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Influence of *in Ovo* Injection of Phytochemicals and Post-Hatch Feeding on Hatching Traits, Physiological Aspects, Productive Performance, Duodenal Morphology, and Digestibility of Broiler Chickens

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Abstract

In poultry research, many phytochemicals extracted from various plants proved their nutritional and physiological functions by inhibiting stressful conditions. This study aimed to examine the effect of *in ovo* injection (IOI) of limonene (LN), cinnamaldehyde (CN) and menthol (ML) followed by post-hatch feeding method (FM), including early feeding (EF) and late feeding (LF) of broiler chicks on hatching, physiological and productive responses up to 35 days. Nine hundred eggs were used and divided into five groups, and each group had four replicates (45 eggs/replicate). One group was without IOI (negative control), whereas other groups were IOI on day 18 of embryogenesis with 100 µL of dimethyl sulfoxide or 5 mg/100 µl each of LN, CN and ML. After hatching, 760 birds were equally divided into 10 treatments of 4 replicates based on FM into EF in the hatchery or LF on the farm. A completely randomized design was conducted and the main effects of IOI×FM were analyzed in a 5×2 factorial arrangement at $P \leq 0.05$. The results showed that IOI of ML increased hatchability and chick length. LN group caused to increase body weight at hatch and villus dimensions whereas CN group shortened incubation period. All IOI groups depressed mortality and lipid hydroperoxide and elevated levels of thyroxine and nutrient digestibility. Both ML and CN groups enhanced body weight and total antioxidant capacity with lowered malondialdehyde, cholesterol, aspartate transaminase and gamma-glutamyl transferase values . Low feed intake and high catalase were obtained by both CN and LN groups. Moreover, low levels of corticosterone, glucose and creatine phosphokinase were acheived by ML and LN groups with lowered uric acid in CN group. The main effect of EF improved all determined characteristics compared with LF and each interactive treatment between IOI and FM behaves in specific manner to exhibit its desirable effect. It has been concluded that IOI of these phytochemicals could improve hatching results and their synergetic effects with post-hatch EF optimized productive performance due to multiple physiological mechanisms.

Introduction

In ovo injection (IOI) technology is increasingly recognized as one of the routine processes in pre-hatch for delivering nutritive solutions to growing avian embryos. IOI can be performed at different routes of injection inside an egg during various embryonic ages, which also depend on a sufficient dose of injected material separately or in combination (Ranjbar *et al.,* 2019; Kadam *et al.*, 2013). Many conventional supplements are given to avian embryos to improve hatchability, immune system, gut health, and antioxidant properties and prevent disease, which consequently maximize excellent chick quality and overall performance (Das *et al.*, 2021; Ebeid *et al.*, 2023). In recent years, researchers focused on *in ovo* administration of phytogenics as natural compounds

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characterized by their antioxidant, antibacterial, and anti-inflammatory activities with potential long-term impact on hatched chicks' life (Akosile *et al.*, 2023a). It is well documented that IOI of plant extract solutions (El-Kholy *et al.*, 2021; Bozbay & Göneci, 2023; Kpossou *et al.*, 2024a,b), essential oils (Oladokun *et al.*, 2021) and phytochemicals originated from various medicinal plant sources (Khaligh *et al.*, 2017; EL-Saadany *et al.*, 2019; Al-Shammari & Zamil, 2024) have proved their physiological efficiency by diverse mechanisms during embryogenesis and reflected on chick development after hatching under normal or stressful conditions.

Limonene (LN) is the major monoterpene hydrocarbon and secondary plant metabolite, which is a major component in the citrus essential oil of the Rutaceae family. LN is characterized by special aroma and flavor and exerts numerous pharmacological properties because of its antioxidant, anti-inflammatory, antimicrobial, antipyretic, analgesic, anticancer and insecticidal activities (Erasto & Viljoen, 2008). LN content ranged from 91.88% in essential oil extracted from orange peel waste (Gore *et al.*, 2024) to 95.64% in whole fruit of orange (Souza *et al.*, 2021). In broiler chickens, supplementation of various levels of citrus oils due to their high content of LN compound in diet (Erhan & Bolukbaş, 2017; Souza *et al.*, 2021; Elbaz *et al.*, 2022; Christofoli *et al.*, 2023) or drinking water (Alagbe & Ushie, 2022) led to enhanced productivity, antioxidant indices, beneficial gut microbiota, immune state, metabolism and digestive enzymes.

Cinnamaldehyde (CN) is a terpenoid and one of the bioactive volatile compounds which is commonly extracted from the bark oil of cinnamon (Lauraceae family). This compound among other phytochemicals such as cinnamyl acetate, eugenol and carvacrol, is largely found in cinnamon oils and performs very active functions that are basically related to its antioxidant, anti-inflammatory and antimicrobial efficiency with blood purifying characteristics (Saeed *et al*., 2018; Ali *et al.*, 2021). By virtue of its powerful cinnamon composition, it has demonstrated promise as an alternative to synthetic antibiotics in poultry feeding. Growth performance and physiological attributes were all increased in broilers after offering diets supported by CN through a positively direct impact on intestinal absorption, meat quality, gene regulation of intestinal inflammations (Yang *et al*., 2021) and beneficial microbiota growth (Yang *et al*., 2020). Moreover, a dietary blend of CN and vitamin C stimulated barrier function and expression of anti-inflammatory response in broilers' intestines (Huang *et al.*, 2024).

Another terpenoid constituent that has received more attention is menthol (ML), which is derived abundantly from the essential oil of peppermint leaves. Peppermint (*Mentha piperita* L.) contains 4% essential oil and ML makes up around 35-45% of its oil content (Veselin *et al.*, 2021). ML is suggested to be highly advantageous because of its biological properties as an antioxidant, anti-inflammatory, antipruritic, antimicrobial, analgesic, antitussive, anticancer antispasmodic and insecticidal substance and it is an active vehicle for pharmaceutical delivery *in vivo* (Kamatou *et al*., 2013). Abdel-Wareth & Lohakare (2023) claimed that dietary lipid compounds of peppermint consisted of 38.12% ML, 33.35% menthone and different levels of other 11 bioactive compounds and could improve productive traits, carcass criteria, meat quality and blood serum variables. In addition, ML was an effective supplement to increase fertility and digestibility coefficient in laying quail *via* enhancing the antioxidant system, intestinal microflora and metabolic functions of liver and kidney (Aly *et al.*, 2023).

The optimal management during the incubation period of embryos plays a crucial role in the subsequent development of hatchlings regarding resistance to multiple environmental and physiological stressors after hatching till farm placement. In hatchery practice, some chicks are commonly hatched earlier than their counterparts in the same hatching tray which exposes them to dehydration and delaying access to feed from 24 to 36 hours post-hatch. Moreover, manipulation of all hatched chicks during sexing, vaccination, separation, comb dubbing, debeaking, packing and transportation may extend the late feeding (LF) to undesirable extra times up to 72 hours post-hatch (Noy *et al*., 2001; Noy & Uni, 2010; Sarica *et al.*, 2014). Therefore, the use of early feeding (EF) strategy after hatching immediately chicks may accelerate growth rate and muscle development through metabolic impact on fast yolk utilization, enhancing gastrointestinal development, and modulating intestinal permeability (Hollemans *et al.*, 2020), stimulating the immune system (Madej *et al*., 2024) and inhibiting unfavourable bacterial populations of the digestive system (Li *et al*., 2022). Moreover, previous studies have indicated that EF could improve the digestibility of metabolized nutrients (Sarica & Corduk, 2013), intestinal histology, gene expression of tight junction proteins in the intestine (Li *et al*., 2022) and activate serum antioxidant capacity and blood biochemistry (Al-Shammari, 2023a) which resulted in finally improved productivity and welfare of poultry.

According to our information, there are no existing findings on poultry species in relation to the use of the phytochemicals (LN, CN and ML) as *in ovo* injected solutions to counteract the detrimental effect of LF in post-hatch. Therefore, the possibly important impacts of IOI by these natural compounds synchronized with post-hatch EF on hatching

variables, physiological response, nutrient digestibility, intestinal histomorphology and overall productive performance of hatched chicks were extensively investigated in the current experiment.

Materials and methods

Ethics approval

The guidelines of experimental procedures with respect to animal management developed by the Scientific Committee in the Department of Animal Production Techniques of the Al-Musaib Technical College, Al-Furat Al-Awsat Technical University (Babylon, Iraq) were applied precisely.

Egg incubation

The experiment utilized fertile eggs which were laid by a uniform flock of broiler breeders (Ross 308) at 41 weeks of age. Clean eggs were selected accurately based on evenly initial weight (58.23±0.57 g) and incubated under regulated temperature at 37.7 °C and relative humidity at 60-65% with the automatic turning of eggs from the 1st day till the 18th day of incubation. Thereafter, eggs were transferred from the setter to the hatcher compartment and placed in hatcher baskets at 37.0°C and 80-85% of temperature and relative humidity, respectively, until hatching day.

IOI protocol and experimental treatments

On day 18 of embryonic development, eggs were candled to determine the amniotic sac which is considered the site of IOI. The site of IOI from the blunt end direction of the individual egg was disinfected by 76% ethyl alcohol and then accurately injected using a 1 mL disposable insulin syringe (25 gauge). The IOI site was disinfected again and closed with paraffin wax using a glass stirring rod to avoid microbial contamination of eggs after the termination of IOI. Thereafter, all injected eggs were placed back quickly in the hatcher baskets to complete the required incubation period. Prior to the implementation of IOI, some eggs were utilized to prove that injected materials were taken up by the amniotic cavity through injection of phenyl blue stain as a positive marker for IOI. All eggs were exposed to uniform environmental conditions. The injected solutions were gradually warmed before injection at identical temperature degree of hatcher to avoid any probable thermal shocks to growing embryos during IOI. The antioxidant compounds of LN, CN and ML were used for IOI. These compounds were in finely powdered form (97% purity) provided by a commercial source (Naturalin Bio-Resources) and dissolved in dimethyl sulfoxide (DMSO; ≥ 99.9 purity, Sigma Aldrich) to prepare their solutions at a concentration of 5%. Totally, 900 eggs were used and assigned randomly into five groups, each group contained 180 eggs divided into four replications. 1st group was left with no IOI and treated as negative control (NC), whereas 2nd group was IOI with 100 µl of DMSO as positive control (PC). In 3rd, 4th and 5th groups, each egg received 5 mg/100 µl of LN, CN and ML solutions, respectively. After hatching, chicks were redistributed into 10 treatments based on their basic IOI groups (NC, PC, LN, CN and ML) and feeding method (FM). In total, 760 birds were used and each treatment included four replicates with 19 birds each. First part of the chicks that resulted from IOI was fed immediately post-hatch in the hatchery (early feeding, EF) whereas 2nd part of birds that resulted from IOI was fed after 24 hours post-hatch on the farm (late feeding, LF).

Birds management

Chicks were reared according to their respective treatments in the Poultry Farm of Al-Musaib Technical College for 5 weeks in floor pens under optimal environmental and hygienic requirements. Birds were offered freely balanced diets (NRC, 1994; Table 1). Temperature and humidity were adjusted according to age by providing a continuous lighting schedule.

Hatching and chick quality parameters

These parameters were determined after hatching directly in each replicate. By counting the hatched chicks and total dead embryos out of the injected fertile eggs, the percentages of hatchability and embryonic mortality were determined, respectively. Also, deformed chicks out of the healthy hatched chicks were calculated. A sensitive digital scale was used to measure the absolute body weight (BW) of hatched chicks individually, and from this value, the BW in relation to initial egg weight was taken. Ten chicks were chosen in each replicate to measure their length using a ruler. Moreover, two chicks per replicate were chosen randomly and euthanized to determine the weight of residual yolk in relation to BW and also for determination of the yolk-free BW of chicks. The hatch window is estimated in each particular replicate by calculating the hatching time elapsed between the first and last chick through checking the hatching baskets starting from 460 hours to the predicted time of hatching at 504 hours. The incubation period was recorded hourly from the commencement of incubation till hatching time. By dividing the percentage of the hatch by a number of hours during the hatch window, the hatching rate was achieved (Abioja *et al*., 2022). The eggshell conductance (G) and eggshell conductance constant (K) were both calculated as biomarkers for egg weight loss during whole incubation time based on the formulas of Christensen *et al.* (2001).

Blood collection and serum antioxidant and biochemical indices

At 5 days of age, three birds (one male and two females) in each replicate (n=12/treatment) were selected randomly for blood sampling. From the same chick, blood was sampled twice immediately, firstly from the puncture of a brachial vein and secondly from the jugular vein after slaughtering to obtain an ample amount of blood needed for analyses. Blood collections were kept in serum separator gel tubes. To separate serum, blood tubes were centrifuged for 15 minutes at 1200×*g* and then kept at –20°C until analyses.

Table 1. Diets composition and chemical analysis

Ingredient (%)	Starter (1-7 day)	Grower (8-14 days)	Finisher (15-35 days)
Soybean meal	33	29.2	24.8
Wheat	10	10	23.7
Yellow corn	47.7	51.1	40
Protein concentrate	5	5	5
Sunflower oil	2	2.8	4.6
Limestone	1.1	1	1
Dicalcium phosphate	0.7	0.5	0.5
Premix*	0.2	0.2	0.2
Sodium chloride	0.3	0.2	0.2
Total	100	100	100
Calculated nutrients			
Crude protein (%)	23.02	21.11	20.04
Metabolizable energy (kcal/kg)	3008	3096	3201
Lysine (%)	1.31	1.22	1.11
Methionine (%)	0.54	0.49	0.46
Methionine + cysteine (%)	0.88	0.83	0.78
Calcium (%)	1.05	0.9	0.85
Available phosphorus (%)	0.5	0.45	0.42

* Premix (Provimi 3110, Jordan) supplies per kilogram the following composition: 10% crude protein, 3800 kcal metabolizable energy, 4% lysine, 8.5% methionine, 8.5% methionine + cysteine, 0.55% threonine, 2.1% crude fat, 0.34% crude fiber, 20.08 % calcium, 4.8% sodium, 11% phosphorus, 20000 mg choline chloride, 3680 mg zinc, 9.2 mg selenium, 50 mg iodine, 3680 mg manganese, 2760 mg iron, 20125 IU vitamin D3, 57500 IU vitamin A, 3000 mg vitamin E, 138 mg vitamin B1, 345 mg vitamin B2, 1840 mg vitamin B3, 552 mg vitamin B5, 184 mg vitamin B6, 46 mg vitamin B9, 1000 mg vitamin B12, 138 mg vitamin K3 and 6900 g biotin.

Using a spectrophotometer (Shenzhen, China) and diagnostic kits (Sigma Aldrich) equipped with reagents and a set of calculated concentrations of standards were used for the determination of the redox indicators in serum. The method of Benzie & Strain (1996) was followed to determine total antioxidant capacity (TAC) depending on ferric reducing antioxidant power assay. Catalase (CAT) activity was estimated in accordance with Aebi (1984). Regarding malondialdehyde (MDA) and lipid hydroperoxide (LOOH) determination, the quantitative colourimetric assays using respective kits were run according to Salih *et al.* (1987) and Södergren *et al.* (1998), respectively. The slightly modified analytical method of Levine *et al.* (1990) was used to measure the protein carbonyl (PCA). Concerning the estimation of the biochemical indices, a spectrophotometric analysis using various kits (Biolabo) was used for measuring the total protein (TP), total cholesterol (TC) and triglycerides (TGs) based on steps adopted by Young (2000). Also, values of uric acid and creatinine and alkaline phosphatase (ALP) activity were determined using a commercial kit (Biolabo) according to the analytical method of Burtis & Ashwood (1999). The Cromatest kit was used to quantify the glucose content (Young, 2000). Aspartate transaminase (AST) and alanine aminotransferase (ALT) activities were calculated (Reitman & Frankel, 1957) after following analytical procedures provided by kits (Randox). The method of Tietz (1986) stated in a ready kit (Ortho-Clinical Diagnostics) was used to estimate the activity of both gamma-glutamyl transferase (GGT) and creatine phosphokinase (CPK).

Serum hormonal values

Levels of thyroid stimulating hormone (TSH), triiodothyronine (T3) and thyroxine (T4) were quantified using chicken ELISA kits (MyBioSource). Corticosterone was estimated using a specific enzyme-radioimmunoassay kit (IDS, Boldon). All hormonal analyses in serum depended on the use of an ELISA microplate reader (BK-EL10C) to record the determined absorbance in reference wavelength at a specific level in accordance with the described methods reported by the manufacturer's instructions in each accredited kit.

Apparent nutrient digestibility

On day 5, 12 chicks in treatment assigned to 3 chicks per replicate were selected and housed in 4 metabolism cages per treatment for determination of apparent nutrient digestibility. Chicks were allowed to be adapted for 3 days and subsequently, the trial measurements were implemented after five consecutive days. The quantity of feed offered, feed left over and excreta were weighed as pooled samples in replicated cages. The excreta collected in aluminium foils and dried. Determination of the approximate analysis of the dry matter, crude protein, crude fiber, ether, extract, nitrogen-free extract and crude ash in dried samples of feed and excreta was according to procedures of the AOAC (2001). The coefficient of apparent nutrient digestibility was measured using the formula reported by Nhlane *et al.* (2020).

Duodenal histomorphology

From the same euthanized birds assigned to blood collection, the histomorphological examination of the duodenum segment of the gut was carried out by taking three birds in a replicate with two sections of five readable slides per bird, namely 10 slides per bird in replicate were registered. In this analysis, the villus height (VH), villus width (VW), crypt depth (CD), villus height/crypt depth (VH/CD) and villus surface area (VSA) were measured following the conventionally described method of Rubio *et al*. (2010) and using a microtome (Leica RM 2155) and an optical microscope (DELTA).

Productive performance

The productive parameters were registered weekly and estimated cumulatively from 1 to 3 and 6 weeks of age. BW and feed intake (FI) were taken and from these variables, a feed conversion ratio (FCR) was obtained by monitoring the mortality percentage. Also, by dividing weight gain values by amounts of consumed metabolizable energy and crude protein, the energy efficiency ratio (EER) and protein efficiency ratio (PER) were estimated, respectively. The production efficiency factor (PEF) was calculated according to the formula of Lemme *et al.* (2006). At five weeks of age, one male and two females per replicate (n=12/treatment) were chosen, weighed, euthanized and eviscerated to record the relative weights of abdominal fat and carcass yield without or with edible organs (heart, liver, gizzard) in relation to BW. The relative weights of carcass parts (breast, leg and secondary carcass parts involving wings, back and neck) in relation to eviscerated carcasses were also obtained.

Statistical analysis

The data were arranged in a completely randomized design, subjected to the 1-way ANOVA and analyzed by the general linear model using SAS software (SAS, 2012) to find the main effect of IOI factor (NC, PC, LN, CD and ML) and the main effect of FM factor (EF and LF) and their interactive treatments (5×2) on observed variables. The probability level of ($P \leq 0.05$) was tested to verify the significant differences and comparisons among treatment means based on the Duncan multiple range test (Duncan, 1955) by the following statistical model for hatching performance and chick quality results: $Yij = \mu + t_i + e_{ij}$, where Y_{ij}: observation value, μ: overall mean, t_i: effect of IOI, and e_{ij}: random error. For the rest of results, the following statistical model was applied: $Y_{ijk} = \mu + IOI_i + FM_i + (IOI \times FM)_{ij} + e_{ijk}$, where Y_{ijk}: observation value, μ: overall mean, IOI_i: effect of IOI (j=5), FM_j: effect of FM (i=2), $(IOI \times FM)_{ij}$: interaction between IOI and FM (10 treatments), and e_{ijK}: random error.

Results

Hatching parameters and chick quality

Table 2 shows that high hatchability and low embryonic mortality ($P \leq 0.05$) were obtained by ML compared with NC. No significant differences among groups in hatching rate and K value. CN and PC shortened ($P \leq 0.05$) incubation period, whereas shortening ($P \leq 0.05$) in the hatch window was only in favor of CN. LN reduced ($P \leq 0.05$) G value. Table 3 indicated that LN increased ($P \leq 0.05$) absolute and relative BW at hatch and yolk-free BW at hatch with decreasing ($P \leq 0.05$) in deformed chicks and residual yolk in comparison to NC. ML increased ($P \leq 0.05$) chick length compared with NC.

Table 2. Effect of *in ovo* injection of phytochemicals on hatching performance of broiler chickens

Groups	Hatchability(%)	Embryonic mortality(%)	Hatching rate(% /h)	Incubation period (h)	Hatch window(h)	G(mg/d/ mm/Hg)	K
NC	91.67^{bc}	8.33^{ab}	4.34	501.05^{a}	21.14^{a}	14.75^{a}	5.29^{ab}
PC	91.11^{bc}	8.89^{a}	4.37	494.27^{b}	20.87^{ab}	15.19^{a}	5.38^{ab}
LN	92.52^{b}	7.47^{b}	4.46	499.04^{a}	20.73^{ab}	12.78^{b}	4.55^{b}
CN	90.56^{c}	9.44^{a}	4.53	492.00^{b}	19.93^{b}	15.78^{a}	5.56^{a}
ML	95.56^{a}	4.44^{c}	4.56	495.23^{ab}	20.97^{ab}	14.16^{ab}	5.02^{ab}
SEM	8.53	2.76	1.54	25.76	6.11	4.65	0.99
P-value	0.038	0.019	0.176	0.035	0.022	0.042	0.030

NC: negative control, PC: positive control, LN: limonene, CN: cinnamaldehyde, ML: menthol, G: eggshell conductance, K: eggshell conductance constant. Means within columns with different superscripts (a-c) differ significantly at $P \leq 0.05$. *SEM*: standard error mean.

Table 3. Effect of *in ovo* injection of phytochemicals on hatched chick quality

Groups	BW at hatch (g)	Yolk-free BW at hatch (g)	BW at hatch (%)	Chick length (mm)	Deformed chicks (%)	Residual yolk (%)
NC	43.50^{bc}	38.08^{bc}	74.70^{bc}	176.93^{bc}	1.79^{a}	12.47^{a}
PC	42.75^{c}	37.52^{c}	73.42^{c}	175.40^{c}	1.24^{ab}	12.24^{a}
LN	45.26^{a}	40.55^{a}	77.71^{a}	179.98^{ab}	0.00^{b}	10.38^{b}
CN	43.25^{bc}	38.09^{abc}	74.27^{bc}	177.45^{b}	1.23^{ab}	11.93^{a}
ML	44.27^{ab}	39.15^{ab}	75.99^{b}	180.53^{a}	1.16^{ab}	11.62^{ab}
SEM	7.47	4.85	10.83	21.54	0.004	2.64
P-value	0.044	0.050	0.019	0.032	0.017	0.048

NC: negative control, PC: positive control, LN: limonene, CN: cinnamaldehyde, ML: menthol, BW: body weight. Means within columns with different superscripts (a-c) differ significantly at $P \leq 0.05$. *SEM*: standard error mean.

Serum antioxidant activity and biochemistry

In Table 4, high and low values ($P \leq 0.05$) of serum TAC and MDA, respectively, were obtained by the main effect of CN and ML compared with NC. LN and CN increased ($P \leq 0.05$) CAT level whereas all IOI groups reduced ($P \leq 0.05$) LOOH value with no effect among them in PCA level. Compared with LF, EF increased TAC and decreased MDA and LOOH ($P \leq 0.05$) with no effect on CAT and PCA. Significant differences among interactive treatments (IOI×FM) in all parameters were obtained depending on the group.

Table 5 shows that LN and ML did not differ from NC in serum TP. Lowering ($P \leq 0.05$) in serum TC, AST and GGT was in CN and ML compared with NC. Decreased levels of TGs and uric acid were in CN in comparison to NC. LN and ML reduced ($P \leq 0.05$) glucose and CPK values. Absence of significance regarding creatinine level with inhibited activity of ALT and ALP ($P \leq 0.05$) for LN. In comparison to LF, values of TC, TGs, glucose, uric acid, AST, ALT, ALP, GGT and CPK were reduced by EF. Noticeable differences ($P \leq 0.05$) in these measurements were presented in interactions and a more positive influence was for IOI with EF in comparison to IOI with LF.

Table 4. Effect of *in ovo* injection of phytochemicals and feeding method on serum antioxidant biomarkers of broiler chickens.

Groups	TAC (U/mL)	CAT (U/mL)	MDA (µmol/L)	LOOH (µmol/L)	PCA (nmol/mg protein)
In ovo injection (IOI)					
NC	6.79^{b}	3.55^{b}	9.63^{a}	8.99^{a}	4.71
PC	7.32^{b}	3.88^{b}	8.71^{a}	7.66^{ab}	4.20
LN	7.83^{ab}	4.21^{a}	8.69^{ab}	6.69^{bc}	4.52
CN	8.29^{a}	4.27^{a}	7.51^{b}	5.98^{c}	4.44
ML	9.05^{a}	4.04^{ab}	8.33^{b}	6.89^{bc}	4.52
Feeding method (FM)					
EF	8.11^{a}	4.01	7.77^{b}	6.96^{b}	4.28
LF	7.59^{b}	3.97	9.38^{a}	7.52^{a}	4.67
IOI × FM					
NC × EF	7.23^{bc}	4.10^{a}	8.75^{ab}	8.43^{ab}	5.26^{a}
PC × EF	7.52^{b}	3.53^{ab}	8.05^{b}	6.87^{bc}	4.17^{ab}
LN × EF	7.73^{b}	3.76^{ab}	7.15^{c}	5.85^{cd}	4.04^{ab}
CN × EF	8.38^{ab}	3.75^{ab}	7.18^{c}	6.43^{bcd}	3.84^{b}
ML × EF	9.66^{a}	4.88^{a}	7.73^{bc}	7.22^{b}	4.07^{ab}
NC × LF	6.34^{c}	2.99^{b}	10.51^{a}	9.54^{a}	4.14^{ab}
PC × LF	7.11^{bc}	4.23^{a}	9.37^{a}	8.44^{ab}	4.23^{ab}
LN × LF	7.92^{b}	4.65^{a}	10.24^{a}	7.55^{b}	5.00^{a}
CN × LF	8.21^{ab}	4.77^{a}	7.84^{bc}	5.53^{d}	5.03^{a}
ML × LF	8.43^{a}	3.19^{ab}	8.93^{ab}	6.56^{bc}	4.97^{a}
SEM	2.77	1.82	4.42	2.65	0.89
P-value					
IOI	0.039	0.048	0.030	0.018	0.097
FM	0.013	0.073	0.022	0.042	0.111
IOI × FM	0.046	0.029	0.035	0.042	0.021

NC: negative control, PC: positive control, LN: limonene, CN: cinnamaldehyde, ML: menthol, EF: early feeding, LF: late feeding, TAC: total antioxidant capacity, CAT: catalase, MDA: malondialdehyde, LOOH: lipid hydroperoxide, PCA: protein carbonyl. Means within columns with different superscripts (a-d) differ significantly at $P \leq 0.05$. *SEM*: standard error mean.

Hormonal values

Based on data exhibited in Table 6, lack of significance for the main effect of IOI and FM groups and their interactions in serum TSH and T3 contents. All IOI groups elevated ($P \leq 0.05$) serum T4 compared with NC. Reduced level ($P \leq 0.05$) of corticosterone was recorded by ML and LN in comparison to NC.

Table 5. Influence of *in ovo* injection of phytochemicals and feeding method on serum biochemistry of broiler chickens

Groups	TP (g/dL)	TC (mg/dL)	TGs (mg/dL)	Glucose (mg/dL)	Uric acid (mg/dL)	Creatinine (mgL/dL)	AST (U/L)	ALT (U/L)	ALP (U/L)	GGT (U/L)	CPK (U/L)
In ovo injection (IOI)											
NC	2.69^{a}	111.39^{a}	59.87^{a}	130.61^{a}	4.02^{a}	0.49	129.51^{ab}	7.39^{a}	79.05^{ab}	6.54^{a}	5488.88^{a}
PC	2.46^{b}	112.71^{a}	61.99^{a}	128.43^{ab}	3.98^{ab}	0.48	130.89^{a}	7.61^{a}	80.88^{a}	6.27^{a}	5495.33^{a}
LN	2.55^{ab}	110.35^{ab}	60.13^{a}	125.98^{c}	4.01^{a}	0.46	129.42^{b}	6.35^{b}	76.21^{c}	6.19^{ab}	5305.33^{b}
CN	2.24^{b}	108.35^{b}	57.44^{b}	129.73^{a}	3.86^{b}	0.41	126.59^{c}	6.78^{ab}	80.43^{a}	5.88^{b}	5427.78^{a}
ML	2.82^{a}	107.74^{b}	59.32^{ab}	127.53^{bc}	4.22^{a}	0.48	123.49^{d}	7.62^{a}	78.94^{b}	5.89^{b}	5210.43^{c}
Feeding method (FM)											
EF	2.59	108.90^{b}	55.27^{b}	126.01^{b}	3.69^{b}	0.45	124.39^{b}	6.51^{b}	75.94^{b}	5.08^{b}	5188.97^{b}
LF	2.51	111.30^{a}	64.23^{a}	130.89^{a}	4.35^{a}	0.48	131.57^{a}	7.79^{a}	82.26^{a}	7.22^{a}	5582.13^{a}
IOI × FM											
NC × EF	2.54^{ab}	110.34^{b}	52.76^{e}	126.76^{cd}	3.59^{b}	0.49^{a}	121.26^{e}	6.34^{bc}	74.34^{de}	5.65^{b}	5254.21^{c}
PC × EF	2.43^{ab}	112.16^{a}	58.65^{c}	127.62^{bc}	3.58^{b}	0.47^{ab}	125.45^{d}	7.23^{a}	78.87^{c}	4.99^{c}	5236.43^{cd}
LN × EF	2.72^{a}	109.27^{bc}	54.28^{de}	124.52^{e}	3.56^{b}	0.44^{ab}	127.39^{c}	5.87^{c}	72.54^{e}	5.48^{bc}	5123.54^{de}
CN × EF	2.35^{b}	107.47^{cd}	54.89^{d}	125.62^{d}	3.72^{ab}	0.38^{b}	128.53^{c}	6.35^{b}	76.53^{d}	4.51^{c}	5243.23^{c}
ML × EF	2.91^{a}	105.28^{d}	55.76^{d}	125.52^{de}	3.99^{a}	0.45^{ab}	119.34^{f}	6.75^{b}	77.43^{cd}	4.79^{c}	5087.43^{e}
NC × LF	2.83^{a}	112.43^{a}	66.98^{a}	134.45^{a}	4.45^{a}	0.48^{a}	137.76^{a}	8.43^{a}	83.76^{a}	7.43^{a}	5723.54^{a}
PC × LF	2.49^{ab}	113.26^{a}	65.34^{a}	129.23^{b}	4.14^{a}	0.49^{a}	136.34^{a}	7.98^{a}	82.88^{ab}	7.54^{a}	5754.23^{a}
LN × LF	2.37^{b}	111.42^{ab}	65.98^{a}	127.43^{bc}	4.47^{a}	0.47^{ab}	131.45^{b}	6.83^{b}	79.87^{c}	6.89^{ab}	5487.11^{b}
CN × LF	2.13^{b}	109.22^{c}	59.98^{c}	133.83^{a}	4.23^{a}	0.46^{ab}	124.65^{d}	7.22^{ab}	84.33^{a}	7.26^{a}	5612.32^{ab}
ML× LF	2.72^{a}	110.19^{bc}	62.87^{b}	129.53^{b}	4.44^{a}	0.50^{a}	127.65^{c}	8.49^{a}	80.45^{bc}	6.99^{a}	5333.43^{bc}
SEM	0.36	32.39	17.65	6.87	1.54	0.00	42.23	2.65	8.87	3.22	123.76
P-value											
IOI	0.027	0.023	0.034	0.039	0.018	2.107	0.031	0.042	0.029	0.038	0.030
FM	0.089	0.035	0.042	0.030	0.049	1.623	0.038	0.048	0.018	0.025	0.041
IOI × FM	1.021	0.026	0.019	0.050	0.042	0.013	0.029	0.039	0.022	0.044	0.029

NC: negative control, PC: positive control, LN: limonene, CN: cinnamaldehyde, ML: menthol, EF: early feeding, LF: late feeding, TP: total protein, TC: total cholesterol, TGs: triglycerides, AST: aspartate transaminase, ALT: alanine aminotransferase, ALP: alkaline phosphatase, GGT: gamma-glutamyl transferase, CPK: creatine phosphokinase. Means within columns with different superscripts (a-f) differ significantly at $P \leq 0.05$. *SEM*: standard error mean.

Moreover, EF presented high T4 and low corticosterone ($P \leq 0.05$) in serum compared with LF. Higher T4 and lower corticosterone values ($P \leq 0.05$) were more obvious in IOI×EF than IOI×LF (interactions).

Duodenal histology

Table 7 shows that LN increased VH and VW ($P \leq 0.05$) with reduced CD ($P \leq 0.05$) by CN, ML and PC compared with NC. No differences among IOI groups in VH/CD with increased VSA ($P \leq 0.05$) by LN and CN. EF presented an improvement ($P \leq 0.05$) in all these parameters compared with LF. Additionally, there were high differences ($P \leq 0.05$) in interactions between IOI and EF compared with IOI and LF.

Nutrient digestibility

In comparison to NC, ML improved ($P \leq 0.05$) dry matter digestibility. CN improved crude protein and crude ash digestibility. The digestibility of crude fiber was increased ($P \leq 0.05$) by both LN and ML. IOI groups improved ($P \leq 0.05$) ether extract digestibility with increasing ($P \leq 0.05$) nitrogen-free extract digestibility by CN and ML. EF caused to improvement in all nutrient digestibility compared with LF. Better significant differences in these traits were also reported for interactive treatments of IOI with EF compared with IOI with LF (Table 8).

Productive response and carcass criteria

Table 9 showed that improved BW ($P \leq 0.05$) was noticeable in LN at 3 weeks and in CN and ML at 5 weeks. Reduced amount of FI ($P \leq 0.05$) was in PC at 1-3 weeks and a high amount of FI was in LN and CN at 1-5 weeks. Superiority in FCR ($P \leq 0.05$) was obtained for LN at 1-3 weeks. No significance was observed among the main effect of IOI groups in PER. LN and ML provided better EER at 1-3 weeks with no change in this trait among CN, ML, PC and NC at 1-5 weeks. IOI groups reduced mortality compared with NC. EF increased BW and FI and reduced mortality in all periods with an increase in PER and EER at 1-3 weeks ($P \leq 0.05$) compared with LF. An improvement for IOI×EF *vs.* IOI×LF was observed in these parameters.

Table 10 indicated that there were no differences among the main effects of IOI groups and NC in carcass yields. There was an absence of significance among IOI groups with regard to relative weights of breast, legs, and abdominal fat. ML registered high PEF and low secondary carcass parts ($P \leq 0.05$) compared with NC. The main effect of FM did not show any differences in carcass quality; however, EF increased PEF in comparison to LF. Moreover, there were higher values in most of the traits among interactive treatments of IOI×EF than IOI×LF.

Table 6. Effect of *in ovo* injection of phytochemicals and feeding method on serum hormone values of broiler chickens

Groups	TSH (ng/mL)	T3 (ng/mL)	T4 (ng/mL)	Corticosterone (ng/mL)
In ovo injection (IOI)				
NC	0.06	1.29	69.89^{d}	9.39^{a}
PC	0.06	1.28	67.74^{d}	8.95^{ab}
LN	0.06	1.19	78.03^{b}	7.83^{bc}
CN	0.08	1.25	75.37^{c}	8.75^{ab}
ML	0.07	1.37	81.09^{a}	7.05^{c}
Feeding method (FM)				
EF	0.08	1.33	76.49^{a}	7.51^{b}
LF	0.05	1.22	72.35^{b}	9.28^{a}
IOI × FM				
NC × EF	0.08	1.32	70.43^{e}	8.34^{bc}
PC × EF	0.06	1.33	68.52^{ef}	8.35^{bc}
LN × EF	0.07	1.25	80.34^{b}	6.32^{d}
CN × EF	0.09	1.33	79.62^{bc}	7.65^{cd}
ML × EF	0.08	1.41	83.54^{a}	6.87^{d}
NC × LF	0.04	1.25	69.34^{ef}	10.45^{a}
PC × LF	0.05	1.23	66.95^{f}	9.54^{a}
LN × LF	0.05	1.14	75.72^{d}	9.34^{ab}
CN × LF	0.06	1.17	71.11^{e}	9.84^{a}
ML × LF	0.06	1.32	78.65^{c}	7.22^{cd}
SEM	0.00	0.13	7.54	1.86
P-value				
IOI	0.069	0.112	0.015	0.031
FM	0.354	0.363	0.034	0.019
IOI×FM	0.417	0.432	0.027	0.044

NC: negative control, PC: positive control, LN: limonene; CN: cinnamaldehyde, ML: menthol, EF: early feeding, LF: late feeding, TSH: thyroid stimulating hormone, T3: triiodothyronine, T4: thyroxine. Means within columns with different superscripts (a-f) differ significantly at $P \leq 0.05$. *SEM*: standard error mean.

Table 7. Effect of *in ovo* injection of phytochemicals and feeding method on duodenal histomorphology of broiler chickens

Groups	VH (μm)	VW (μm)	CD (μm)	VH/CD	VSA ($\times 10^3$ μm^2)
In ovo injection (IOI)					
NC	859.77^{b}	150.92^{b}	162.98^{a}	5.29	408.94^{c}
PC	839.58^{c}	144.81^{c}	155.36^{bc}	5.43	383.66^{d}
LN	892.16^{a}	158.86^{a}	161.16^{a}	5.54	446.49^{a}
CN	885.95^{ab}	150.49^{b}	154.28^{c}	5.76	421.52^{b}
ML	855.57^{bc}	148.78^{bc}	157.12^{b}	5.45	400.48^{cd}
Feeding method (FM)					
EF	924.09^{a}	158.93^{a}	153.97^{b}	6.00^{a}	461.74^{a}
LF	809.11^{b}	142.60^{b}	162.38^{a}	4.97^{b}	362.69^{b}
IOI × FM					
NC × EF	924.12^{b}	157.41^{b}	156.54^{d}	5.90^{ab}	456.99^{b}
PC × EF	891.65^{c}	155.24^{bc}	148.36^{e}	6.01^{a}	434.86^{c}
LN × EF	955.11^{a}	165.42^{a}	160.67^{bc}	5.95^{a}	496.35^{a}
CN × EF	965.13^{a}	161.24^{a}	150.24^{de}	6.42^{a}	488.89^{a}
ML × EF	884.48^{c}	155.32^{bc}	154.04^{de}	5.74^{ab}	431.58^{c}
NC × LF	795.42^{ef}	144.42^{d}	169.41^{a}	4.69^{b}	360.89^{e}
PC × LF	787.51^{f}	134.38^{e}	162.36^{b}	4.85^{b}	332.46^{f}
LN × LF	829.21^{d}	152.25^{c}	161.65^{bc}	5.13^{ab}	396.62^{d}
CN × LF	806.76^{e}	139.73^{de}	158.31^{cd}	5.09^{ab}	354.15^{e}
ML × LF	826.65^{d}	142.23^{d}	160.19^{bc}	5.16^{ab}	369.37^{e}
SEM	52.52	24.18	15.82	2.50	72.01
P-value					
IOI	0.039	0.047	0.028	0.088	0.041
FM	0.030	0.026	0.034	0.021	0.019
IOI × FM	0.016	0.042	0.023	0.035	0.032

NC: negative control, PC: positive control, LN: limonene; CN: cinnamaldehyde, ML: menthol, EF: early feeding, LF: late feeding, VH: villus height, VW: villus width, CD: crypt depth, VH/CD: villus height/crypt depth, VSA: villus surface area. Means within columns with different superscript (a-f) differ significantly at $P \leq 0.05$. *SEM*: standard error mean.

Table 8. Effect of *in ovo* injection of phytochemicals and feeding method on apparent nutrient digestibility (%) of broiler chickens

Groups	Dry matter	Crude protein	Crude fiber	Ether extract	Nitrogen free extract	Crude ash
In ovo injection (IOI)						
NC	78.59^{b}	73.40^{b}	54.87^{c}	77.54^{c}	86.43^{b}	70.14^{b}
PC	77.08^{b}	74.20^{ab}	54.49^{c}	79.59^{bc}	85.31^{b}	70.69^{ab}
LN	79.03^{ab}	74.75^{ab}	59.61^{a}	81.41^{ab}	87.00^{ab}	71.67^{ab}
CN	78.81^{ab}	75.87^{a}	56.03^{bc}	82.85^{a}	87.44^{a}	73.08^{a}
ML	80.86^{a}	73.53^{b}	56.73^{b}	83.15^{a}	89.48^{a}	71.75^{ab}
Feeding method (FM)						
EF	80.06^{a}	75.38^{a}	58.37^{a}	82.40^{a}	87.99^{a}	72.32^{a}
LF	77.68^{b}	73.32^{b}	54.32^{b}	79.41^{b}	86.27^{b}	70.61^{b}
IOI × FM						
NC × EF	80.34^{b}	73.31^{c}	57.86^{b}	82.32^{ab}	88.38^{a}	71.52^{b}
PC × EF	77.84^{c}	76.53^{ab}	56.45^{bc}	79.34^{c}	86.23^{bc}	71.39^{b}
LN × EF	79.43^{b}	75.52^{b}	60.24^{a}	82.43^{ab}	87.24^{b}	73.58^{a}
CN × EF	80.24^{b}	77.35^{a}	57.48^{b}	84.26^{a}	88.23^{ab}	74.48^{a}
ML × EF	82.45^{a}	74.17^{b}	59.81^{a}	83.65^{a}	89.87^{a}	70.63^{bc}
NC × LF	76.83^{d}	73.49^{c}	51.87^{d}	72.76^{d}	84.47^{c}	68.76^{c}
PC × LF	76.32^{d}	71.87^{d}	52.54^{d}	79.83^{bc}	84.38^{c}	69.98^{c}
LN × LF	78.63^{bc}	73.98^{bc}	58.98^{ab}	80.39^{bc}	86.76^{b}	69.76^{c}
CN × LF	77.37^{cd}	74.39^{b}	54.57^{c}	81.43^{b}	86.65^{b}	71.68^{b}
ML × LF	79.26^{b}	72.89^{cd}	53.64^{cd}	82.65^{ab}	89.09^{a}	72.87^{ab}
SEM	25.76	17.36	8.99	10.54	20.27	13.52
P-value						
IOI	0.043	0.039	0.040	0.021	0.032	0.046
FM	0.050	0.045	0.027	0.039	0.031	0.020
IOI × FM	0.038	0.049	0.017	0.032	0.016	0.045

NC: negative control, PC: positive control, LN: limonene; CN: cinnamaldehyde, ML: menthol, EF: early feeding, LF: late feeding. Means within columns with different superscripts (a-d) differ significantly at $P \leq 0.05$. *SEM*: standard error mean.

Table 9. Effect of *in ovo* injection of phytochemicals and feeding method on productive traits of broiler chickens

Groups	BW (g)		FI (g)		FCR (g/g)		PER (g/g)		EER (g/100 kcal)		Mortality (%)
	3 weeks	5 weeks	1-3 weeks	1-5 weeks	1-3 weeks	1-5 weeks	1-3 weeks	1-5 weeks	1-3 weeks	1-5 weeks	1-5 weeks
In ovo injection (IOI)											
NC	760.66^{bc}	2061.14^{bc}	945.09^{a}	3318.98^{bc}	1.32^{a}	1.65^{ab}	3.55	2.84	24.46^{c}	19.59^{a}	5.47^{a}
PC	743.34^{c}	1950.70^{c}	888.98^{b}	3241.34^{c}	1.27^{a}	1.70^{ab}	3.68	2.75	25.38^{b}	18.96^{ab}	4.69^{a}
LN	840.04^{a}	2113.41^{ab}	923.14^{a}	3575.13^{a}	1.16^{b}	1.73^{a}	4.02	2.70	27.71^{a}	18.64^{b}	2.34^{b}
CN	751.65^{bc}	2127.90^{a}	915.39^{ab}	3515.05^{a}	1.29^{a}	1.69^{ab}	3.63	2.78	25.01^{bc}	19.12^{a}	0.00^{c}
ML	798.57^{ab}	2160.09^{a}	912.11^{ab}	3370.85^{ab}	1.22^{ab}	1.59^{b}	3.86	2.93	26.61^{ab}	20.23^{a}	0.78^{c}
Feeding method (FM)											
EF	814.25^{a}	2147.94^{a}	925.45^{a}	3446.63^{a}	1.21	1.64	3.89^{a}	2.85	26.82^{a}	19.68	1.56^{b}
LF	743.45^{b}	2017.36^{b}	908.43^{b}	3361.91^{b}	1.30	1.70	3.60^{b}	2.74	24.84^{b}	18.93	3.75^{a}
IOI × FM											
NC × EF	763.32^{b}	2134.63^{b}	955.73^{a}	3392.43^{b}	1.33^{a}	1.62^{b}	3.52^{b}	2.88	24.28^{bc}	19.87^{a}	4.68^{ab}
PC × EF	754.42^{bc}	2004.54^{c}	895.52^{ab}	3283.43^{cd}	1.26^{ab}	1.67^{a}	3.71^{ab}	2.79	25.59^{b}	19.25^{a}	3.12^{bc}
LN × EF	895.51^{a}	2225.38^{a}	953.83^{a}	3612.48^{a}	1.12^{b}	1.66^{ab}	4.17^{a}	2.82	28.72^{a}	19.45^{a}	0.00^{d}
CN × EF	791.43^{ab}	2156.38^{ab}	892.43^{b}	3532.47^{a}	1.19^{b}	1.67^{a}	3.92^{a}	2.79	27.04^{a}	19.29^{a}	0.00^{d}
ML × EF	866.57^{a}	2218.76^{a}	929.73^{a}	3412.32^{b}	1.13^{b}	1.57^{b}	4.13^{a}	2.98	28.50^{a}	20.54^{a}	0.00^{d}
NC × LF	757.99^{b}	1987.65^{cd}	934.45^{a}	3245.52^{cd}	1.31^{a}	1.67^{a}	3.57^{b}	2.80	24.65^{b}	19.31^{a}	6.25^{a}
PC × LF	732.26^{c}	1896.87^{d}	882.43^{b}	3199.24^{d}	1.28^{ab}	1.73^{a}	3.65^{ab}	2.71	25.17^{b}	18.68^{bc}	6.25^{a}
LN × LF	784.56^{b}	2001.43^{c}	892.44^{b}	3537.77^{a}	1.21^{b}	1.78^{a}	3.87^{a}	2.58	26.69^{ab}	17.82^{c}	4.69^{ab}
CN × LF	711.87^{c}	2099.42^{c}	938.36^{a}	3497.62^{ab}	1.40^{a}	1.70^{a}	3.33^{b}	2.75	22.98^{c}	18.95^{abc}	0.00^{d}
ML × LF	730.56^{c}	2101.43^{bc}	894.48^{b}	3329.38^{bc}	1.30^{a}	1.62^{b}	3.59^{ab}	2.89	24.72^{b}	19.91^{a}	1.56^{cd}
SEM	67.39	111.29	87.54	125.43	0.02	0.09	0.76	0.43	5.66	6.28	0.14
P-value											
IOI	0.016	0.031	0.028	0.037	0.030	0.028	0.087	0.206	0.043	0.034	0.021
FM	0.041	0.040	0.033	0.022	0.062	0.196	0.017	0.165	0.026	0.203	0.032
IOI × FM	0.019	0.038	0.041	0.049	0.023	0.035	0.030	0.099	0.029	0.017	0.046

NC: negative control, PC: positive control, LN: limonene, CN: cinnamaldehyde, ML: menthol, EF: early feeding, LF: late feeding, BW: body weight, FI: feed intake, FCR: feed conversion ratio, PER: protein efficiency ratio, EER: energy efficiency ratio. Means within columns with different superscripts (a-d) differ significantly at $P \leq 0.05$. *SEM*: standard error mean.

Table 10. Effect of *in ovo* injection of phytochemicals and feeding method on carcass quality and production efficiency factor of broiler chicken

Groups	Carcass yield (%)*	Carcass yield(%)**	Breast (%)	Legs (%)	Secondary carcass parts (%)	Abdominal fat (%)	PEF
In ovo injection (IOI)							
NC	74.29^{ab}	79.22^{ab}	38.39	32.41	29.19^{a}	0.42	338.62^{bc}
PC	73.01^{b}	77.75^{b}	39.34	31.82	28.84^{ab}	0.40	312.89^{c}
LN	74.43^{ab}	79.47^{a}	38.41	32.92	28.56^{ab}	0.39	342.51^{b}
CN	74.83^{a}	79.89^{a}	38.55	32.68	28.61^{ab}	0.44	360.68^{ab}
ML	74.53^{a}	79.73^{a}	39.21	32.71	27.96^{b}	0.11	384.54^{a}
Feeding method (FM)							
EF	74.74	79.88	38.78	32.70	28.54	0.31	369.19 ^{a}
LF	73.69	78.54	38.77	32.31	28.72	0.39	326.50 ^{b}
IOI × FM							
NC × EF	74.64^{a}	79.69^{a}	38.41	32.54^{ab}	29.04^{ab}	0.33	358.32^{b}
PC × EF	73.48^{b}	78.41^{bc}	39.14	32.07^{ab}	28.79^{ab}	0.41	331.42^{bc}
LN × EF	74.75^{a}	79.91^{a}	38.42	33.10^{a}	28.53^{ab}	0.35	383.67^{ab}
CN × EF	75.44^{a}	80.75^{a}	38.67	32.89^{a}	28.51^{ab}	0.43	368.64^{b}
ML × EF	75.38^{a}	80.64^{a}	39.27	32.92^{a}	27.85^{b}	0.03	403.92^{a}
NC × LF	73.95^{ab}	78.75^{b}	38.38	32.28^{ab}	29.33^{a}	0.51	318.93^{c}
PC × LF	72.53^{b}	77.09^{c}	39.53	31.57^{b}	28.89^{ab}	0.39	294.38^{d}
LN × LF	74.11^{ab}	79.02^{ab}	38.39	32.73^{a}	28.59^{ab}	0.42	301.33^{cd}
CN × LF	74.22^{a}	79.04^{ab}	38.42	32.47^{ab}	28.71^{ab}	0.45	352.71^{b}
ML × LF	73.67^{ab}	78.81^{b}	39.14	32.50^{ab}	28.06^{b}	0.18	365.14^{b}
SEM	6.73	10.54	12.94	8.99	9.43	0.09	28.98
P-value							
IOI	0.023	0.043	0.316	0.439	0.040	0.218	0.033
FM	0.211	0.444	0.212	0.099	0.326	0.084	0.026
IOI × FM	0.047	0.030	0.111	0.041	0.037	0.764	0.042

NC: negative control, PC: positive control, LN: limonene; CN: cinnamaldehyde, ML: menthol, EF: early feeding, LF: late feeding, PEF: production efficiency factor. Means within columns with different superscripts (a-d) differ significantly at $P \leq 0.05$. *SEM*: standard error mean. * without giblets, ** with giblets.

Discussion

IOI of ML solution supported the hatching of embryos by increasing hatchability and decreasing mortality, which might be associated with maintaining the energy reserve and reducing the oxidative stress that is crucial during the last phase of embryonic development (Ebeid *et al*., 2023). It, therefore, seems that the growing embryo markedly benefited from exogenous ML. It was reported that day 18 of embryogenesis is the best time for embryo feeding through IOI because the metabolic ability to consume amniotic fluid is accompanied by the beginning of pulmonary respiration and high assembly of pectoral and hepatic glycogen stores, and glycogenolysis happens around that time (Kadam *et al.*, 2013). In general, data with respect to the IOI of our extracts are unfortunately missing. However, similarly, Ranjbar *et al.* (2019) indicated that IOI of naringin at 15 mg/egg into the amnion sac on the 17.5th day of embryonic age increased hatchability and decreased mortality. Akosile *et al.* (2023b) observed that high and low hatchability were induced by IOI in air cells of 0.5 mL of distilled water containing 2 and 4 mg cinnamon, respectively, on day 17.5 of incubation. The short time of incubation period and hatch window induced by CN might be related to the high metabolic rate of embryos through swallowing the amniotic fluid content after IOI of this solution. Accordingly, IOI of 0.5 µg/mL of *Manihot esculenta* leaf extract in an air chamber at 18 days of incubation led to a lower total duration of incubation and improved chick quality with no changed hatchability and mortality (Ngueda *et al.*, 2021). Decreasing G and K values for the LN group is a good indicator for reducing unfavorable water loss inside the egg through the eggshell during incubation. Both these values basically depend on initial weight of the egg, final weight of the hatchling and the incubation period (Christensen *et al.,* 2001). Consequently, this would explain the superior absolute and relative BW and yolk-free BW at the hatch for the LN group (Table 3). Moreover, the reason behind the increased weight of hatchlings in LN is due to declined amounts of residual yolk. A low remaining yolk is speculated to be linked to trigger yolk utilization and energy assimilation. Therefore, the nutritive value in the residual yolk is suggested to reinforce the chick's livability during LF after hatching (Ngueda *et al.*, 2021; Das *et al.*, 2021). Compatible results were achieved by Kpossou *et al.*

(2024a), who found that relative and absolute BW of hatched chicks were increased by IOI of 0.5µg/mL of *Citrus aurantiifolia* seeds containing a moderate amount of limonins at a dose of 0.2 mL per egg in air chamber on day 18 of incubation. In a study of IOI on the 18th day of incubation with 0, 3, 9, and 12 mg/egg each of cinnamon, anise and ginger, Bozbay & Göneci (2023) confirmed that no main effect of IOI on chick weight, but high hatchability and chick quality and low pipped chicks were all achieved by IOI of cinnamon and ginger. Decreased relative deformed chicks in LN might attributed that the essential oils of oranges are considered an effective compound to enhance bone characteristics by specific metabolic mechanisms because of their lipophilic properties in the bloodstream (Souza *et al.*, 2021) with multiple therapeutic benefits that support skeletal system development (Erasto & Viljoen, 2008). Similar to EL-Saadany *et al.* (2019), high activity and low malformation of chicks which correlated with high hatchability and chick length, were all induced through IOI of 50 and 25 µg of resveratrol per egg in yolk sac at 14 days of embryo age. In the current study, ML increased chick length which perhaps is associated with its antioxidant potentiality and activation of the thyroid gland that supports growth and osteogenesis (Aly *et al*., 2023). Consistent with this result, Ranjbar *et al.* (2019) concluded that high chick length and BW at hatch were obtained by IOI of naringin (30 mg/egg) at the 17.5th day of incubation. Differently, it was documented that IOI into an amnion sac of essential oils blends on day 18 of incubation decreased chick length and hatchability (Oladokun *et al.*, 2021).

The powerful antioxidant capacity was exhibited based on IOI and FM type-dependent manner and their interactions by increasing TAC and CAT and decreasing lipid peroxidation indicators (MDA and LOOH) and PCA. The avian embryos feeding through IOI of various doses of bioactive phytochemicals could develop the cellular and molecular antioxidant defence system and then protect the embryo from free radicals attack. Thus, IOI might be proposed as an active method to decrease embryonic sensitivity to oxidative stress of polyunsaturated fatty acids in growing tissues and reduce the consequences of LF after hatching (Akosile *et al*., 2023a). Undoubtedly, in current data (Table 5), improving or stability of serum metabolites and enzymatic activity is a direct reflection of improving of antioxidant system and protecting metabolic pathways in liver, kidney and muscle tissues from oxidative stress status. The permeability of hepatic cells, liver injury and skeletal muscle damage were usually determined by high concentrations of blood ALT, AST, ALP, GGT and CPK, which are important markers that are associated with oxidative stress and toxicity in poultry (Al-Shammari, 2023b). In agreement with the results of Kpossou *et al*. (2024a), MDA was inhibited in chicks' serum, which resulted from IOI of 1µg *Citrus aurantiifolia* with no influence on glucose, creatinine and TP levels under effect of IOI of 0.5 and 0.75 µg of the same extract. Similarly, El-Kholy *et al*. (2021) reported that IOI of 0.1 mL of cinnamon, thyme, and clove extracts resulted in inhibiting MDA, AST, ALT, ALP and TC values at 35 days of broilers' age. The substantially reduced LOOH and PCA levels in the present findings might be linked to the high activity of the hydroxyl group in LN, CN and ML which is able to deactivate the production of harmful oxygen-reactive species. Differently, Oladokun *et al*. (2021) indicated that IOI of the mixture of essential oils did not change levels of plasma TAC, TP, uric acid, glucose, TC, ALP, CPK, AST and GGT. More recently, Al-Shammari & Zamil (2024) pointed out that IOI of 5 mg of oleuropein and epigallocatechin-3 gallate in the yolk sac and air cell at the 12th day of incubation boosted the serum antioxidant and biochemical traits by inhibiting levels of MDA, glucose, TC and AST with no effect on TP, creatinine, uric acid and ALT on day 2 of heat-stressed chicks.

Lowered glucose, ALT, ALP and CPK values exhibited by the main effect of LN is due to its pharmacological impacts against hyperglycemia through reducing insulin resistance and regulating blood sugar with overall hepatoprotective effects (Chen *et al*., 2024). CN is involved in hypocholesterolemic and lipid-lowering effects by preventing the activity of 3-hydroxy-3-methylglutaryl coenzyme A reductase, which is considered a principal enzyme in the metabolic synthesis of TC (Abd El-Hack *et al*., 2020) with potent hepatoprotective advantages (Saeed *et al*., 2018). It is widely known that post-hatch EF is a successful procedure to modulate oxidation levels and boost the immune system in gut microbiota. These changes will reinforce the active enhancement of nutrient utilization and synthesis of antioxidant-related genes in the gut and other different organs (Noy & Uni, 2010; Jha *et al*., 2019). No data is available about the influence of EF on blood antioxidative indices in broiler chickens. In Japanese quail, consistent findings were confirmed by Al-Shammari (2023a), that immediate EF in post-hatch with/without dietary date syrup and vitamin C augmented ferric-reducing ability of plasma and lowered MDA, LOOH, TC, AST, ALT, creatinine and uric acid in serum compared with post-hatch LF at 24 hours. In discordance with Kang *et al.* (2019), who mentioned that feeding broilers in different periods at 3, 12, 24, 36 and 48 hours after hatching did not affect serum TP, TC, TGs and AST measured at 7, 21 and 35 days of age.

Enhancing in T4 value for the main effect of IOI and EF, as well as their interactions, is an indicator of the post-hatch development of chicks. Thyroid hormones fundamentally participate in regulating muscle metabolism, thermogenesis, hatching development and mobilization of fat and glycogen (Das *et al*., 2021). Although, TSH and T3 levels remained constant in serum (Table 6). These results align with the previous observations which found that IOI of different levels of naringin (Ranjbar *et al.*, 2019), clove (El-Kholy *et al.* 2021) and *Citrus aurantiifolia* (Kpossou *et al.*, 2024a) extracts could elevate levels of blood plasma/serum T3 and T4 *via* activating the thyrotrophic axis in newly hatched chicks until marketing age. Conversely, Noy *et al*. (2001) indicated that plasma T3 level was higher in early-fed poults than feed-deprived ones. Marked decrease in corticosterone levels for the same main effects of IOI and EF might be associated with attenuating physiological stressors and endocrine disruption (Al-Shammari, 2023b) through upgraded antioxidant activity and biochemical profile in serum (Tables 4 and 5). Identical previous results showed that IOI of oleuropein and epigallocatechin-3 gallate (Al-Shammari & Zamil, 2024) did not show any stress reaction in chickens as a result of low serum corticosterone in circulating blood. These data are also congruent with the recent findings of Madej *et al.* (2024), who stated that early access to feed and water inhibits the pituitary-adrenal axis and led to decreased values of serum corticosterone of chicks almost two-fold on day 1 after hatching compared with delayed fed chicks.

In this study, an increased villus morphology including VH, VW and VSA in duodenum under the main effect of IOI of LN is a pivotal biomarker for the assessment of the absorptive capacity, digestive efficiency and gut health. LN motivates cell proliferation and provides suitable protection for intestinal architecture from oxidative injury (Chen *et al*., 2024). It was demonstrated that essential oil of orange at 400 mg/kg (Souza *et al*., 2021) or 200 mg/kg of lemon with 200 mg/kg of garlic (Elbaz *et al.*, 2022) added to the diet increased VH with no effect on VH/CD. The great VSA in CN might be correlated with high VH which belongs to the antioxidant power of CN as a hydrogen donor to maintain the intestinal mucosa from free radicals damage *via* activation of antioxidant enzymes, preventing pathogenic bacteria and stimulating the gut immune system (Ali *et al*., 2021). Improved CD in the ML group could be ascribed to antioxidative properties in the gut environment and maintaining intestinal function efficiently (Kamatou *et al.*, 2013). Due to the high content of peppermint from ML and menthone constituents, Ahmed *et al.* (2016) stated that were desirable effects of dietary leaves (1.5, 3 g/kg) and oil (250 mg/kg) peppermint to augment CD and VH in the ileum of broilers. Accordingly, Kpossou *et al.* (2024b) confirmed the synergetic effect of IOI in an air chamber at 18 days of incubation of 0.75 mg/mL of *Citrus aurantiifolia* with dietary supplementation (5 g/kg) after hatching enhanced VH in small intestines at 42 days of age in broilers. Moreover, identical results found that high duodenal VH was increased through a combination of essential oils injected *in ovo* without a noticeable effect on jejunum and ileum morphology on day 28 of age in chicks (Oladokun *et al*., 2021). All duodenal histological parameters in our results were enhanced by the main effect of EF. This could be likely explained by the importance of early onset of feeding in the enlargement of villi absorptive area with enhancing gut immunity that is affected by gene expressions of tight junction proteins and immune-related genes in intestines of broilers (Hollemans *et al*., 2020; Li *et al*., 2022). LF is involved in depressing intestinal development *by* inhibiting the proliferation, differentiation and dimensions of enterocytes and villi (Noy et al., 2001). Therefore, it is well-reported that possibly immediate EF in post-hatch could reverse these deleterious phenomena in the intestine and directly accelerate intestinal morphology (Jha *et al*., 2019). In contrast with Kang *et al.* (2019), withholding feed after hatching from 3 to 48 hours did not substantially change VH and CD in jejunum at 35 d of the age of the broiler.

Also, it is probable that improvement in duodenal morphology led to a concomitantly positive modification in nutrient digestibility (Table 8). LN was suggested to be an excellent regulator for microbial content of lactobacillus and *E.coli,* and promotes the digestive enzymes (amylase, trypsin, lipase) which enhances nutrient digestibility in broiler's intestine (Elbaz *et al.* 2022). Besides, the apparent ileal digestibility of nutrients was improved by CN supplemented in the diet of broilers because of the functionality of CN to support gut barrier function and nutrient transporters (Yang *et al*., 2021) with reducing pathogenic microbiota in the gut (Yang *et al.*, 2020). ML could exert the ability to improve nutrient digestibility through modulation of bacterial community growth in the intestine *via* lowering *E.coli* and Salmonella with increasing lactobacillus (Aly *et al.*, 2023). Syed *et al.* (2021) proved that better ileal digestibility of dry matter was achieved during feeding broilers on a diet containing ML and other combined phytochemicals in the encapsulated form at 65 g/tone up to 42 days. This would efficiently stimulate endogenous digestive enzyme activity that supports intestinal digestion, absorption and utilization (Ahmed *et al*., 2016). As a result, it seems that IOI could help support nutrient digestibility depending on developing gut health by enhancing antioxidative protection levels and useful microbial population by lowering intestinal pH,

increasing intestinal morphology and reinforcing gut-associated lymphoid tissues (Kadam *et al*., 2013; Akosile *et al*., 2023a; Ebeid *et al*., 2023). Moreover, these favorable alterations in digestibility of the main effect of EF might explain the substantial importance of EF in enhancing RNA expression of nutrient transporters and brush-border enzymes as well as preferential maturation of goblet cells and mucosal enzyme activity (Noy & Uni, 2010). The present results concurred with the findings of Obun & Osaguona (2013), who concluded that nutrient digestibility on day 7 was enhanced in chicks fed at 12, 24 and 36 hours after hatching in comparison to those delayed fed chicks at 48, 60 and 72 hours.

It is possible that an improvement in most of the productive attributes of newborn chicks under main effects of IOI and FM and interactive treatments (Table 9) was related to previous results shown in this study. Each IOI×FM treatment behaves in specific mode of action to show its long-lasting effect on periodical and final productive response. This was evidenced in improved BW, FI and EER and depressed FCR and mortality. In addition, there was an increased PEF (Table 10). A growing body of similar data refers previously that final and periodical BW and FCR were improved with altered amounts of FI in post-hatch broilers resulting from IOI of different active solutions of plant extracts. The positive alterations in this productivity could be translated into enhanced antioxidant capacity, hormonal changes, gut health and liver activity, which reflect on stimulation of intestinal absorption and nutrient utilization (El-Kholy *et al*., 2021; Ngueda *et al*., 2021; Akosile *et al*., 2023c; Kpossou *et al.*, 2024b). However, Khaligh *et al.* (2017) stated that productive parameters involving FI, BW, BW gain and FCR were not changed up to 11 days of age in hatched chicks resulting from IOI of 4.5 mg of either chrysin or quercetin in the amniotic cavity on day 18 of embryonic age. Low total mortality in IOI groups is probably associated with the importance of aqueous solutions of phytogenics injected *in ovo* in the escalated generation of immunoglobulins M and G from immune organs (EL-Saadany *et al.*, 2019; El-Kholy *et al.*, 2021; Akosile *et al.*, 2023a). Likewise, early first access to feed and water was particularly important to enhance productive parameters. These outcomes are consistent with the research of Obun & Osaguona (2013), who indicated that post-hatch EF stimulated FI at 28 days of age by early development of the digestive system that led to improved BW and FCR in broilers fed after 12, 24, 36 hours of hatching compared with 48, 60 and 72 hours. Also, BW was found to be superior in broilers at 3, 4 and 14 days (Sarica *et al*., 2014; Hollemans *et al.*, 2020) with improved FCR in hatched chicks through their EF in comparison to post-hatch fasting for 24, 48 or 72 hours. Nevertheless, Kang *et al.* (2019) concluded that final BW and FCR at 35 days did not change in broilers offered different periods of feeding in post-hatch, starting from 3 to 48 hours. Lowered mortality in the main effects of EF *vs*. LF could be an obvious indication of EF in the development of the immune system. EF provides immunocompetence for chicks in the post-hatch period due to its effect on immunoglobulin production by either providing substrates for immunomodulation and immunogenicity or by conferring micronutrients for cell differentiation and proliferation (Jha *et al.*, 2019). This assumption was confirmed recently by Madej *et al.* (2024), who proposed that EF of chick affects immunostimulation by increasing the number of $CD4^+$ cells in the cecal tonsil and high relative weight of the bursa of Fabricius on day 7 post-hatch.

Conclusion

The present experiment reveals that IOI at 5 mg/100 µl of ML and LN solutions enhanced hatchability and chick quality, respectively, while IOI of the same dose of CN reduced the time required for hatching. In the post-hatch period, the main effect of IOI and immediate EF in hatchery increased antioxidant properties, biochemical parameters and T4 hormone and lowered corticosterone in serum. Moreover, there was developed intestinal function due to boosting the duodenal histology and apparent nutrient digestibility at 5 days. These positive modifications led to improvement in productive performance up to 5 weeks of broilers' age. The synergistically interactive influence between IOI and EF showed better or equivalent effect than IOI and LF in investigated variables based on IOI of phytochemicals and feeding method after hatching-dependent manner.

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