period, QNPs enhanced phospholipids, total phenolics and flavonoids, total antioxidant activity (T-AOC) simultaneous with decreased MDA content in eggs. Furthermore, enhanced immune response was detected in both in serum and intestine of QNPs fed hens as reflected by higher lysozymes activity, IgM, IgG and phagocytic index and demotion of NO together with AvBD 6–12, IL-10, IgM and ATg 5-7-12 upregulation and downregulation of IL-1β and TNF-α especially at QNPs200 and 300. Intestinal redox balance was modified via decreasing H_2O_2 and MDA simultaneous with upregulation of catalase, SOD, GSH-Px, HO-1 and NQO1 in groups fed higher doses of QNPs.

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Conclusions QNPs supplementation provides a new nutritional strategy towards increasing hen performance, fortification of eggs with natural antioxidants that prevents egg quality deterioration during storage.

Keywords Quercetin nano formulated, Laying performance, Egg quality, Storage, Antioxidant, Lipid peroxidation

Background

The poultry antioxidant defence system is strongly related to their health and immune status, and gut antioxidant status is a key marker for their performance [1]. During the laying period, the intestinal epithelium of hens is particularly susceptible to inflammation and infection [2]. This susceptibility can lead to disruptions in barrier functions and immune homeostasis, exacerbate oxidative stress, and disturb gut flora. Consequently, these issues negatively affect host metabolism and productive performance [3].

Today's modern laying hens, with their increased productivity, are more exposed to oxidative stress, particularly during peak and late egg production periods. This exposure can lead to adverse effects on both intestinal health and overall productive performance [4]. Under normal conditions, cellular homeostasis is maintained by eliminating excessive ROS through the enhancement of antioxidant defence mechanisms. This includes increasing the activities of enzymes such as GPX-1, SOD-1, and CAT [5]. However, excessive oxidative stress during the peak of egg production can lead to the overproduction of reactive oxygen species (ROS) or reactive nitrogen species, which disrupts the cell's physiological functions. These free radicals are responsible for irreversible changes, including mitochondrial damage, protein breakdown, lipid peroxidation, reduced egg production, and increased disease susceptibility [6, 7]. Intestinal inflammation, resulting from intensive metabolism and prolonged egg production periods, is identified as a significant issue that exacerbates oxidative stress and disrupts immune homeostasis. This, in turn, negatively impacts egg production performance [8].

On the other hand, eggs are a highly valued source of animal protein due to their affordability compared to other protein sources. In addition to being cost-effective, eggs are rich in bioactive compounds with high biological value and are abundant in essential amino acids and polyunsaturated fatty acids (PUFAs [9]. However, during storage, eggs are highly susceptible to deterioration due to protein denaturation and lipid peroxidation, which adversely impact their quality [10]. Cholesterol is required by humans for cell membranes formation and synthesis of the bile acids, sex hormones and vitamin D. However, unnecessary cholesterol intake may persuade a series of cardiovascular diseases, comprising hypertension, hyperlipidaemia, and heart diseases [11]. Phospholipids content accounts for nearly 30% of lipids of egg yolk [12], , consisting of lecithin, phosphoinositide and cephalin. Lecithin, recognized as the "third nutrient," is a polyunsaturated phosphatidylcholine, which has a key role in the development of brain and improving memory [13]. Moreover, the research sector has been partly focused on providing the market with highly nutritious eggs. However, eggs contain high levels of polyunsaturated fatty acids (PUFAs), which are particularly susceptible to peroxidation during storage [14]. Lipid peroxidation in egg yolk can adversely affect stability during storage and alter the nutritional quality of eggs. Additionally, incorporating flavonoids into the feed for laying hens is widely regarded as an effective method for improving egg quality [15].

Thus, searching for nutritional interventions by incorporating natural antioxidants with unique properties into laying hens' diets holds promising potential. Such interventions can strengthen the hens' antioxidant capacity, improve intestinal health, enhance egg quality, and extend the shelf life of eggs. In this sense, flavonoids can exert greater biological antioxidants activity in tissues even at lower concentrations [16]. Quercetin is one of the most potent antioxidants, protecting cells by modulating endogenous antioxidant activities to reduce apoptosis [17]. Dietary quercetin, as a natural flavonoid, has beneficial effects on intestinal functions and health, which subsequently enhances production performance in broilers and laying hens under normal conditions [18]. The antioxidative properties of quercetin are associated with its ability to scavenge free radicals, reduce MDA levels, and enhance the expression of endogenous antioxidant enzymes, such as GSH-PX, catalase, and SOD [19]. Nuclear factor erythroid-2 related factor 2 (Nrf2) regulates the expression of antioxidant-related enzymes, providing defense for the body's antioxidant function. Under oxidative stress, Nrf2 forms a complex with specific proteins that recognize key elements of the antioxidant response. This interaction modifies the expression of Nrf2-mediated phase II detoxifying enzyme genes, such as *GSH-Px*, *SOD*, *CAT*, and *HO-1* [20]. HO-1 can reduce oxidative stress via boosting scavenging activity, preventing lipid oxidation and maintaining the antioxidant enzymes during the egg-storage period [21]. NRF2, HO-1, and NQO1 are considered transcription factors with significant regulatory roles in the oxidative stress response of cells. They enhance the expression of antioxidant and detoxifying enzymes, thereby helping to manage oxidative stress [22]. Thus, using of natural feed additive with antioxidant capability could restore Nrf2 levels and regulate the expression of detoxifying enzyme

linked genes, and adjust the antioxidant defence capacity [23]. Additionally, free quercetin can reduce excessive inflammatory response via lowering the production of pro-inflammatory cytokines (TNF-α, IL-1β, and IL-6) [24]. Nevertheless, the application of quercetin is still limited due to its poor stability, low bioavailability, limited aqueous solubility, and insufficient permeability [25]. Therefore, developing effective delivery nano-systems for quercetin can enhance its water solubility and bioavailability, thereby promoting its anti-inflammatory and antioxidant functions [26]. Unfortunately, no information is available on dietary intervention of quercetin loaded nanoparticles (QNPs) on laying hens' performance and eggs quality. Therefore, the current study aimed to evaluate the effects of quercetin nanoparticles (QNPs) at different concentrations on laying hens. Specifically, it focused on their performance, gene expression related to immune response, antioxidant status, as well as the quality of stored eggs and yolk fatty acid content.

Methods

ARRIVE guidelines; husbandry and rearing management for laying hens were in accordance with the Institutional Animal Care and Use Committee of the Faculty of Veterinary Medicine at Zagazig University (ZU-IACUC/2/F/258/2024).

Quercetin nano particles preparation and characterization

Chitosan (9012-76-4, Sigma Aldrich, St. Louis, MO, USA) nanoparticles were prepared following the method of Zhang et al. [27]. Briefly, electrostatic gelation of chitosan was done by dissolving 2 mg of chitosan in solution of glacial acetic (one mL, 1% v/v), with overnight stirring at 500 rpm and centrifugation for 20 min at 9000 rpm. After that, 4 mL of sodium tripolyphosphate (TPP) solution (7758-29-4, Sigma Aldrich, St. Louis, MO, USA) was added and mixed with 10 mL of chitosan gel solution. Lastly, 3 mg of quercetin (6151-25-3, Sigma Aldrich, St. Louis, MO, USA) was loaded into the previously prepared solution of chitosan nanoparticles, then, under magnetic stirring (2000 rpm) TPP solution was added to the mixture then centrifuged and lyophilized. The physicochemical properties of QNPs were examined by transmission electron microscopy (Fig. 1A) and zeta potential analysis (Fig. 1B).

Entrapping efficiency (EE) of QNPs was calculated AS previously described by [28, 29] as following:

$$\text{EE}\,(\%) = \frac{\text{Total quercetin amount} - \text{Free quercetin amount}}{\text{Total quercetin amount}} \times 100$$

Hens, experimental design and formulated dies

Four hundred 12-week age Hy-line brown laying hens were purchased commercially from local producers, with initial body weight of 1.08±0.25 kg, were weighed and randomly divided in to equal four experimental groups, with ten replicates pens of ten hens each. All birds were housed in a battery housing system in their corresponding replicates (width: 1.20-meter, depth: 45 cm and height: 45 cm) with relative temperature between 25 °C and 27 °C, the relative humidity between 60 and 70%, and with photoperiod 16 h/day. Water and feed were allowed ad libitum access during the day. Four diets were offered for laying hens during the feeding trial in pelleted form as following: developer ration (12 to 15 weeks), pre-layer ration (16 to 17 weeks), laying ration (18 to 45 weeks) and late laying ration (46 to 60 weeks). The feed ingredients were thoroughly mixed then pelleted under a pressure of 1.5 kg/cm^2 in a steam pellet mill (Koppers Junior C40, Koppers Company, Pittsburgh, PA, USA) to approximately 2.5 mm as feed pellet size according to [30]. All diets were formulated to furnish the Hy-line brown laying hens nutrient requirements in accordance with its nutritional specification catalogue [30]. The experimental groups were established as following: control group; hens received a basal diet without additives. Groups 2, 3 and 4 attained a basal diet supplemented with QNPs at

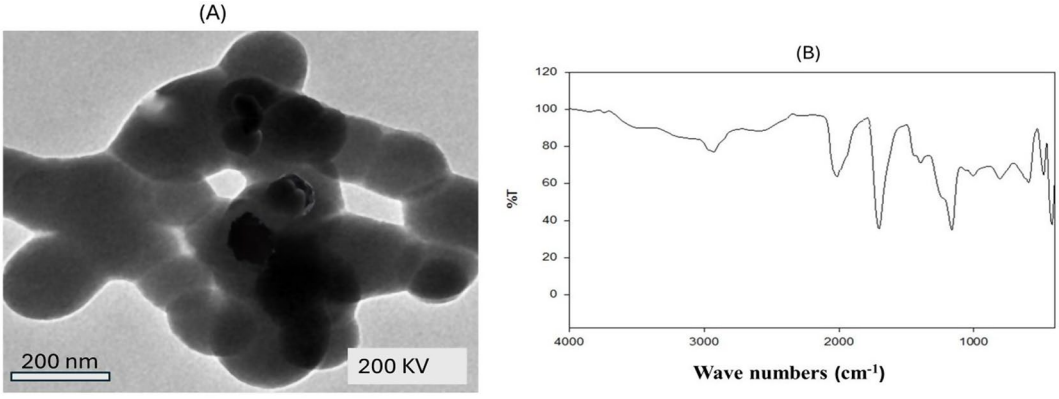

Fig. 1 Scanning electron microscopy (**A**) and Fourier-transform infrared spectroscopy (FTIR, **B**) of quercetin loaded nanoparticles

concentrations of 100, 200, and 300 mg/kg diet, respectively. The duration of study lasted for 48 weeks, starting with Hy-Line Brown laying hens at 12 weeks of age and ending at 60 weeks of age. The levels of feed ingredient and basal diets chemical composition are presented in Table 1. The proximate assessment of the feed components was made along with the standard measures of the Association of Official Agricultural Chemists [31].

Laying hen's performance

All performance parameters was performed according to Hy-line management catalogue [30]. The egg collection was done three times daily (8.00 am, 1.00 pm and 6.00 pm) for each replicate, and we recorded the eggs number and weight and the amount of feed intake per replicate daily to calculate the following: hens' days egg production % (HDEP)=the number of eggs produced during a definite period/number of live birds during the same period. Average egg mass of the period % = (HDEP × average weight of eggs)/100. Total feed intake=sum of the daily feed intake in specific period/number of live birds in each replicate at the same time. Feed conversion ratio (FCR)=overall feed intake (g)/overall weight of eggs (g). Net feed efficiency index (NFEI) = (egg mass (g)+the differences in hen body weight (g)) × 100/total feed intake (g).

Table 1 Ingredients and nutrient levels of laying hens' experimental diets (as dry matter)

Ingredients g/kg	Developer ((12 to 15 week)	Pre-layer (16 to 17 week)	Layer (18 to 45 week)	Late layer (46 to 60 week)
Yellow corn grain	66.00	62.00	61.70	64.00
Soybean meal, 47.5%	17.00	21.50	14.00	15.00
Corn gluten, 60%	--	--	8.70	6.20
Wheat bran	13.00	5.50	--	--
Soybean oil	--	3.25	4.50	4.00
Calcium carbonate	1.25	5.00	7.50	7.30
Dicalciumphosphate	1.65	1.65	2.50	2.50
Common salt	0.30	0.30	0.30	0.30
Premix*	0.50	0.50	0.30	0.30
DL- Methionine, 98%	0.10	0.10	0.10	0.10
Lysine, Hcl, 78%	0.10	0.10	0.30	0.20
Anti-mycotoxin	0.10	0.10	0.10	0.10
Nutrient levels				
Metabolic energy, Kcal/Kg**	2809.46	2975.47	3150.91	3111.14
Crude protein, %	15.88	16.61	17.03	16.09
Ether extract, %	2.78	5.76	7.01	6.55
Crude fiber, %	3.58	2.83	2.09	2.14
Calcium, %	1.00	2.42	3.57	3.50
Available phosphorus, %	0.42	0.42	0.55	0.55
Lysine, %	0.77	0.92	0.94	0.87
Methionine, %	0.33	0.36	0.47	0.43

*Vitamin and mineral premix provided per each kg of diet as follow: Vitamin D3, 2900 IU, Vitamin A, 12 500 IU; Vitamin K3, 5.80 mg; Vitamin E, 60 IU; Thiamin, 3.33 mg; Vitamin B12, 0.5 mg; Riboflavin, 6.62 mg; Pantothenic acid, 13.87 mg; Vitamin B6, 1.6 g; Niacin, 50 mg; Biotin, 0.73 mg; Folic acid, 1.15 mg; Calcium 1.38 g, Fe, 68.9 mg; Mn, 55 mg; I, 0.9 mg; Cu, 8.8 mg; Se, 0.40 mg; Co, 0.25 mg; Zn, 76.8 mg. ** calculated according to NRC [104]

Sampling

To evaluate the interior and exterior egg quality, a total of 100 fresh eggs from each group (10 eggs from each replicate) collected in 10 batches at 35 (peak of egg laying) and 60 weeks (late of egg laying) and labelled for monitoring the external and internal eggs quality parameters. Other eggs' samples (n=50 eggs from each experimental group (5 eggs per replicate) which apparent clean and non- cracked were used for chemical analysis of DM, CP, EE and Ash in eggs albumen and yolk at the last week of experiment. For eggs yolk cholesterol and fatty acids analysis, yolk samples from 50 other set of eggs were collected at the last week of experiment. For estimating of yolk phospholipids, antioxidants and oxidation markers, 100 eggs (50 fresh and 50 stored eggs) were collected (5 eggs/ replicates) at 40 weeks of lying period while the storge of eggs was done for 45 days at adjusted room temperature (18±2 ∘C).

At the 60 weeks, blood samples were collected under aseptic condition from hen's wing vein (10 samples / group) allotted into two equal parts, anticoagulant (EDTA) was added to the first one for phagocytosis whereas blood samples second parts were collected without anticoagulants for sera separation that was used in immunological and lipid profile analysis.

Intestinal tissues were collected at the end experimental period (at 60 weeks), by random choosing birds (n=10/group) then anesthetized through intra-peritoneal injection of sodium pentobarbital (50 mg/kg) infused intravenously into the wing vein after that, euthanized by cervical dislocation for sampling the technique was in accordance to Institutional Animal Care and Use Committee of the Faculty of Veterinary Medicine at Zagazig University for the euthanasia of animals. The collected intestinal samples were thoroughly homogenized for assessing the oxidative and antioxidant markers. For molecular analysis, samples were collected from intestinal, splenic and abdominal adipose tissues at the end of the experiment (60 weeks) from the same slaughtered birds (n=10/group) directly on TRIzol reagent (Invitrogen Life Technologies, Carlsbad, CA, USA) according to the manufacturer's instruction and stored at – 80 ∘C until further gene expression analysis.

Monitoring eggs quality indicators

For external quality parameters evaluation, eggs were weighted individually and egg shape index (%) is calculated by dividing egg width (mm) by egg length (mm)

×100 [32]. For interior egg's quality, the eggs from replicates were weighed individually on a sensible electrical scale and gently cracked then its contents carefully transferred into a clean plate to assess the their yolk height and diameter and albumen height (mm) using sensitive electronic calliper in accordance with Reddy et al. [32]. The yolk was gently separated from the albumen and then weighed, whereas the albumen weight was assessed by subtracting the yolk weight and eggshell from weight of eggs, also the shell thickness was determined with a shell thickness micrometre (TSS, England) at the equatorial area after removal of its membranes. The quality features of the eggs were approximated along with the procedure of Romanoff and Romanoff [33] utilizing the subsequent formulas:

Yolk Index = height of yolk, mm / diameter of yolk mm,

Yolk ratio (%) = [weight of yolk, g / weight of egg, g] x100,

Albumen ratio (%) = [weight of albumen, g / weight of egg, g] x100,

Shell ratio (%) = [weight of shell, g / weight of egg, g] x100.

Whereas Haugh unit (HU) was assessed via the next formula:

Haugh unit = 100 log (H + 7.57–1.7 x W0.37) where as "H" and "W" indicate albumen height in mm and egg weight in g, correspondingly [34].

Proximate analysis of eggs, cholesterol and fatty acid profile of egg yolk

Proximate chemical analysis of DM, CP, EE and Ash in egg's albumen and yolk in the last week of the experiment were estimated according to AOAC [35]. For eggs yolk cholesterol evaluation, one gram from each yolk was mechanically homogenized with 1:9 w: v of Absolut ice-cold ethanol in ice bath then centrifuged for 10 min at 2500 r/min. The supernatant was separated, and the amount of cholesterol was verified via K Washburn and D Nix [36] using cholesterol commercial assay kit corresponding to producer's technique (Baolai Biotechnology Co. Ltd., Yancheng, China). For yolk fatty acids analysis, fatty acids extraction was done as following: fatty acids methyl esters were done by benzene: methanol: concentrated sulfuric acid (10: 86: 4 by volume) and methylation was prepared for 1 h at 80–90 °C according to Stahl [37]. The sodium hydroxide was used to saponify the extracted fat samples then methylated with boron trifluoride after that and then extracted with heptane. Then total saturated, mono-unsaturated, total poly unsaturated fatty acids (PUFAs), omega-3 and omega-6-fatty acids contents were analysed by gas chromatography utilizing methyl esters boron trifluoride approach [35].

Determination of yolk phospholipids, antioxidants and oxidation markers of fresh and stored egg

Yolk phospholipids

Fresh egg yolk samples were used for quantification of total phospholipids (PLs) according to method described by Palacios and Wang [38]. In brief, ethanol followed by hexane was used to extract the PLs and total lipids from fresh yolk as the two-phase extraction using two solvents resulted extraction of both polar and neutral fractions. For precipitation from the extracted lipids acetone was used. For quantification of the extracted PLs, HPLC-ELSD was used through application of two mobile phases degraded program involving methanol-chloroform-ammonium hydroxide (19:80:1,v/v), and methanol-chloroform-water–ammonium hydroxide (48:50:1:1, v/v) as the procedure of D Restuccia, UG Spizzirri, F Puoci, G Cirillo, G Vinci and N Picci [39].

Total phenol determination

For total phenols content of fresh or stored egg yolk, 100 mg of fresh or stored eggs were extracted in 5 mL acetone, then 1 mL of acetone extracted yolk was thoroughly mixed with of Folin–Ciocalteu reagent (0.5 mL), then Na_2CO_3 solution (2.5 mL, 20% w/v) were poured and completed to 25 mL deionized water. After that the solution was incubated at 60 ∘C for 40 min before assessing, the absorbance was determined at 750 nm against a blank. Total phenolics content was quantified as mg equivalents gallic acid (GAE/g yolk) Senevirathne et al., [40]

Flavonoid determination

Approximately 100 mg of fresh and stored egg yolk samples were extracted in diethyl (5 mL), centrifuged at 2000 rpm for 15 min then the precipitate was extracted in 5 mL methanol (80%) for 5 h and filtered by Wathman filter paper. In the next step, the prepared filtrate was completed to 50 mL distilled water and then 2.5 mL of it mixed with $NaNO_2$ (0.15 mL, 5%). After that, aluminium chloride (0.15 mL, 10%) was added after 5 min for six minutes followed by addition of NaOH (one mL) and distilled water (1.2 mL). The absorbance was verified at 510 nm against blank. The flavonoid content was stated as mg of quercetin for each gram of yolk. Meda et al., [41]

Total antioxidant activity

Ferric reducing antioxidant power assay was used for evaluating the total antioxidant activity (T-AOC), corresponding to Benzie and Szeto [78]. Fresh and stored egg yolk samples (0.5 g) were diluted in sodium chloride (10 mL, 2%) for preparation of egg yolk aqueous solutions. An aliquot of 150 µL of prepared yolk was stirred with distilled water (2.4 mL), of HCl (0.75 mL), of ethanol (0.45 mL), potassium ferric cyanide (0.75 mL of 1%), and

ferric chloride (0.25 mL of 0.2%), incubated in water bath for 20 at 50 ∘ C min then settled for cooling at room temperature. The capacity antioxidant power was conveyed as mg Equivalents of gallic acid for each gram yolk.

The malondialdehyde (MDA)

Lipid oxidation marker of fresh and stored egg yolk was estimated as described by HH Draper and M Hadley [42]. Briefly, egg yolk (0.5 g) diluted in trichloro acetate (5 mL, 20%) with 0.5 mL of butylated hydroxytoluene (1%) in 99% of ethanol then 2.95 mL of thiobarbituric acid (50 mM) were poured to 1.5 mL of prepared solution, incubated, cooled and then centrifuged for 10 min at 3500 rpm. The thiobarbituric acid reactive substances (TBARS) are defined as µg of MDA for each gram of yolk.

Blood immunological and lipid profile measurements

The serum total cholesterol (TC), triacylglycerol (TAG), low-density lipoprotein cholesterol (LDL-C), high-density lipoprotein cholesterol (HDL-C), very-low-density lipoprotein cholesterol (VLDL-C), were assessed using analytical kits (Spinreact Co., Santa Coloma, Spain).

A phagocytosis assay was performed according to Bos et al. [43], . Briefly, directly after blood collection a mononuclear cell layer was separated, washed and then added to a Roswell Park Memorial Institute medium (RPMI-164), then fetal calf serum (FCS, 15%) was added. After that, monolayer macrophages cells were gained by planting them on chambers then stained and incubated at 37 °C for 1 h under 99% humidity 5 and % CO2. After that, three times washing were applied for removal of non-adherent cells and then 1 mL of Candida Albicans (107/mL of RPMI with 15% FCS) was added and incubated with the adherent macrophages were under the same conditions then washed three -times, fixed, and stained. Ultimately, 100 macrophages were enumerated to determine the followings:

The intracellular killing capacity (ICK) was assed as the total number of ghost cells which exist in 100 phagocytized candida.

Phagocytic %= (number of phagocytic cells engulfing No. of labelled C. albicans / total number of phagocytic cells) x 100.

Phagocytic index = [(total number of engulfed cells / number of macrophages containing engulfed cells) x (number of macrophages containing engulfed cells / total number of counted macrophages)] x 100.

Immunoglobulins (IgM, REF; 035071190) and (IgG, REF; 03507432) were measured in the serum using ELISA commercial kits (Roche Diagnostics Co., Indiana, USA). Nitric oxide (NO) and lysozyme levels in serum samples were determined utilizing commercial kits (Jiancheng Biotechnology Institute, China).

Intestinal tissue oxidative and antioxidant markers

The collected intestinal samples were thoroughly homogenized for assessing the oxidative and antioxidant markers. Lipid oxidation marker malondialdehyde (MDA) was evaluated through the thiobarbituric acid-reactive assay (TBARS) value as demarcated by D Ahn, D Olson, C Jo, J Love and S Jin [44] and MDA values were stated as nmol/g tissue. For determination of total antioxidant capacity (T-AOC) commercial kits (Sigma-Aldrich, MAK187) were used. The levels of Hydrogen peroxide (H_2O_2) were measured following the instruction of F Loreto and V Velikova [45] and their values were stated as µmoL/g of tissue. Additionally, reactive oxygen species(ROS) was evaluated according to the technique of CP LeBel, H Ischiropoulos and SC Bondy [46].

Genes expression by reverse transcription quantitative real-time PCR (RT-qPCR)

At the end of the experiment (60 weeks), samples were collected from the intestinal, splenic, and abdominal adipose tissues ($n=10$/group) and immediately transferred to TRIzol reagent (Invitrogen Life Technologies, Carlsbad, CA, USA) following the manufacturer's instructions. Splenic and abdominal adipose tissues were used for the expression levels of autophagy encoding genes; mechanistic target of rapamycin (*mTOR*) and autophagy-related genes 5, 7&12 (*Atg5*, *Atg7* and *Atg12*), and lipogenesis genes; fatty acid synthase gene (*FAS*) and acetyl–co A carboxylase (*ACC*). Intestinal samples were used for evaluation of immune related genes: interleukin, *IL-1β* and *IL-10*, immunoglobulin M (IgM), and Avian β-defensin 6 and 12 (*AvBD6* and *AvBD612*). antioxidant related genes: catalase (*CAT*), superoxide dismutase (*SOD*), glutathione peroxidase (*GSH-Px*), heme oxygenase-1 (HO-1) and NAD (P) H quinone dehydrogenase 1 (*NQO1*) genes expression. The separation of total RNA was prepared by QIAamp RNeasy Mini kit (Qiagen, Germany) and its concentration was measured using spectrophotometer. One-step RT-qPCR assay was accomplished by Stratagene MX3005P real time PCR employing a Kit of QuantiTect SYBR Green RT-PCR (Qiagen, Germany). The distinction of each PCR amplification assay was confirmed through ultimate melting curve analysis. The Various levels of transcripts were normalized via glyceraldehyde 3-phosphate dehydrogenase (*GAPDH*). Primer sequences engaged in RT-qPCR assay for all study related genes are represented in Table 2. The data regarding the relative mRNA expression of examined genes were assessed utilizing $^{2-\Delta\Delta}$Ct approach [47].

Statistical analysis

The general linear method (GLM) of SPSS was applied for statistical analysis of experimental data. The homogeneity among all experimental groups was anticipated by

Table 2 Primer sequences employed for qPCR assay

Specificity/ Target gene	Primer sequence (5′-3′)	Accession No.
Autophagy genes		
mTOR	F: CATGTCAGGCACTGTGTCTATTCTC R: CTTTCGCCCTTGTTTCTTCACT	XM_417614.5
Atg5	F: TCACCCCTGAAGATGGAGAGA R: TTTCCAGCATTGGCTCAATTC	NM_001006409
Atg7	F: ACTGGCAATGCGTGTTTCAG R: CGATGAACCCAAAAGGTCAGA	NM_001030592
Atg12	F: GCACCCGCACCATCCA R: GAGGCCATCAGCTTCAGGAA	XM_003643073
Immune related genes		
IL-10	F: GCTGAGGGTGAAGTTTGAGG R: AGACTGGCAGCCAAAGGTC	XM_025143715.1
IgM	F GCA-TCA-GCG-TCA-CCG-AAA-GC R: TCC-GCA-CTC-CAT-CCT-CTT-GC	X01613.1
IL-1β	F-GCTCTACATGTCGTGTGTGATGAG R-TGTCGATGTCCCGCATGA	NM_204,524
AVBD6	F: GCCCTACTTTTCCAGCCCTATT R: GGCCCAGGAATGCAGACA	NM 001001193.1
AVBD12	F: TGTAACCACGACAGGGGATTG R: GGGAGTTGGTGACAGAGGTTT	NM 001001607.2
Lipogenic related genes		
FAS	F: GCAGCTTCGGTGCCTGTGGTT R: GCTGCTTGGCCCACACCTCC	NM205155
ACC	F: TGCCTCCGAGAACCCTAA R: TCCAGGCTTGATACCACA	JQ080306
Antioxidants genes		
GSH-Px	F- GCTGTTCGCCTTCCTGAGAG R- GTTCCAGGAGACGTCGTTGC	NM_001277853.1
SOD	F- CACTGCATCATTGGCCGTACCA R- GCTTGCACACGGAAGAGCAAGT	NM_205064.1
CAT	F- TGGCGGTAGGAGTCTGGTCT R- GTCCCGTCCGTCAGCCATTT	NM_001031215.1
HO-1	F-AAGAGCCAGGAGAACGGTCA R-AAGAGCCAGGAGAACGGTCA	NM_205344
NQO1	F-TCGCCGAGCAGAAGAAGATTGAAG R-CGGTGGTGAGTGACAGCATGG	NM_001277620.1
Housekeeping genes		
GAPDH	F: GGTGGTGCTAAGCGTGTTA R: CCCTCCACAATGCCAA	NM205518

mTOR: mechanistic target of rapamycin ; Atg: autophagy-related genes; *IL-10*: interleukin-10; *IgM*: immunoglobulin M; interleukin, *IL-1β* and *IL-10*, Avian β-defensin 6 and 12 (*AvBD6* and *AvBD12*), *FAS*: fatty acid synthase gene; *ACC*: acetyl–co A carboxylase; catalase (*CAT*), superoxide dismutase (*SOD*), glutathione peroxidase (*GSH-Px*), heme oxygenase – 1 (*HO-1*) and *NAD* (P) H quinone dehydrogenase 1 (*NQO1*) glyceraldahyde-3-phosphate dehydrogenase (*GAPDH*)

Levene's test and normality was measured via the test of Shapiro–Wilk. Tukey's test was accomplished to perceive the mean values as the changes were significant. Data differences were stated as standard error of the mean (SEM) and the statistical significance was modified at P value less than 0.05. All experimental graphs were designed by GraphPad Prism software Version 8.

Results

Efficiency of quercetin loaded nanoparticles on eggs laying performance

The impact of experimental diets fortified by QNPs different levels on different laying periods was shown in Table 3. At the peak of eggs production period, HDEP % displayed the maximum improvement ($P<0.05$) in groups fed QNPs200 and QNPs300. Remarkably, the group fed QNPs300 had the significantly highest ($P<0.05$) egg mass % and lowest FCR. Regardless the late laying periods, groups supplemented with QNPs200 and QNPs300 exhibited a promoted ($P<0.05$) HDEP, egg mass % and FCR compared to control one. In peak and late phases of eggs production NFEI data were significantly increased ($P<0.05$) in QNPs200 and QNPs300 received groups however, cumulative feed intake showed no significant differences among all experimental groups.

Efficiency of quercetin loaded nanoparticles on external and internal eggs quality

Table 4; shows the effect of various levels of QNPs fortified diets on egg quality related parameters. At the end of 35 weeks, compared to the control, hens fed QNPs200 and QNPs300 had higher egg weight, shell thickness, egg yolk and albumen weight than those fed other dietary treatments. Albumen height and yolk height and index and Haugh unit recorded the highest values ($P<0.05$) in QNPs300 supplemented group.

At the end of 60 weeks of laying cycle, groups supplemented with QNPs200 and QNPs300 showed higher yolk ratio and weight and egg weight in comparison with other experimental groups. Additionally, experimental dietary treatments had no effect ($P>0.05$) on the albumen weight, Haugh unit and shell weight and thickness.

Efficiency of quercetin loaded nanoparticles on chemical composition of egg yolk and albumen and egg yolk lipids analysis

The chemical composition of the eggs (DM, CP, EE, and ash) and fatty acids of eggs yolk is stated in Table 5. Notably, EE content in eggs yolk was dramatically reduced ($P<0.05$) in groups supplemented with QNPs in a dose dependent way. However, DM, CP and ash in eggs albumen and yolk showed no significant differences ($P>0.05$) among all experimental groups. The content of total cholesterol in yolk was prominently decreased ($P<0.05$) in group fed QNPs300 followed by QNPs100 and QNPs200 unlike control non-supplemented group as shown in Table 6. Among all experimental groups, the lowest ($P<0.05$) total SFA % was detected in group fed QNPs at the level 300 mg/kg diet. Total PUFAs and omega −3 FAs were significantly increased ($P<0.05$) in all groups supplemented with QNPs especially at higher levels.

Table 3 Productive performance of Hy-line brown laying hens consuming diets fortified with varying levels of quercetin loaded nanoparticles (QNPs)

Experimental Group						
Parameter	**Control**	**$QNPs_{100}$**	**$QNPs_{200}$**	**$QNPs_{300}$**	***p*-value**	**SEM**
Peak laying period (23–36 week)						
HDEP %	92.78 ^{b}	93.12 ^{ab}	93.55 ^{a}	93.55 ^{a}	0.030	0.17
Egg mass %	52.80 ^{c}	52.88 ^{c}	55.20 ^{b}	56.13 ^{a}	< 0.001	0.34
Total FI (g/bird)	10,648	10,638	10,644	10,629	0.510	4.46
FCR	2.06 ^{a}	2.05 ^{a}	1.97 ^{b}	1.93 ^{c}	< 0.001	0.01
NFEI	50.90 ^{b}	50.93 ^{b}	53.23 ^{a}	54.03 ^{a}	< 0.001	0.34
Late laying period (46–60 week)						
HDEP %	86.46 ^{b}	86.36 ^{b}	87.07 ^{a}	87.22 ^{a}	< 0.001	0.10
Egg mass %	54.47 ^{b}	54.41 ^{b}	55.72 ^{a}	55.82 ^{a}	< 0.001	0.16
Total FI (g/bird)	11,218	11,186	11,215	11,224	0.481	8.98
FCR	1.96 ^{a}	1.96 ^{a}	1.92 ^{b}	1.92 ^{b}	< 0.001	0.01
NFEI	48.55 ^{b}	48.76 ^{b}	49.82 ^{a}	49.87 ^{a}	< 0.001	0.14

HDEP %= hen-days egg production %; FI=feed intake; FCR=feed conversion ratio; NFEI=net feed efficiency index. $^{a\text{-}c}$ Means within a row donating varying superscript letters represent significant variations ($p < 0.05$). QNPs: quercetin loaded nanoparticles. Control: group fed basal diet free from QNPs, QNPs100: group fed basal diet enriched with 100 mg/kg diet of QNPs, QNPs200: group fed basal diet enriched with 200 mg/kg diet of QNPs, QNPs300: group fed basal diet enriched with 300 mg/kg diet of QNPs

Efficiency of quercetin loaded nanoparticles on egg yolk phospholipids, egg phenols and flavonoids and oxidation biomarkers in fresh and stored eggs

Notably, QNPs300 supplemented hens had the richest phospholipids content in egg yolk lipids (232.15 µmol/g fresh egg yolk) Table 7. All collected experimental eggs showed higher total phenols, flavonoids and antioxidant activity and inversely lower MDA content in fresh eggs than after 45 days storage. Remarkably, the levels of total phenols, flavonoids and antioxidant activity in eggs fed QNPs remained high even after 45 days storage in comparison with those from control group. Moreover, with increasing the levels of QNPs in hens' diets MDA levels were significantly decreased ($P < 0.05$) and total phenols, flavonoids and antioxidant activity were significantly increased ($P < 0.05$) in a dose dependent pattern.

Efficiency of quercetin loaded nanoparticles on serum biochemical parameters and intestinal oxidative and antioxidant biomarkers

Data regarding the impact of QNPs on serum immunological parameters, lipids profile and oxidative and antioxidant attributes are represented in Table 8. The levels of TAG, total cholesterol, LDL-C and VLDL were reduced ($p < 0.05$) and the concentrations of HDL were significantly elevated with increasing levels of QNPs in hens' diets. Remarkably, supplementation of QNPs had improved the tested serum immune parameters. Besides, groups fed QNPs especially at the level of 300 mg/kg showed the highest ($p < 0.05$) immune response as proven by elevated levels of phagocytic percent and index, serum IgM, IgG and lysozyme activity and lowered levels of NO.

A noticeable higher concentration of T-AOC ($P < 0.05$) and lower ROS contents in intestinal tissues was observed in groups fed QNPs supplemented diets in a dose dependent manner. Significant lower lipid peroxidation biomarkers (MDA) ($P < 0.05$) were identified with increasing the supplemental levels of QNPs in intestinal tissues. A remarkable decrease in H_2O_2 production was detected in intestinal tissue of group fed QNPs at the level of 300 (mg/kg diet).

Expression of immune and autophagy related genes

The expression patterns of proinflammatory cytokines (IL-1β and TNF-α), anti-inflammatory cytokine (IL-10) and IgM, *AvBD6* and *AvBD612* genes are illustrated in Fig. 2. Notably, group supplemented with QNPs at the level of 300 mg/kg exhibited the highest($P < 0.05$) expression levels of *AvBD6* (increased by 1.66-fold), *AvBD612* *(*(increased by 1.47-fold),, *IL-10* (increased by 1.80-fold), and IgM (increased by 1.53-fold) genes. In contrast, groups fed QNPs300 and QNPs200 expressed significantly lower levels of *IL-1β* and *TNF*-α than other experimental groups (Fig. 2).

The transcriptional levels of mTOR and autophagy related genes (*atg5*, *atg7*, and *atg12*) are clarified in Fig. 3. All groups fed QNPs showed higher expression levels of *atg5* and *atg7* whereas the highest ones were achieved in group fed QNPs300. In contrast with increasing dietary inclusion of QNPs, mTOR expression pattern was significantly reduced ($P < 0.05$) when compared with control group. Concerning *atg12* gene, group fed QNPs300 significantly expressed higher levels ($p < 0.05$) of such gene followed by group fed QNPs200 and QNPs100 comparing with control group.

Table 4 Egg quality at 35 and 60 weeks of Hy-line brown laying hens consuming diets fortified with varying levels of quercetin loaded nanoparticles (QNPs)

Experimental Group						
Parameter	**Control**	**$QNPs_{100}$**	**$QNPs_{200}$**	**$QNPs_{300}$**	***p*-value**	**SEM**
At the end of 35 weeks of age						
Egg weight, g	56.68 [b]	57.04 [b]	59.08 [a]	60.08 [a]	< 0.001	0.36
Egg shape index	74.36	75.06	74.50	74.83	0.730	0.23
Yolk weight, g	15.71 [b]	15.76 [b]	16.47 [a]	16.75 [a]	< 0.001	0.12
Yolk height, mm	14.63 [b]	14.55 [b]	15.10 [b]	16.08 [a]	< 0.001	0.16
Yolk width, mm	35.45	35.27	35.16	35.59	0.558	0.11
Yolk ratio %	27.71	27.62	27.88	27.87	0.200	0.05
Yolk index %	41.27 [c]	41.27 [c]	42.96 [b]	45.17 [a]	< 0.001	0.41
Albumen weight, g	34.70 [b]	35.04 [b]	36.16 [a]	36.77 [a]	< 0.001	0.21
Albumen height, mm	5.06 [b]	5.58 [ab]	5.42 [ab]	6.17 [a]	0.024	0.14
Albumen ratio %	61.22	61.43	61.21	61.21	0.509	0.06
Haugh unit	70.17 [b]	74.48 [ab]	72.37 [ab]	77.63 [a]	0.049	1.03
Shell weight, g	6.28 [b]	6.24 [b]	6.45 [ab]	6.56 [a]	0.006	0.04
Shell thickness, mm	0.42 [b]	0.43 [b]	0.47 [a]	0.48 [a]	0.001	0.01
Shell ratio %	11.07	10.94	10.91	10.92	0.355	0.03
At the end of 60 weeks of age						
Egg weight, g	63.08 [b]	63.04 [b]	64.02 [a]	64.18 [a]	< 0.001	0.11
Egg shape index	75.27	75.61	74.63	75.23	0.238	0.17
Yolk weight, g	18.07 [b]	18.06 [b]	18.97 [a]	19.05 [a]	0.001	0.07
Yolk height, mm	16.65	16.56	16.64	16.67	0.960	0.07
Yolk width, mm	40.20	39.90	40.46	40.14	0.762	0.17
Yolk ratio %	28.65 [b]	28.66 [b]	29.64 [a]	29.68 [a]	0.003	0.18
Yolk index %	41.44	41.51	41.14	41.54	0.847	0.16
Albumen weight, g	37.87	38.16	37.75	37.81	0.794	0.15
Albumen height, mm	8.59	8.52	8.66	8.68	0.809	0.06
Albumen ratio %	61.77	56.85	59.01	60.48	0.541	1.20
Haugh unit	91.79	91.45	91.92	92.00	0.932	0.30
Shell weight, g	7.14	6.82	7.30	7.32	0.391	0.11
Shell thickness, mm	0.54	0.54	0.54	0.55	0.946	0.01
Shell ratio %	11.32	10.82	11.41	11.41	0.587	0.17

QNPs: quercetin loaded nanoparticles. Control: group fed basal diet free from QNPs, QNPs100: group fed basal diet enriched with 100 mg/kg diet of QNPs, QNPs200: group fed basal diet enriched with 200 mg/kg diet of QNPs, QNPs300: group fed basal diet enriched with 300 mg/kg diet of QNPs. [a–c] Means within a row donating varying superscript letters represent significant variations ($p < 0.05$)

Table 5 Proximate chemical analysis, of Hy-line brown laying hens' eggs consuming diets fortified with varying levels of quercetin loaded nanoparticles (QNPs)

Experimental Group						
Parameter	**Control**	**$QNPs_{100}$**	**$QNPs_{200}$**	**$QNPs_{300}$**	***p*-value**	**SEM**
Chemical analysis of egg albumen						
DM %	12.07	12.07	12.07	12.04	0.526	0.01
CP %	10.97	11.04	11.02	11.01	0.928	0.04
EE %	0.09	0.09	0.09	0.09	0.662	0.001
Ash %	0.64	0.64	0.63	0.63	0.926	0.01
Chemical analysis of egg yolk						
DM %	44.42	44.44	44.48	44.37	0.931	0.06
CP %	13.61	13.60	13.44	13.46	0.397	0.04
EE %	28.31 [a]	28.13 [ab]	27.55 [b]	26.74 [c]	< 0.001	0.16
Ash %	1.26	1.27	1.27	1.26	0.868	0.003

DM: dry matter; CP: crude protein; EE: ether extract. QNPs: quercetin loaded nanoparticles. Control: group fed basal diet free from QNPs, $QNPs_{100}$: group fed basal diet enriched with 100 mg/kg diet of QNPs, $QNPs_{200}$: group fed basal diet enriched with 200 mg/kg diet of QNPs, $QNPs_{300}$: group fed basal diet enriched with 300 mg/kg diet of QNPs. [a-c] Means within a row donating varying superscript letters represent significant variations ($p < 0.05$)

Table 6 Total cholesterol and fatty acids (percent of total fatty acids) evaluation of Hy-line brown laying hens' eggs consuming diets fortified with varying levels of quercetin loaded nanoparticles (QNPs)

Experimental Group						
Parameter	**Control**	**$QNPs_{100}$**	**$QNPs_{200}$**	**$QNPs_{300}$**	***p*-value**	**SEM**
Total cholesterol mg/100 g	94.24 [a]	91.56 [b]	90.65 [b]	88.96 [c]	< 0.001	0.47
Total SFA %	31.46 [a]	31.53 [a]	31.02 [ab]	30.77 [b]	0.019	0.11
Total MUSFA %	42.82	42.14	41.98	41.71	0.150	0.18
Total PUSFA %	25.73 [c]	26.33 [bc]	27.00 [ab]	27.52 [a]	< 0.001	0.19
Total omega – 6 FA %	24.58 [c]	24.95 [bc]	25.64 [ab]	26.19 [a]	< 0.001	0.17
Total omega – 3 FA %	0.63 [b]	0.63 [b]	0.66 [a]	0.67 [a]	< 0.001	0.005

SFA: saturated fatty acids; MUSFA: monounsaturated fatty acids; PUSFA: poly unsaturated fatty acids; FA: fatty acids. QNPs: quercetin loaded nanoparticles. Control: group fed basal diet free from QNPs, $QNPs_{100}$: group fed basal diet enriched with 100 mg/kg diet of QNPs, $QNPs_{200}$: group fed basal diet enriched with 200 mg/kg diet of QNPs, $QNPs_{300}$: group fed basal diet enriched with 300 mg/kg diet of QNPs. [a-c] Means within a row donating varying superscript letters represent significant variations ($p < 0.05$)

Table 7 Egg yolk phospholipids and eggs' antioxidants of Hy-line brown laying hens consuming diets fortified with varying levels of quercetin loaded nanoparticles (QNPs)

Parameter	**Phospholipids (µmol /g fresh egg yolk)**		**Total phenols, mg GAE/g**		**Flavonoids, mg CE/g**		**Total antioxidant activity, mg gallic acid equivalent (GAE/g yolk)**		**Malondialdehyde (MDA), µg/g**	
Egg yolk	**Fresh**	**Stored**	**Fresh**	**Stored**	**Fresh**	**Stored**	**Fresh**	**Stored**	**Fresh**	**Stored**
Control	196.60 [d]	117.77 [d]	1.76 [d]	1.47 [d]	1.97 [d]	1.49 [d]	3.20 [d]	1.33 [d]	0.34 [a]	0.39 [a]
QNPs100	207.91 [c]	171.51 [c]	2.09 [c]	1.87 [c]	2.16 [c]	2.06 [c]	3.81 [c]	2.01 [c]	0.19 [b]	0.31 [b]
QNPs200	213.73 [b]	184.98 [b]	2.18 [b]	2.03 [b]	2.35 [b]	2.25 [b]	4.21 [b]	2.78 [b]	0.24 [c]	0.27 [c]
QNPs300	223.15 [a]	202.36 [a]	2.33 [a]	2.22 [a]	2.92 [a]	2.63 [a]	4.79 [a]	3.23 [a]	0.19 [d]	0.21 [d]
p-value	< 0.001	< 0.001	< 0.001	< 0.001	< 0.001	< 0.001	< 0.001	< 0.001	< 0.001	< 0.001
SEM	2.22	7.28	1.05	0.06	1.08	0.09	0.13	0.17	0.01	0.01

QNPs: quercetin loaded nanoparticles. Control: group fed basal diet free from QNPs, QNPs100: group fed basal diet enriched with 100 mg/kg diet of QNPs, QNPs200: group fed basal diet enriched with 200 mg/kg diet of QNPs, QNPs300: group fed basal diet enriched with 300 mg/kg diet of QNPs. Eggs were stored for 45 days egg at room temperature. [a–d] Means within a row donating varying superscript letters represent significant variations ($p < 0.05$)

Table 8 Serum lipid profile and serum immune and intestinal oxidative/antioxidant parameters of laying of Hy-line brown laying hens consuming diets fortified with varying levels of quercetin loaded nanoparticles (QNPs)

Experimental Group						
Parameter	**Control**	**$QNPs_{100}$**	**$QNPs_{200}$**	**$QNPs_{300}$**	***p*-value**	**SEM**
Serum Lipid profile						
Total cholesterol, mg/dl	130.85 [a]	127.08 [b]	123.23 [c]	119.73 [d]	< 0.001	0.96
Triacylglycerol, mg/dl	61.80 [a]	58.24 [b]	55.06 [c]	51.56 [d]	< 0.001	0.88
HDL-cholesterol, mg/dl	88.46 [d]	93.72 [c]	96.09 [b]	99.91 [a]	< 0.001	0.96
LDL-cholesterol, mg/dl	30.04 [a]	21.71 [b]	16.13 [c]	9.51 [d]	< 0.001	1.73
VLDL-cholesterol, mg/dl	12.36 [a]	11.65 [b]	11.01 [c]	10.31 [d]	< 0.001	0.18
Serum immune parameters						
phagocytic %	55.40 [d]	59.80 [c]	63.60 [b]	67.20 [a]	< 0.001	1.07
Phagocytic index	2.62 [d]	3.41 [c]	3.97 [b]	4.56 [a]	< 0.001	0.17
Intra-cellular killing capacity %	67.41 [d]	71.93 [c]	76.50 [b]	80.30 [a]	< 0.001	1.12
IgM(mg/dL)	12.87 [d]	14.50 [c]	16.80 [b]	18.33 [a]	< 0.001	0.48
IgG(mg/dL)	1.56 [d]	1.81 [c]	1.93 [b]	2.11 [a]	< 0.001	0.05
NO (µmol/L)	0.67 [a]	0.59 [b]	0.55 [c]	0.49 [d]	< 0.001	0.02
Lysozymes (U/Ml)	1.11 [d]	1.31 [c]	1.65 [b]	1.87 [a]	< 0.001	0.07
Intestinal oxidative/antioxidant parameters						
T-AOC (U/mg of protein)	1.14[c]	1.25 [b]	1.31 [b]	1.39 [a]	< 0.001	0.021
MDA (nmol/g tissue)	15.12[a]	13.65 [b]	12.95 [c]	12.01 [d]	< 0.001	1.32
ROS	58.18[a]	56.12 [b]	53.10 [c]	50.69 [d]	< 0.001	5.13
H_2O_2 (µmoL/g tissue)	2.25[a]	1.97 [b]	1.77 [bc]	1.60 [c]	< 0.001	0.52

Immunoglobulins M and G (IgM and IgG); nitric oxide (NO); total antioxidant activity (T-AOC); malondialdehyde (MDA); hydrogen peroxide (H_2O_2). QNPs: quercetin loaded nanoparticles. Control: group fed basal diet free from QNPs, QNPs100: group fed basal diet enriched with 100 mg/kg diet of QNPs, QNPs200: group fed basal diet enriched with 200 mg/kg diet of QNPs, QNPs300: group fed basal diet enriched with 300 mg/kg diet of QNPs. [a–d] Means within a row donating varying superscript letters represent significant variations ($p < 0.05$)

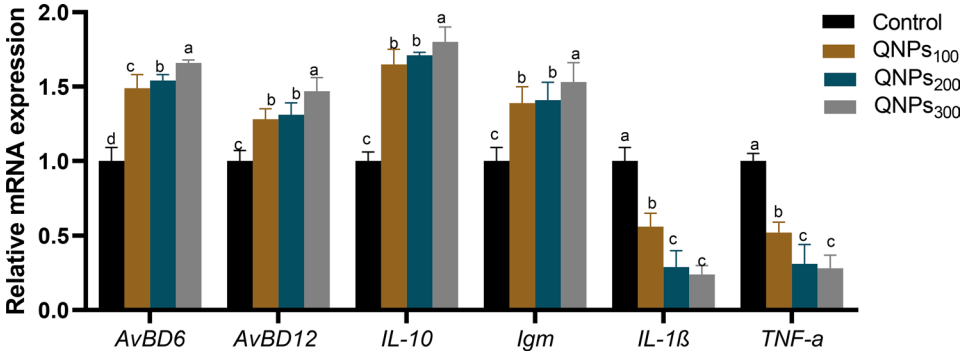

Fig. 2 Efficacy of varying levels of dietary quercetin loaded nanoparticles (QNPs) enrichment for laying hens' diets on mRNA expression of intestinal immune related genes; Avian beta defensin 6, 12 (*AvBD 6* and *12*), immunoglobulin M (*IgM*), interleukin-10, 1β (*IL-10* and *IL-1β*) and tumor necrosis factor-α (*TNF*-α) genes.QNPs: quercetin loaded nanoparticles. Control: group fed basal diet free from QNPs, QNPs100: group fed basal diet enriched with 100 mg/kg diet of QNPs, QNPs200: group fed basal diet enriched with 200 mg/kg diet of QNPs, QNPs300: group fed basal diet enriched with 300 mg/kg diet of QNPs. [a–d] Means within a column denoting varying superscript letters represent significant variations ($p < 0.05$)

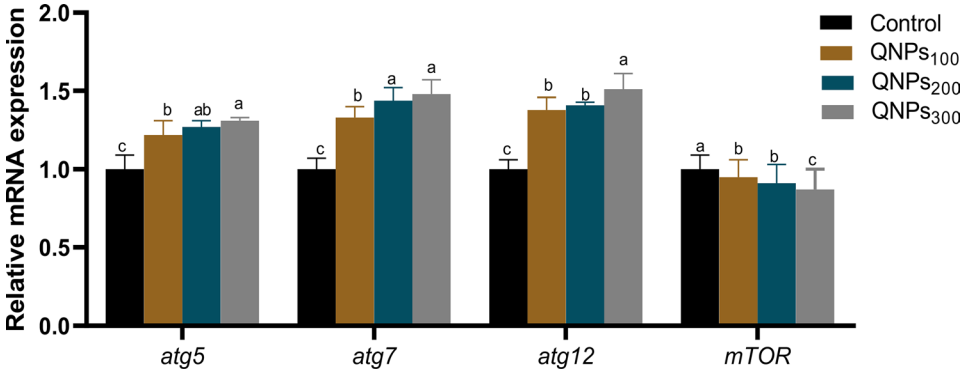

Fig. 3 Efficacy of varying levels of dietary quercetin loaded nanoparticles (QNPs) enrichment for laying hens' diets on mRNA expression of autophagy (*atg5*, *atg7*, and *atg12*) related genes; and mechanistic target of rapamycin (*mTOR*). QNPs: quercetin loaded nanoparticles. Control: group fed basal diet free from QNPs, QNPs100: group fed basal diet enriched with 100 mg/kg diet of QNPs, QNPs200: group fed basal diet enriched with 200 mg/kg diet of QNPs, QNPs300: group fed basal diet enriched with 300 mg/kg diet of QNPs. [a–c] Means within a column denoting varying superscript letters represent significant variations ($p < 0.05$)

Intestinal antioxidants gene expression

The effect of supplementing diets with various levels of QNPs on expression of antioxidants associated genes was illustrated in Fig. 4. Intestinal *CAT*, *SOD*, *GSH-Px*, *HO-1* and *NQO1* genes expression were significantly upregulated ($p<0.05$) in QNPs group when compared control group. The mRNA expression of *CAT*, *SOD* was significantly higher ($p<0.05$) in groups fed QNPs300 QNPs200 followed by QNPs100 unlike control group. Moreover, the height expression ($P<0.05$) of GSH-Px was noticed in QNPs300 supplemented group. Regarding the expression of *HO-1* and *NQO1*, QNPs supplementation significantly increased ($P<0.05$) their levels dose dependently.

Expression of lipogenesis related genes

Data concerning transcriptional levels of FAS and ACC genes are displayed in Fig. 5. The results revealed that mRNA expression of FAS gene achieved its minimal level ($P<0.05$) in groups supplemented with QNPs300 and QNPs200 in comparison with other groups. Groups fed QNPs300 significantly down-regulated ($p<0.05$) the mRNA expression of ACC followed by QNPs 200 unlike other experimental groups.

Discussion

The effective role of functional feed additives containing flavonoids has recently been robustly studied for their beneficial health effects on poultry growth performance, meat, and egg quality. Nutritional interventions with phenolic compounds that have strong natural antioxidants, derived from plant extracts, not only improve the antioxidant capacity of laying hens and prolong their laying lifespan but also enhance egg production rates. Additionally, eggs fortified with these natural antioxidants have improved quality [48].

Quercetin, a ubiquitous flavonoid, is known for its powerful antioxidant, anti-inflammatory, antimicrobial, anti-obesity, anti-hypercholesterolemic, and anti-aging properties [49]. Quercetin plays a fundamental role in improving the performance and egg quality of laying

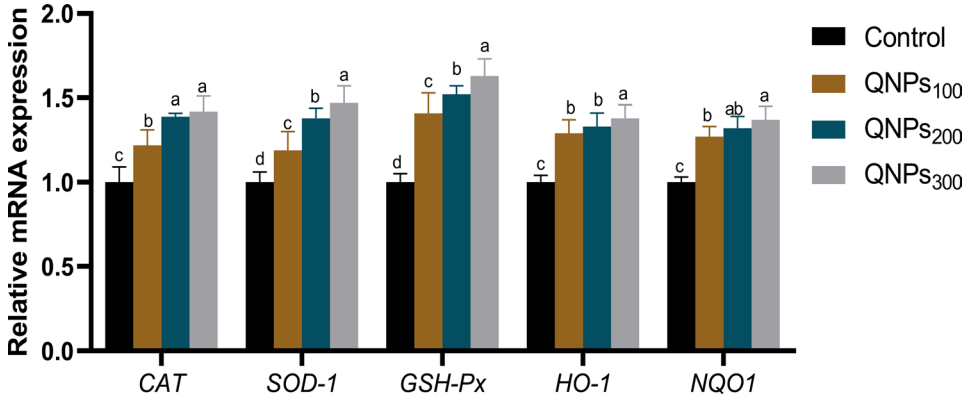

Fig. 4 Effect of supplementation of layer hens' diets with QNPs at levels of 100, 200 and 300 mg/kg on mRNA expression of intestinal antioxidant related genes; catalase (CAT), superoxide dismutase (*SOD*), glutathione peroxidase (*GSH-Px*), heme oxygenase-1 (*HO-1*) and NAD (P) H quinone dehydrogenase-1 (*NQO1*) genes.QNPs: quercetin loaded nanoparticles. Control: group fed basal diet free from QNPs, QNPs100: group fed basal diet enriched with 100 mg/kg diet of QNPs, QNPs200: group fed basal diet enriched with 200 mg/kg diet of QNPs, QNPs300: group fed basal diet enriched with 300 mg/kg diet of QNPs. [a–d] Means within a column denoting varying superscript letters represent significant variations ($p < 0.05$)

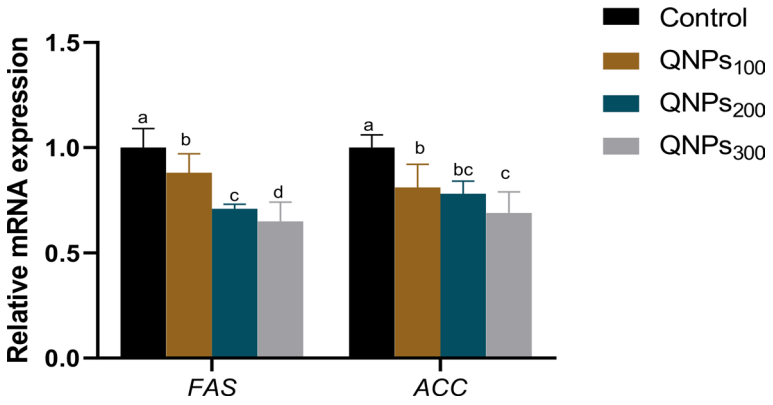

Fig. 5 Effect of supplementation of layer hens' diets with QNPs at levels of 100, 200 and 300 mg/kg on abdominal adipose tissues mRNA expression of fatty acid synthase (*FAS*) and acetyl-coA carboxylase (*ACC*) genes. QNPs: quercetin loaded nanoparticles. Control: group fed basal diet free from QNPs, QNPs100: group fed basal diet enriched with 100 mg/kg diet of QNPs, QNPs200: group fed basal diet enriched with 200 mg/kg diet of QNPs, QNPs300: group fed basal diet enriched with 300 mg/kg diet of QNPs. [a–d]Means within a column denoting varying superscript letters represent significant variations ($p < 0.05$)

hens by regulating their antioxidant and immune systems, which in turn enhances their production rates [50]. In the modern poultry industry, advances in nanotechnology have led to the use of quercetin nanoparticles in laying hens' feed. This innovative approach provides several benefits, including improved aqueous solubility of quercetin and enhanced stability in the gastrointestinal tract (GIT). In addition, quercetin nanoparticles can improve intestinal permeation, enable controlled release in the gastrointestinal tract (GIT), and facilitate intracellular and transcellular delivery, ultimately enhancing their arrival in eggs. However, a review of the existing literature reveals a lack of data on the potential effects of quercetin nanoparticles (QNPs) on cellular redox status, immune function, and the productivity and quality of laying hens' eggs during storage.

In this study, the laying performance of hens supplemented with quercetin nanoparticles (QNPs) was enhanced, as evidenced by increased HDEP% (Hen-day egg production percentage), egg mass percentage, and NFEI (net feed efficiency index). Additionally, feed conversion ratio (FCR) was reduced, particularly at higher supplementation levels, throughout the entire productive cycle of egg production. In consistence with these results Liu et al. [51] reported an increase in laying rate and egg mass and decrease in feed conversion ratio after feeding on higher free quercetin levels (500 mg/kg) in late laying period. In the same line, Amevor et al. [52], proofed that supplementation with dietary quercetin (400 mg/kg) in Tianfu laying hens at 52 week old had enhanced the laying rate, feed intake, egg mass and increased the ratio between feed and egg weight. The improvement in laying performance, such as increased laying rate, following quercetin supplementation may be attributed to its phytoestrogenic effects. Quercetin mimics estrogen by competing with and binding to estrogen receptors. This

action stimulates the production of estrogen-responsive hormones and genes, which in turn promotes follicular maturation and ovulation, ultimately enhancing laying performance [53]. Regarding both external and internal egg quality, our results indicated that supplementation with quercetin nanoparticles (QNPs) significantly improved egg quality characteristics. Specifically, QNPs supplementation at levels of 200 and 300 mg/kg diet enhanced egg weight, yolk index, albumen weight, Haugh unit, and shell thickness compared to the control diet. A previous study reported that supplementation with free quercetin significantly improved egg quality, including metrics such as the Haugh unit, eggshell strength and thickness, and albumen height [54]. In consistency with these findings, quercetin supplementation at a level of 0.4 g/kg was shown to enhance egg quality traits, including the Haugh unit, eggshell strength and thickness, and yolk ratio, when compared to the control [55].

The concentrations of antioxidant-related compounds in fresh eggs were significantly influenced by dietary treatments. Over the storage period, eggs can be exposed to both protein and lipid peroxidation, which adversely affects their functional and physicochemical properties, significantly impacting their overall quality [14, 56]. In the current study, we evaluated the antioxidant activity of quercetin nanoparticles (QNPs) in laying hens' egg quality for the first time. The results demonstrated that QNPs effectively enhanced egg quality during storage by increasing the total antioxidant capacity and reducing the concentration of malondialdehyde (MDA) in eggs, with these benefits becoming more pronounced at higher dietary QNPs levels. In accordance with this, nutritional interventions using natural antioxidants for laying hens have been aimed at mitigating oxidative stress and reducing the peroxidation rates of lipids and proteins. These interventions help improve egg quality traits and extend the shelf life of eggs [57]. In agreement, Lee et al., [58] described that plant extract enriched flavonoids can reduced TBARS and increase DPPH radical scavenging activity and thus enhanced antioxidant capacity in stored eggs. Moreover, eggs from natural polyphenol catechin fed layers had higher total T-AOC, oxygen free radical absorbance ability, decreasing power, and reduce albumen and yolk MDA contents [59]. Additionally, quercetin improved the oxidative stability of eggs stored at room temperature for 28 days. These findings demonstrate that free quercetin, at a level of 800 mg/kg, may help preserve freshness and extend the shelf life of eggs, primarily by reducing MDA (malondialdehyde) content in the egg yolk as its concentration increases [60]. Flavonoids can stabilize the anion superoxide by donating a hydrogen ion (H+) to it, thereby inhibiting the oxidation of lipoproteins and DNA [61]. Herein, over a 45-day storage period, the total phenolics and flavonoids content in stored eggs remained at higher concentrations in groups receiving quercetin nanoparticles (QNPs) in a dose-dependent manner. This effect may be attributed to their ability to block free radicals [62, 63]. Accordingly, the concentration of polyphenols was elevated in both egg yolk and egg albumen in experimental groups fed a diet containing 3% grapeseed meal enriched with antioxidants [64]. A diet enriched with flavonoids (hesperidin, naringin, and quercetin at 0.5 g/kg) improved egg albumen quality after 8 weeks of supplementation. This enhancement was achieved by boosting the eggs' antioxidant capacity and regulating the MAPK/Nrf2 signalling pathway [59]. Interestingly, in the current study, the promising effects of quercetin on egg quality can be further enhanced by incorporating it into a nanocarrier system. This approach offers the advantages of high absorption, increased bioavailability, and more effective delivery to the target tissues [65].

Eggs are rich in polyunsaturated fatty acids (PUFAs), which are highly susceptible to peroxidation during storage [14]. Lipid oxidation in egg yolk can adversely affect stability during storage and alter the nutritional quality of eggs. The fatty acid composition in eggs can be modified through dietary manipulation of hens' diets [66]. Besides, incorporation of flavonoids in laying hens feed industry is strongly considered as an appropriate approach for improving eggs quality [15]. Protecting egg yolk PUFAs from auto-oxidation can be achieved by enriching the diet with natural, strong antioxidants like quercetin. In the current study, we observed that dietary quercetin nanoparticles (QNPs) enhanced the nutritional quality of laid eggs by reducing the total amount of saturated fatty acids (SFAs) and increasing the deposition of polyunsaturated fatty acids (PUFAs), particularly in the groups fed QNPs at 200 mg/kg and 300 mg/kg. In agreement, a diet enriched with flavonoids, including anthocyanins, can significantly increase the polyunsaturated fatty acid content in eggs compared to a control diet [67]. It has been described that phytogenic antioxidants can extend the shelf life of omega-3 fatty acids in egg yolk for up to 35 days of storage [68]. Previous studies have reported that natural antioxidants can help reduce lipid peroxidation in egg powder during storage [69]. The enhancing effects of quercetin nanoparticles (QNPs) on polyunsaturated fatty acids (PUFAs) may be attributed to their protective role in preserving PUFAs in eggs, thereby contributing to higher product quality [70] and these results are further supported by the reduction in MDA levels and the increase in total antioxidant concentrations in eggs from hens fed QNPs. Moreover, quercetin nanoparticles (QNPs) may exert their effects on polyunsaturated fatty acids (PUFAs) indirectly by promoting bioenergetic stability and influencing fatty acid metabolism [71]. Additionally, the positive effects of quercetin nanoparticles

(QNPs) can be attributed to their stability, extended release in the GIT, and effective intracellular delivery to the eggs, which allow for prolonged and beneficial impact [72].

Notably, after dietary intervention with higher levels of quercetin nanoparticles (QNPs), the cholesterol content in egg yolk was reduced, while phospholipid levels increased. These positive results suggest that QNPs, as natural flavonoids, regulate lipid metabolism by increasing lipolysis and decreasing fat content and cholesterol deposition in egg yolk. Consequently, this enhances the nutritional value of the eggs. Other researchers have observed similar results with free forms of flavonoids; however, the effects of nano-loaded flavonoids have not been investigated until now. In agreement with our findings, a diet supplemented with 0.4–1.2% mulberry leaf flavonoids has been shown to decrease total cholesterol (TC) and triglyceride (TG) contents in egg yolk [73]. Moreover, TC content in egg yolk was reduced by dietary addition with quercetin and hesperidin (0.5 g/kg) for 28 weeks old laying hens [74]. Also, 0.04% of dietary quercetin significantly reduced the contents of TC and TG and increased the contents of phospholipids and lecithin in the egg yolk of laying hens [13].

Furthermore, poor egg quality resulting from lipid metabolism disorders, along with the period after the peak laying stage, makes laying hens more vulnerable to lipid accumulation [54]. The ovaries of laying hens do not synthesize lipids; instead, the liver is responsible for synthesizing over 90% of the cholesterol. The liver is considered the main organ for cholesterol synthesis and its transfer to the blood in laying hens [75]. Utmost of the serum cholesterol is transported to the egg yolk by lipoproteins and LDL which is the key a lipoprotein responsible for transportation of cholesterol and triglyceride to liver and formation of bile acids or for re-circulation [76]. VLDL is the chief carrier of cholesterol in the serum, transporting it to the ovary, where it is then absorbed by the oocyte to form egg yolk. In this manner, VLDL accounts for nearly 95% of the cholesterol found in egg yolk [77]. In this context, the levels of total cholesterol (TC), triglycerides (TG), low-density lipoprotein (LDL), and very-low-density lipoprotein (VLDL) in the serum of laying hens were used as indicators for assessing normal lipid metabolism. Feeding on QNPs300 resulted in a significant reduction in the levels of total cholesterol (TC), triglycerides (TG), low-density lipoprotein (LDL), and very-low-density lipoprotein (VLDL) in the serum of laying hens, while HDL levels were not significantly elevated. This suggests that incorporating quercetin into an efficient nano carrier system contributes to improved lipid metabolism in laying hens. Similarly, feeding chickens higher doses of free quercetin (0.04% and 0.06%) reduced the levels of total cholesterol (TC), triglycerides (TG), and low-density lipoprotein (LDL) in the serum, thereby improving cholesterol metabolism [78]. Another benefit of including flavonoids in the diets of laying hens is their ability to form insoluble complexes with cholesterol in the digesta, which slows the intestinal absorption of both endogenous and exogenous cholesterol [50]. Additionally, a diet fortified with fermented Ginkgo biloba leaves, enriched with flavonoids, reduced the concentrations of total cholesterol (TC), triglycerides (TG), and low-density lipoprotein cholesterol (LDL-C) in the serum of 49-week-old laying hens. This, in turn, decreased egg cholesterol content and improved egg quality [79]. These enhanced effects of dietary QNPs may also result from their role in inhibiting the activity of 3-hydroxy-3-methylglutaryl coenzyme A reductase, which contributes to reduced cholesterol levels in the serum and liver, and consequently lowers cholesterol deposition in egg yolk [80]. In the same context, the reduction in serum cholesterol and triglycerides was associated with a significant downregulation of fatty acid synthase (FAS) and acetyl-CoA carboxylase (ACC) expression, especially in the group supplemented with QNPs300. The possible mechanism by which QNPs reduce cholesterol synthesis may involve their inhibitory effect on the enzyme 3-hydroxy-3-methylglutaryl-CoA reductase or their ability to trigger the catabolism of lipoproteins [81]. Likewise, results stated by [82] clarified that the addition of flavonoids decreased cholesterol and triglyceride levels by enhancing excretion in animals, which inhibits the intestinal absorption of cholesterol and bile. Similarly, quercetin significantly reduced the expression of the lipogenic enzyme fatty acid synthase (FAS), which in turn decreased fat accumulation [83]. Moreover, the role of QNPs is demonstrated by their ability to restore the balance disrupted by dysregulation of lipogenesis and lipolysis, which is primarily linked to redox imbalances which came in agreement with Gentile et al., [84].

The current study demonstrated that intestinal antioxidants and oxidative stress-related markers were significantly improved with increased dietary QNPs intervention, as evidenced by decreased levels of MDA, H_2O_2, and ROS, along with upregulation of the genes *SOD1*, *CAT*, GSH-Px, *NRF2*, *HO-1*, and *NQO1*. These findings highlight the potential of QNPs to protect against intestinal inflammation and oxidative stress in laying hens at the peak of egg production [85]. Similarly, the antioxidant impacts of quercetin offered the strong protection to birds with a healthy intestinal environment [86]. Therefore, maintaining a healthy intestine through nutritional interventions is crucial for the health of laying hens and extends the laying cycle in commercial flocks. Iskender et al., [87], informed a noteworthy increase in antioxidant enzymes, involving the activities of GSH-Px and SOD of laying hens fed a diet enriched with 0.5 g/

kg free form of quercetin. Also, green tea polyphenol enriched diet increased the expression of Nrf2 and HO-1 in liver of lying hens [59]. Additionally, the dietary inclusion of herbal plants enriched with antioxidants resulted in higher the transcriptional levels of intestinal *GPX-1*, *SOD-1*, *CAT*, *HO-1*, *NQO1*, and *NRF2* genes in chickens' [88]. The antioxidant properties of quercetin are associated with its ability to scavenge free radicals, enhance the expression of endogenous antioxidant enzymes (such as *GSH-Px*, catalase, and *SOD*), and reduce MDA level [26]. Furthermore, quercetin can modulate intracellular redox status by scavenging reactive oxygen species (ROS), increasing intracellular glutathione (GSH) levels, and enhancing the expression of anti-apoptotic proteins through Nrf2-dependent regulation [26]. Remarkably, the enhanced efficacy of QNPs, even at lower levels, can be attributed to their higher bioavailability and sustained release over a longer period, ensuring prolonged functionality [89].

The immune system displayed a significant role in controlling laying hens' inflammatory disorders associated with oxidative stress during the peak of egg production. In this regard, dietary quercetin positively affected the laying hen's immune system by stimulating the production of immunoglobulins and enhancing the expression of immune-related genes [54]. In this context, the addition of QNPs as a dietary supplement improved serum immune parameter, as evidenced by increased lysozyme activity, elevated levels of IgM and IgG, and a higher phagocytic index, while also decreasing nitric oxide (NO) levels, particularly with higher doses of QNPs. Moreover, cytokines are endogenous mediators of the immune system and play a key role in regulating the mechanisms of inflammatory reactions [90]. Consistent with the immune-boosting effects of QNPs on serum levels, there was a notable downregulation of proinflammatory cytokines (*IL-1β* and *TNF-α*) and an increased expression of immune-boosting genes (*IL-10* and *IgM*) in the intestinal tissues of laying hens following QNPs administration throughout the feeding cycle, thereby reducing oxidative stress. The fundamental role of proinflammatory cytokines is their regulatory impact on acute-phase inflammation, which is linked to general and metabolic alterations [91]. Additionally, defensin are indispensable peptides, responsible for host defence mechanism against invasion of pathogenic microbes. Avian β-defensin 6 and 12 demonstrated chemotactic role and lipopolysaccharide-neutralizing impact for birds' macrophages. Furthermore, *AvBD12* evinced to be embraced in promotion of migration of murine immature dendritic cells to the site of inflammation [92]. Herein, up-regulating expression of *AvBD6* and 12 after dietary supplementation of QNPs suggested its defensive role against augmenting the host immune response. Similarly, Yang et al. [93]. , showed that using of free quercetin as have potent immunomodulatory and anti-inflammatory impact on laying hens. Dietary antioxidants can suppress pro-inflammatory secretion, thereby reducing intestinal inflammation [94]. Consistent with this, dietary polyphenolics can enhance the immune system of birds through various mechanisms: modulating the host's immune response against pathogenic microorganisms, increasing the production of anti-inflammatory cytokines to strengthen resistance to infections, and interacting with immune cell receptors to alter cell signalling pathways [95]. Furthermore, supplementation with free dietary quercetin at higher levels (0.4–0.6 g/kg) during the late laying period has been shown to increase egg production and boost immunity by modulating cytokine levels and enhancing estrogenic immunoglobulin production [55]. Autophagy is key pathway that preserves cellular homeostasis and physiological functions involving growth, reproduction [96, 97] and immunity [98]. Additionally, it assists as a cellular defence mechanism antagonistic outer harmful stimuli through protein degradation of aggregates, injured organelles, and even pathogens inside cells [99]. Defective autophagy is linked to many metabolic syndrome and inflammatory diseases [97], along with, autophagy initiation be governed by the coordination of a sequences of autophagy-correlated genes like *mTOR*, *atg5*, *atg7*, *atg12*, and *atg12* and the expression of *mTOR* gene would trigger protein synthesis and inhibit initiation of autophagy [100]. Herein, the transcriptional levels of autophagy related genes were significantly upregulated and mTOR expression was downregulated in QNPs supplemented groups. These findings supported the beneficial impact of QNPs on modifying the autophagy mechanisms in hen's body. In accordance, quercetin inducing autophagy via inhibiting of protein phosphatase 2 A activation responsible for phosphorylation *mTOR* [101] and by increasing the efflux of autophagy related genes. The mechanisms underlying the effects of QNPs may be attributed to their incorporation into an efficient nanocarrier system, which enhances their bioavailability and targeted delivery to tissues involved in regulating immune homeostasis [102, 103].

Conclusion

The current research data further supports the use of QNPs supplementation in laying hen diets. This approach is associated with their integration into an effective nano-delivery system, which shields them from adverse external and internal conditions, thereby enhancing targeted delivery to specific tissues. Additionally, QNPs protect the bioactive components of eggs from deterioration, thus improving egg quality and extending their storage time effectively. Collectively, our findings indicated that diets fortified with QNPs can maximize the benefits of flavonoids by improving laying hens' performance and

egg quality nutritive value. Additionally, QNPs protected the bioactive composition of eggs from deterioration and extended their storage time. This effect can be attributed to enhanced intestinal health, immune function, and antioxidant status in laying hens, particularly when supplemented with QNPs. Overall, the study's findings recommend the use of QNPs as a promising nutritional intervention in the laying industry to enhance egg productivity and increase their marketability by providing consumers with health-promoting nutrients.

Abbreviations

QNPs	Quercetin nano particles
BW	Body weight
BWG	Body weight gain
FI	Feed intake
FCR	Feed conversion ratio
NFEI	Net feed efficiency index
HDEP	Hen-days egg production
DM	Dry matter
CP	Crude protein
EE	Ether extract
TAG	Triglycerides
LDL	Low-density lipoprotein
VLDL	Very-low-density lipoprotein
HDL	High-density lipoprotein
SEM	Standard error of the mean
PUFAs	Polyunsaturated fatty acids
T-AOC	Total antioxidant capacity
SOD	Superoxide dismutase
GSH-Px	Glutathione peroxidase
CAT	Catalase
HO-1	Heme oxygenase-1
NQO1	NAD(P)H dehydrogenase quinone 1
ROS	Reactive oxygen species
GSH-Px	Glutathione peroxidase
SOD-1	Superoxide dismutase 1
CAT	Catalase
MDA	Malondialdehyde
GAE	Gallic acid equivalent
NO	Nitric oxide
H_2O_2	Hydrogen peroxide
AvBD	Avian β-defensin
IL-1β	Interleukin-1 beta
TNF-α	Tumour Necrosis Factor alpha
ACC	Acetyl–co A carboxylase
FAS	Fatty acid synthase gene
mTOR	Mechanistic target of rapamycin
Atg	Autophagy genes
Ig	Immunoglobulin
GAPDH	Glyceraldehyde 3-phosphate dehydrogenase
RT-qPCR	Reverse transcription-quantitative polymerase chain reaction

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Data availability
No datasets were generated or analysed during the current study.

Declarations

Ethics approval and consent to participate
ARRIVE guidelines, husbandry and rearing management for laying hens were in accordance with the Institutional Animal Care and Use Committee of the Faculty of Veterinary Medicine at Zagazig University (ZU-IACUC/2/F/62/2024). In the current study, the animals were purchased so the written informed consent from owners was not applicable. In this study, the birds were purchased so the written informed consent from owners was not applicable.

Consent for publication
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Competing interests
The authors declare no competing interests.

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