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Evaluation of Microencapsulation of Herbal Essences and Probiotics on Egg Characteristics, Antioxidant and Liver Enzymes and Expression of the Calbindin Gene in Intestine and Ovary in Hy-Line W80 Laying Hens

Research Article

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ABSTRACT

In poultry nutrition, finding an environmentally friendly alternative additive to increase poultry production, is a major challenge. Therefore, the effect of microencapsulation of herbal essences and probiotics on quantitative and qualitative egg traits, antioxidant and liver serum enzymes was considered in laying hens. A total of 420 Hy-line W80 laying hens were designed by 7 treatments, 6 replicates, and 10 laying hens in each, in a completely randomized design (CRD). The experimental treatments included 1- control (corn-soybean meal) (C), and treatments 2, 3 and 4, respectively, the control diet supplemented with 100 mg/kg of microencapsulated essential oil, *Zataria multifllora* (Z.EO), *Foeniculum vulgare* (F.EO) and *Origanum vulgare* (O.EO), and treatments 5 and 6, control diet supplemented with 150 mg/kg of Bio-poul probiotic (P.B) and Parsilact probiotic (P.PL) respectively, and treatment 7 including the control diet plus 100 mg/kg of a mixture of microencapsulated essential oils of each of the plants and 150 mg/kg of Parsilact and Bio-poul probiotics (Z.F.O)EO+(B.PL)P. The results have shown that egg quality was effected by treatments (P<0.05). The positive effect of antioxidant enzymes, except malondialdehyde, was shown by experimental treatments (P<0.05). Protected the liver cells and expression of the Calbindin gene in the intestine were occurred in laying hens in this respect. Therefore, essential oils could be a suitable alternative to growth-promoting antibiotics to improve production and performance in layers.

KEY WORDS calbindin D28k, egg production, microcapsulation herbal, probiotics, serum enzymes.

INTRODUCTION

The global demand for animal and poultry products has surged in recent years (Liu *et al.* 2019). Historically, antibiotics were a common choice in feed additives due to their economic benefits (Wang and Chai, 2022). However, this widespread use has unfortunately led to an increase in antibiotic-resistant bacteria, posing significant threats to livestock, human health, and the environment (Wang *et al.* 2024). This has spurred the search for safer alternatives. Probiotics have emerged as a promising solution, offering effective benefits like producing antimicrobial substances and competing with pathogenic bacteria (Obianwuna *et al.* 2022). Numerous studies have demonstrated their positive impact on laying hens, improving egg production, feed conversion ratio, egg quality, and disease resistance (Khan *et al.* 2020; Bindari *et al.* 2022; Jiang *et al.* 2022).

Another natural alternative comes in the form of phytobiotics, also known as plant chemicals or phytogenics. There's a growing trend towards using herbal medicines over chemical ones, thanks to their natural availability and suitability as feed additives (Gholami-Ahangaran *et al.* 2021). Phytobiotics beneficially modify gut microflora and reduce the population of harmful bacteria, possibly by altering membrane permeability to hydrogen ions (Gholami-Ahangaran *et al.* 2020).

To maximize the efficacy of these beneficial compounds, microencapsulation is gaining traction in poultry production. This innovative technology involves enclosing active ingredients within a protective capsule, shielding them from environmental factors like light, temperature, and pH. This also allows for a controlled release at the desired location within the animal's digestive system (Pittia and Gharsallaoui, 2021; Huang *et al.* 2023). Spray drying stands out as a common and cost-effective encapsulation method, efficiently encapsulating oil-in-water emulsions with high evaporation rates (Santos *et al.* 2020). Research indicates that encapsulated probiotics bolster the host's defense against pathogens by strengthening the integrity of the intestinal barrier, limiting mucosal epithelial cell junctions, and modulating the immune response (Fusco *et al.* 2023). Furthermore, they help regulate the composition of gut microorganisms, alleviate negative effects of nutrient intolerances (such as lactose intolerance), boost the absorption of essential nutrients, and reduce allergic reactions (Roobab *et al.* 2020).

Calcium and phosphorus are vital for numerous biological processes, particularly for eggshell formation in laying hens. Maintaining the balance of these two elements is crucial, involving absorption from the small intestine, excretion by the kidneys, mobilization from bones, and incorporation into the eggshell. In pullets, the transfer of calcium and phosphorus to the eggshell can adversely affect the laying hen's health (Eusebio-Balcazar *et al.* 2018). Therefore, efficient calcium and phosphorus metabolism is essential for laying hens.

Calbindin D28k plays a key role in intracellular calcium diffusion within poultry intestinal and kidney tissues. It binds to calcium and transports it from the apical to the basolateral membrane of duodenal cells.

Interestingly, *Origanum vulgare* essential oil has shown the highest antioxidant effect, attributed to its main phenolic compounds like thymol and carvacrol (Gutierrez-Grijalva *et al.* 2017). Its inclusion in poultry feed has been linked to improved egg production and quality (Abdel-Wareth *et al.* 2020).

Researchers have also found that *Origanum vulgare* can enhance the feed conversion ratio, promote digestive health, increase antioxidant activity, and reduce bacterial counts in meat and eggs (Denli *et al.* 2019). Specifically, carvacrol and thymol present in *Zataria multiflora* have been observed to inactivate insulin sites in the liver, leading to an increased feed conversion ratio (Youssef *et al.* 2021). Additionally, *Foeniculum vulgare* extract in the diet of 36-week-old laying hens has been shown to reduce eggshell breakage over an 8-week period (Hadavi *et al.* 2017).

While several studies exist on probiotics in laying hens, our current research takes a novel approach. For the first time, we are investigating the simultaneous use of microencapsulated essential oils from three plants (*Zataria multiflora*, *Foeniculum vulgare*, and *Origanum vulgare*) alongside two probiotics (Biopoul and Parsilact) in laying hens.
The objective of this study is to determine the impact of these microencapsulated plant essential oils and probiotics on the quantitative and qualitative characteristics of eggs, antioxidant levels, liver enzymes, and the expression of the Calbindin gene in the intestine and ovary of commercial Hy-line W80 laying hens.

MATERIALS AND METHODS

This study was approved by the Research Ethics Committee of Bu Ali Sina University- Hamedan- Iran, with ID (IR.BASU.REC.1402.055). This experiment included 7 treatments, 6 replicates, and 10 laying hens in each. Hy-line W80 laying hens was managed in this study. The experimental treatments included 1- control (corn-soybean meal) control (C), 2- control diet + 100 mg/kg *Zataria multifllora* essential oil (Z.EO), 3- control diet + 100 mg/kg *Foeniculum vulgare* essential oil (F.EO), 4- control diet + 100 mg/kg *Origanum vulgare* essential oil (O.EO), 5- control diet + 150 mg/kg Bio-poul probiotic (P.B), 6- control diet+150 mg/kg Parsilact probiotic (P.PL), 7- control diet + 100 mg/kg of a mixture of mixture of *Zataria multifllora*, *Foeniculum vulgare*, *Origanum vulgare* essential oil + 150 mg/kg of Bio-poul + Parsilact probiotics.

The experiment started at 24 weeks of age and lasted for 12 weeks. This experiment was located in Sepid Morgh farm. The basal diet was formulated based on the digestible amino acid requirements of Hy-line W80 laying hens by WUFFDA software. During the experiment, birds were located in cages, at a 24 ± 2 °C temperature and 30 to 40% humidity with a 16-hour light/day. Feed (110 grams per hen per day) was distributed at 5 am and 2 pm and eggs were collected at 4 pm, as well as birds had free access to drinking water.

Microencapsulation

Microencapsulated essential oils were sourced from the Research Institute of Food Science Industries in Mashhad. The spray drying technique was selected due to its cost-effectiveness and its ability to produce particles around 40 micrometers in diameter. To start, 150 mL of distilled water was combined with 15 g of maltodextrin (Dextrose Equivalent=18), and this mixture was stirred using an IKA RCT Basic stirrer at 1500 rpm for two hours. After mixing, 3.75 mL of essential oil-calculated to achieve 25% dry matter-was added. The blend was then processed with an Ultra-Turrax homogenizer (IKA T25 Digital, disperser S 25N) at 12000 rpm for three minutes. Following homogenization, the emulsion was dried using a Buchi Mini Spray Dryer B-191 (Switzerland) with an inlet temperature of 180 °C, outlet temperature of 85 °C, and a pump power set at 20%.

Quantitative egg Characteristics

Feed intake is calculated as the total feed consumed per day, in grams, divided by the number of hens (as described by Chen *et al*. 2019). Egg weight is determined by weighing the eggs from each experimental unit, then dividing by the number of eggs in that unit (see Zhan *et al*. 2019). Egg production, expressed as a percentage, involves dividing the number of eggs produced by the number of hens in the experimental unit and multiplying by 100 (Vakili and Majidzadeh Heravi, 2016). Feed conversion ratio represents the daily feed intake per hen (in grams) divided by the egg mass produced per hen per day (Wang *et al*. 2020).

Qualitative egg characteristics

To assess egg quality parameters, ten eggs are randomly collected from each treatment group on the final two days of each four-week period. The collected eggs are then analyzed for eggshell strength, thickness, weight, Haugh Unit, and yolk color.

Eggshell strength, thickness, and weight

Eggshell strength was determined in kg/cm² by an FHK model strength tester (Fujihara Industrial Co., Ltd., Tokyo, Japan, 2002). The thickness of the eggshell was determined at three points (top, bottom, and middle) by a micrometer, and the mean of these readings was taken as the ultimate eggshell thickness. For the determination of eggshell weight, the white was removed from the shell first, then the shell was dried and weighed using a scale sensitive of 0.01 g (Abdel-Wareth *et al*. 2020).

Haugh Unit

The Haugh unit was determined as an index of internal egg quality. To this end, the albumen height was measured in three locations using a height meter. The results derived from egg weight and albumen height, gives rise to the Haugh unit which was computed by the Emt5300 device according to the formula below (Ye *et al*. 2020):

$$\text{Haugh unit} = 100 \times \log [(7.57+\text{albumen height (mm)}) - (1.7\times\text{egg weight (g)}^{0.37})]$$

Blood parameters

At the end of the study, 5 mL blood were collected from the wing vein by 5 ml syringes for blood parameters measurements and transferred to numbered heparin-containing test tubes. To separate the plasma, the samples were centrifuged at 2500 rpm for 10 minutes, and the resulting plasma was transferred to numbered microtubes and stored at -20 °C until transport to the laboratory. Subsequently, the activities of liver enzymes aspartate aminotransferase (AST), alanine aminotransferase (ALT), alkaline phosphatase (ALP), and lactate dehydrogenase (LDH) were measured by standard kits from Abcam, on the other hands the activities of anti-oxidant enzymes catalase (CAT), glutathione peroxidase (GSH-PX), superoxide dismutase (SOD) were measured using a PerkinElmer Lambda 25 UV/Vis Spectrometer and German Roch technology.

In order to measure malondialdehyde (MDA) concentration in serum samples, 0.5 mL plasma was combined with 1 ml thiobarbituric acid (TBARS) and 2.5 mL trichloroacetic acid and incubated in a water bath at 95 °C for 30 minutes. The mixture was cooled and 4 ml n-butanol was added and centrifuged at 2000 rpm for 10 minutes. Then the upper liquid was read for absorbance at 532 nm using a spectrophotometer and MDA concentration was expressed using a standard curve (Habibian *et al*. 2016).

Expression calbindin gene in the intestine and ovary

RNA extraction from ovarian and intestinal tissues

This includes 4 steps:

1. Homogenization: Here, tissues were properly homogenized in TRIzol by using a homogenizer, and their RNA was extracted. Homogenization was done with chemical lysis.
2. Separation Phase: In this step, the sample was composed of three phases: the top aqueous phase with RNA, the middle white DNA phase that was precipitated, and the bottom phase with TRIzol and other proteins and chemicals. This step is called separation.
3. RNA Precipitation: In this step, the upper layer was taken with a pipette, put into a new microtube, and an equal amount of isopropanol alcohol was added. This step is called RNA precipitation.
4. RNA Wash: 20 µl of DEPC water was added to the samples with pure RNA. Afterwards, the samples were measured quantitatively and qualitatively with a NanoDrop device.

cDNA synthesis steps

cDNA synthesis involved three mixing stages:

First mixture: RNA + H_2O

Second mix: first mix + dNTP (reaction nucleotides) + Primer (random hexamer)
Third mix: second mix + DEPC water + buffer + RNase inhibitor (to inhibit RNA activity) + RTase (reverse transcriptase for reverse transcription).

After that, the sample was put in a PCR machine for 50 minutes at 45 °C, such that cDNA was formed from single-stranded RNA by primers. Then the temperature was increased to 80 °C for 5 minutes in order to inactivate the reverse transcriptase enzyme, and lastly, the temperature was adjusted to 25 °C, the ambient temperature. Lastly, by setting the proper threshold level, CT amount was exported from the Real-Time machine in Excel form.

Data analysis

At the end of the experiment, data were analyzed using the SAS 9.4 statistical software package. The data normality was tested by the Shapiro-Wilk test. Comparison among means was made by Tukey's test at α= 0.05 significance level. The model equation for the experimental design was as follows:

$$Y_{ij}= \mu + \alpha_i + e_{ij}$$

Where:
Y_{ij}: value of each observation.
μ: population mean for each trait.
α_i: treatment effect.
e_{ij}: residual value.

RESULTS AND DISCUSSION

The effects of experimental treatments on feed intake are presented in Table 1. The maximum feed intake (106.93 g/day) was seen in control treatment that did not differ significantly compared to the fennel microencapsulated essential oil treatment. Conversely, the minimum feed intake was recorded by (105.06 g/day) diet with microencapsulated essential oils of *Zataria multiflllllora*, *Foeniculum vulgare*, and *Origanum vulgare*, Bio-poul and Parsilact probiotics in laying hens (P<0.05).

In fact, the natural addition of feed showed beneficial effects in stimulating and activating the digestive system. This leads to an improve in the palatability of the diet, an increase in the appetite of the birds, and consequently an increase feed intake. In addition, reduce undesirable gut microorganisms, improve digestion, and increase feed intake by the antibacterial and antifungal effects of phytobiotics.

Stimulates the secretion of bile acid and digestive enzymes such as protease, lipase, amylase and maltase facilitate digestion, improve nutrient availability, reduce the viscosity of intestinal contents and create a healthy and stable intestinal environment. Which are the reasons for the increased feed intake in birds supplemented with *Foeniculum vulgare*. According to Saleh *et al*. (2018), an increased feed intake by diet containing *Foeniculum vulgare* in broilers under heat stress conditions.

According to the results presented in Table 1, the highest egg weight (57.45 g) was found by for hens which fed the microencapsulated essential oils of *Zataria multifllora*, *Foeniculum vulgare*, and *Origanum vulgare*, Bio-poul and Parsilact probiotics. In addition of other treatments in the laying hen diet did not differ significantly from each other in terms of egg weight. On the other hand, the lowest egg weight was shown by the control treatment (55.33 g) (P<0.05). Higher egg weight was exhibited with the diet supplemented with microencapsulated essential oils of *Zataria multifllora*, *Foeniculum vulgare*, and *Origanum vulgare*, Bio-poul and Parsilact probiotics compared to the control compared to other treatments because of the synergistic effect between active compounds in the plant essential oils and easier access to nutrients. The findings of the current study were in line with those of Wang *et al*. (2024), in that the addition of combined probiotics to the diet elevated egg weight, suggesting that combined probiotics as feed additives can enhance egg-laying performance. The reason might be that probiotics, once in the digestive tract, speed up the use of feed nutrients by elevating fiber digestion and enzyme activity. He *et al*. (2017) have demonstrated that significantly higher average egg weight relative to the control by adding 100 mg/kg *Zataria multifllora* essential oil to Hy-Line hens diet.

Enhanced egg production percent through supplementation of microencapsulated essential oils of *Zataria multifllora*, *Foeniculum vulgare*, and *Origanum vulgare* in the diet of laying hens combined with Bio-poul and Parsilact probiotics. Conversely, the control diet had the least impact on egg production percent (Table 1) (P<0.05).

The increase in egg production can improve nutrient intake (Souza *et al*. 2021), which has a positive impact on the production of lactic acid and other organic acids, can minimize pH, and form an unfavorable environment for pathogenic bacteria, and also boost the production of short-chain fatty acids in the intestine, which not only serve as a source of energy for colonocytes, They also contribute to a healthy and balanced gut microbiome, which plays a role in production performance in laying hens (Song *et al*. 2019; Xu *et al*. 2022).

This fact is substantiated by earlier experiments on laying hens, wherein it appears that probiotics enhance physiological parameters, with regard to immune indices, intestinal morphology, and antioxidant potential, and significantly boost egg production.

In the context of the findings of Pan *et al.* (2022); Wang *et al.* (2021); Darsi and Zaghari, (2021). This enhancement indicates that probiotics are natural growth promoters through the utilization of nutrients. Also, Bacillus strains boosted egg production by 8% in comparison to the control (Yang *et al.* 2020). In accordance with the report of Guo *et al.* (2017), *Bacillus subtilis* included in a dietary supplement positively influenced the cecal microbiota, with a significant increase in the egg production of laying hens. According to Wang *et al.* (2017), increasing egg production by incorporating *Bacillus licheniformis* into the diet is through systemic improvement, elevated intestinal barrier function, and regulation of reproductive endocrine hormones. Ghanima *et al.* (2020) have found that egg production increased by thymol in layer hen diet. Supplementation of thymol (250 mg/kg) enhanced the performance of laying hens (Abdel-Wareth *et al.* 2018). Essential oils play an important role in feed conversion ratio since they stabilize the microbial population and enhance the absorption of nutrients. Moreover, activate digestive enzymes and high digestion was exhibited by essential oils (Li *et al.* 2018).

According to Table 1, there was a significant differences between the different experimental treatments in terms of feed conversion ratio (P<0.05). The results showed that, by microencapsulated essential oils of Zataria *multifllora*, *Foeniculum vulgare*, and *Origanum vulgare* along with Bio-poul and Parsilact probiotics wss indicated significantly improved feed conversion ratio, while the highest feed conversion ratio was observed in the control treatment.

As reported by several studies, probiotics enhance feed conversion ratio by antagonistic mechanisms, competition for colonization sites and nutrient uptake, inhibition of toxic production, and immune system strengthening (Mahfuz *et al.* 2019; Muhammad *et al.* 2020). By *Bacillus subtilis* supplementation along with herbal essential oils in the diet of laying hens Improved feed conversion ratio and egg laying rate significantly (Wang *et al.* 2018; Chen *et al.* 2019). This feed efficiency improvement may be attributed to the fact that probiotics can improve intestinal morphology and health by villi height enhancement for more nutrient uptake and the production of bacteriocins and bacteriostatic volatiles, which inhibit pathogen invasion (Wang *et al.* 2020). Furthermore, this improvement may be due to several enzymes like amylase, cellulase, and protease, which are secreted by the digestive system through stimulation by BS or herbal essential oils (Teixeira *et al.* 2019).

Preservation of internal components (albumin and yolk) and external (shell quality) components of eggs is a top priority in the laying hen industry to satisfy consumers while meeting market needs. In this study, The effect of microencapsulated essential oils of *Zataria multifllora*, *Foeniculum vulgare*, and *Origanum vulgare* and Bio-poul and Parsilact probiotics on eggshell thickness, strength, and weight is shown in Table 2. A significant difference, in eggshell weight was indicated by microencapsulated essential oils of the three herbs along with the two probiotics in the diet. The highest eggshell weight (7.15 g) was observed by (microencapsulated essential oils of *Zataria multifllora*, *Foeniculum vulgare*, and *Origanum vulgare* along with Bio-poul and Parsilact probiotics) treatments. In contrast the lowest eggshell weight (4.84 g) was observed by control treatment (P<0.05). The highest eggshell thickness (0.38 mm), significantly (P<0.05) increased compared with the control diet and other treatments (P<0.05) by microencapsulated essential oils of *Zataria multifllora*, *Foeniculum vulgare*, and *Origanum vulgare* along with Bio-poul and Parsilact probiotics to the diet. In fact, there is a positive correlation between eggshell thickness and strength. The highest (4.47 kg/cm) and lowest values of this trait (3.55 kg/cm) were observed by treatments and control, respectively.

Probiotics were found to enhance the growth of beneficial bacteria and the fermentation rate, which accumulates short-chain fatty acids (SCFA) and lowers luminal pH. Moreover, short-chain fatty acids enhance the growth and nutrition of intestinal villi to enhance the absorption rate. Reduction of the pH in the gastrointestinal tract can solubilize water-insoluble calcium and transform it into a more soluble ionic form, enhancing the bioavailability of calcium in laying hens (Zou *et al.* 2019; Ye *et al.* 2020). Thus, the enhancement of eggshell quality may be attributed to the ability of probiotics to enhance absorption and retention of calcium in the serum of laying birds (Attia *et al.* 2020), making more calcium available for deposition on the shell-forming glands. Furthermore, according to Khan *et al.* (2022) and others, have indicated that medicinal plants in poultry diets result in greater deposition of calcium on the eggshell.

The reason is that medicinal plants enhance the anatomical status of the intestine for the absorption of various nutrients, including calcium. According to Bozkurt *et al.* (2016), *Origanum vulgare* in the diet increased eggshell thickness in 82- to 106-week-old hens.

A significant rise in the weight and thickness of eggshells was exhibited by hens fed rosemary or thyme powder at 0.9% in 36- to 52-week-old hens in comparison to the control treatment.

Table 1 Effect of different experimental treatments on performance and quantitative egg traits in Hy-Line W80 laying hens

Dietary treatment	Feed intake (g/day)	Egg weight (g)	Egg production (%)	Feed conversion ratio
C	109.93[a]	55.33[d]	93.40[d]	1.98[a]
Z.EO	106.91[c]	55.91[c]	94.70[c]	1.89[cd]
F.EO	109.59[a]	55.82[c]	94.27[c]	1.97[b]
O.EO	106.66[cd]	55.69[c]	95.83[b]	1.88[d]
P.B	106.38[d]	55.87[c]	95.34[b]	1.89[cd]
P.PL	106.58[cd]	55.92[c]	95.47[b]	1.88[d]
(Z.F.O)EO+(B.PL)P	105.06[e]	57.45[a]	96.67[a]	1.79[e]
SEM	0.97	0.51	0.50	0.001
P-value				
Diet	<0.001	<0.001	<0.001	<0.001
Time	<0.001	<0.001	<0.001	<0.001
Diet × Time	<0.001	<0.001	<0.001	<0.001

C: Control; Z.EO: microencapsulated *Zataria multifllora* essential oil; F.EO: microencapsulated *Foeniculum vulgare* essential oil; O.EO: microencapsulated origanum vulgare essential oil; P.B: Bio-poul probiotic; P.PL: parsilact probiotic and (Z.F.O)EO+P(B.PL): microencapsulated essential oils of *Zataria multifllora*, *Foeniculum vulgare*, and *Origanum vulgare* combined with Bio-poul and Parsilact probiotics.
The means within the same column with at least one common letter, do not have significant difference (P>0.05).
SEM: standard error of the means.

Table 2 Effect of different experimental treatments on egg quality in Hy-line W80 laying hens

Dietary treatment	Eggshell weight (g)	Eggshell thickness (mm)	Eggshell strength (kg/cm)	Haugh unit
C	4.84[e]	0.24[c]	3.55[d]	83.20[d]
Z.EO	6.59[b]	0.35[b]	4.19[b]	93.79[b]
F.EO	6.31[bcd]	0.33[b]	3.93[bc]	87.41[c]
O.EO	6.42[bc]	0.33[b]	3.88[c]	86.95[c]
P.B	5.87[d]	0.32[b]	3.99[bc]	87.12[c]
P.PL	6.06[cd]	0.33[b]	3.91[c]	87.37[c]
(Z.F.O)EO+(B.PL)P	7.15[a]	0.38[a]	4.74[a]	96.29[a]
SEM	0.13	0.009	0.09	0.82
P-value	0.0001	0.0001	0.0001	0.0001

C: Control; Z.EO: microencapsulated *Zataria multifllora* essential oil; F.EO: microencapsulated *Foeniculum vulgare* essential oil; O.EO: microencapsulated origanum vulgare essential oil; P.B: Bio-poul probiotic; P.PL: parsilact probiotic and (Z.F.O)EO+P(B.PL): microencapsulated essential oils of *Zataria multifllora*, *Foeniculum vulgare*, and *Origanum vulgare* combined with Bio-poul and Parsilact probiotics.
The means within the same column with at least one common letter, do not have significant difference (P>0.05).
SEM: standard error of the means.

The Haugh unit (Hu) reflects the quality of the egg. Egg quality is largely dependent on the egg white. In addition, the quality of albumin is increasing for the food and health processing industries, which use albumin as a raw material for the production of food and pharmaceuticals. Hence, in this the effect of different experimental treatments on egg internal quality is shown in Table 2. Based on the results, the highest Haugh unit value (96.292) was observed in the diet containing microencapsulated essential oils of *Zataria multifllora*, *Foeniculum vulgare*, and *Origanum vulgare* along with Bio-poul and Parsilact probiotics. In contrast the lowest value (83.208) of this trait was observed in the control diet. Increased the Haugh unit in 15.75% compared to the control diet by medical plants and probiotic in diets containing microencapsulated essential oils (P<0.05).

According to the Haugh unit formula, in addition to egg white quality, egg weight also plays a role in increasing egg white quality. According to Table 2, the average egg weight in the (microencapsulated essential oils of *Zataria multifllora*, *Foeniculum vulgare*, and *Origanum vulgare* along with Bio-poul and Parsilact probiotics) treatment was higher than in the other treatments. Probiotics improve synthesis due to significant increase in nutrients. Probiotics have been reported to stimulate the activity of digestive enzymes, leading to increased nutrient utilization and protein digestibility (Ahiwe *et al*. 2020). Evidence showed that *Bacillus subtilis* (Yang *et al*. 2020) and *B. velezensis* (Ye *et al*. 2020) improved the Hu unit and albumin elevation in broiler breeders and laying hens. In another study, *Bacillus* strains improved Hu unit and protein index in laying hens

(Mazanko *et al*. 2018). The increased Hu values could be due to better bioavailability of nutrients and better gross digestible energy due to probiotics. Anethole is the main ingredient in fennel and reduces or stops spasms of the digestive system and improves nutrient digestion, which subsequently leads to increased nutrient intake. By Carvacrol and thymol in *Zataria multifllora* essential oil also Increase nutrient metabolism in liver cells. Increased nutrient metabolism provides more ovalbumin for egg formation, resulting in a larger Haugh unit (Vakili and Majidzadeh Heravi, 2016). Increases the size of the oviduct, which makes them more active in producing albumin proteins by *Foeniculum vulgare* (Rezae *et al*. 2018).

Imbalance between reactive oxygen species and the antioxidant system disturbs homeostatic equilibrium. The reasons for oxidative stress can be a rise in ROS production rates, a lack of low molecular weight antioxidants, and inactivation of certain enzymes that have antioxidant properties. Oxidative stress, due to ROS synthesis, causes a degradation of reproductive function in laying hens and also leads to lipid peroxidation of the yolk and albumen of eggs. It is confirmed that probiotics can cover up the adverse effects of oxidative stress, enhance the activity of dietary CB and antioxidant enzymes; enrich the activity of GSH-Px, CAT, and T-SOD enzymes with the corresponding decrease in serum MDA content (Zhan *et al*. 2019).

In the current research, through diet supplemented with microencapsulated essential oils of three plants (*Zataria multifllora*, *Foeniculum vulgare*, and *Origanum vulgare*) and Bio-poul and Parsilact probiotics, in comparison with the control diet, Increased catalase, superoxide dismutase, glutathione peroxidase, and total antioxidant capacity ($P<0.05$). The lowest amounts of these antioxidants were seen in the control diet. Moreover, lower malondialdehyde (MDA) content was exhibited due to the plant and probiotic supplements in the laying hen diet, and the highest MDA content was seen in the control group (Table 3).

Plant supplements can be utilized as natural antioxidants in chicken feed, as stated by Gholami Ahangaran *et al*. (2022). This is because they contain several bioactive metabolites with antioxidant activity, as reported. Reduce ROS-Induced oxidative damage to cell membranes in poultry by increased levels of glutathione peroxidase and superoxide dismutase. Plant supplements contain a wide range of terpenoids (such as linalool, carvone, and 1,8-cineole) (Masyita *et al*. 2022). However, terpenoid consumption has been documented to increase the expression of genes coding for superoxide dismutase and glutathione peroxidase in birds (Song *et al*. 2022). In poultry, more of the antioxidant metabolites present in plant supplements are available. This is because they are absorbed from the intestine through the bloodstream after ingestion.

In addition, plant supplements contain several monoterpenes (limonene, p-cymene, and pinene) that are absorbed into the bloodstream and eliminate ROS. In the present study, this mechanism of action partially explains the low malondialdehyde concentration. This is because lipid peroxidation is reduced due to the decreased presence of ROS (Jose and Alejandro, 2023). Research have shown that probiotics can decrease the negative effects of oxidative stress, increase the activity of antioxidant enzymes, improve the activity of catalase, superoxide dismutase, glutathione peroxidase enzymes, and decrease malondialdehyde content (Zhan *et al*. 2019). According to Jahns *et al*. (2015), reported by probiotics and Brevibacillus Clostridium in the diet of laying hens Improves the activity of antioxidant enzymes and reduces lipid peroxidation activity due to the ability of Brevibacillus Clostridium to synthesize butyrate and H2, which eliminate ROS. Ahmed *et al*. (2023) concluded that broilers receiving a combination of probiotics and clove essential oil have the lowest MDA level and the highest SOD and GPx serum enzyme levels when compared to the control group.

The activity of liver enzymes in the serum, such as aspartate aminotransferase, alanine aminotransferase, alkaline phosphatase, and lactate dehydrogenase, utilized to evaluate liver function.

Increased activity of these enzymes is related to the damage of liver cells. The influence of the treatments on the change in the concentration of liver enzymes is presented in Table 4. The results indicated that the microencapsulated essential oils of *Zataria multifllora*, *Foeniculum vulgare*, *and Origanum vulgare* and Bio-poul and Parsilact probiotics could protected the liver cells ($P<0.05$). The maximum levels of liver enzymes (aspartate aminotransferase, alanine aminotransferase, alkaline phosphatase, and lactate dehydrogenase) were seen by control diet. According to Haque *et al*. (2017), cellular toxicity due to cell membrane lipid peroxidation can be indicated by a significant elevation of liver enzyme activity. Serum aspartate aminotransferase and alanine aminotransferase in chickens receiving a combined probiotic supplement were lower than those of controls (Wang *et al*. 2024). Moreover, according to a study by Chen *et al*. (2017), feeding chickens on a diet supplemented with Lactobacillus cultures significantly decreased ALT and AST levels.

Decreased serum alanine aminotransferase concentration in laying hens by a combination of 100 and 200 mg/kg of herbal essential oils and 300 mg/kg of rosemary or cinnamon essential oil (Abo Ghanima *et al*. 2020). Moustafa *et al*. (2020) have found that broiler chickens reduced serum alanine aminotransferase and aspartate aminotransferase levels by 100 mg/kg of *Zataria multifllora* essential oil in the diet as compared to the control.

Table 3 Effect of different experimental treatments on blood parameters in Hy-line W80 laying hens

Dietary treatment	Catalase (CAT)	Glutathione peroxidase (GPX)	Superoxide dismutase (SOD)	Total antioxidant capacity (TAC)	Malondialdehyde (MDA)
C	163.67[g]	119.50[e]	136.00[f]	30.33[f]	41.33[a]
Z.EO	230.33[b]	149.83[b]	176.67[b]	55.50[b]	24.62[f]
F.EO	197.83[d]	130.50[c]	152.67[d]	44.00[d]	27.97[e]
O.EO	214.67[c]	130.33[c]	170.33[c]	45.83[c]	29.45[d]
P.B	189.17[f]	123.50[d]	147.33[e]	41.00[e]	33.85[c]
P.PL	193.50[e]	123.00[d]	146.83[e]	42.17[e]	36.27[b]
(Z.F.O)EO+(B.PL)P	241.83[a]	165.33[a]	187.67[a]	65.50[a]	20.28[g]
SEM	1.08	0.85	0.84	0.58	0.48
P-value	0.0001	0.0001	0.0001	0.0001	0.0001

C: Control; Z.EO: microencapsulated *Zataria multifllora* essential oil; F.EO: microencapsulated *Foeniculum vulgare* essential oil; O.EO: microencapsulated origanum vulgare essential oil; P.B: Bio-poul probiotic; P.PL: parsilact probiotic and (Z.F.O)EO+P(B.PL): microencapsulated essential oils of *Zataria multifllora*, *Foeniculum vulgare*, and *Origanum vulgare* combined with Bio-poul and Parsilact probiotics.
The means within the same column with at least one common letter, do not have significant difference (P>0.05).
SEM: standard error of the means.

Table 4 Effect of different experimental treatments on liver enzymes in Hy-line W80 laying hens

Dietary treatment	Aspartate aminotransferase (AST) (SGOT)	Alanine aminotransferase (ALT) (SGPT)	Alkaline phosphatase (ALP)	Lactate dehydrogenase (LDH)
C	212.05[a]	52.00[a]	298.33[a]	183.83[a]
Z.EO	159.17[e]	30.17[d]	246.00[e]	152.50[d]
F.EO	182.83[c]	34.67[c]	266.83[c]	158.50[c]
O.EO	171.17[d]	35.33[c]	256.50[d]	160.50[c]
P.B	186.00[b]	44.50[b]	270.83[b]	163.83[b]
P.PL	184.17[bc]	42.67[b]	272.33[b]	166.83[b]
(Z.F.O)EO+(B.PL)P	141.33[f]	28.00[e]	245.33[e]	129.50[e]
SEM	1.04	0.67	1.00	0.48
P-value	0.0001	0.0001	0.0001	0.0001

C: Control; Z.EO: microencapsulated *Zataria multifllora* essential oil; F.EO: microencapsulated *Foeniculum vulgare* essential oil; O.EO: microencapsulated origanum vulgare essential oil; P.B: Bio-poul probiotic; P.PL: parsilact probiotic and (Z.F.O)EO+P(B.PL): microencapsulated essential oils of *Zataria multifllora*, *Foeniculum vulgare*, and *Origanum vulgare* combined with Bio-poul and Parsilact probiotics.
The means within the same column with at least one common letter, do not have significant difference (P>0.05).
SEM: standard error of the means.

In agreement with these results, Tayeb *et al*. (2019) have reported a reduction in alanine aminotransferase when using 10 mg/kg of thyme powder in the diet of broiler chickens.

Moreover, using 1, 1.5, and 2 mg/kg of *Zataria multifllora* essential oil led to a reduction in aspartate aminotransferase compared to the control (Attia *et al*. 2017). These findings are consistent with those of Ahmed *et al*. (2023), who found that the inclusion of probiotics in broiler chickens' diets reduced serum levels of the alanine aminotransferase enzyme.

According to the results obtained from Table (5), a significant effect on the expression of the calbindin gene was shown in the intestine by the encapsulated essential oils of three plants, *Zataria multifllora*, *Foeniculum vulgare, and Origanum vulgare*, along with the Biopoul and Parsilact probiotics in the diet of laying hens, but no significant response was found on the ovary.

The highest level of calbindin gene expression in the intestine was observed diet containing the encapsulated essential oils of the three plants, *Zataria multifllora*, *Foeniculum vulgare, and Origanum vulgare*, along with the Biopoul and Parsilact probiotics, showed in contrast the lowest level of calbindin gene expression was appread in the control treatment.

In intestinal cells, the synthesis of the calbindin D28k protein is the outcome of $1.25(OH)_2$ D_3 -stimulated calcium absorption. The absorption of this vitamin is positively influenced by the lipase enzyme. Based on reports, the utilization of the encapsulated essential oils of three plants, *Zataria multifllora*, *Foeniculum vulgare*, and *Origanum vulgare*, due to the enhanced secretion of the lipase enzyme, resulted in an increase in vitamin D absorption. This positively influenced the synthesis of the calbindin D28k protein, which trapped calcium in the poultry intestine (Attia *et al*. 2020).

Table 5 Effect of different experimental treatments on expression level of the calbindin gene in the intestine and ovaryin Hy-line W80 laying hens

Dietary treatment	Expression of the calbindin gene in the intestine	Expression of the calbindin gene in the ovary
C	1.06[g]	5.01[a]
Z.EO	6.27[b]	5.23[a]
F.EO	4.23[d]	5.14[a]
O.EO	5.21[c]	5.21[a]
P.B	2.03[f]	5.10[a]
P.PL	2.92[e]	5.11[a]
(Z.F.O)EO+(B.PL)P	8.315[a]	5.46[a]
SEM	0.04	0.16
P-value	0.0001	0.61

C: Control; Z.EO: microencapsulated *Zataria multifllora* essential oil; F.EO: microencapsulated *Foeniculum vulgare* essential oil; O.EO: microencapsulated origanum vulgare essential oil; P.B: Bio-poul probiotic; P.PL: parsilact probiotic and (Z.F.O)EO+P(B.PL): microencapsulated essential oils of *Zataria multifllora*, *Foeniculum vulgare*, and *Origanum vulgare* combined with Bio-poul and Parsilact probiotics.
The means within the same column with at least one common letter, do not have significant difference (P>0.05).
SEM: standard error of the means.

Furthermore, based on the reports of Rifat *et al*. (2022), the application of medicinal plants in poultry nutrition leads to more deposition of calcium on the eggshell because medicinal plants enhance the anatomical state of the intestine for the absorption of different nutrients, including calcium.

CONCLUSION

The present study provides definitive evidence that co-administration of dietary probiotics and essential oils in plants has a tremendous inhibitory role on lipid peroxidation in the system of laying hens, and therefore enhances the native antioxidant value. At the same time, this composite intervention succeeded to regulate protein building cascades, evoking the advantageous impact on rotation rate of oviposition and growth of significant oological quality traits. This means that the co-supplementation of probiotics and phytobiotics is a very viable, long-term solution to the traditional antibiotic growth supplementation in the poultry industry. Findings of the current study indicate that more mechanistic and applied studies are needed to explain the long-term health effects and optimize practical implementation of such integrative approaches to nutrition.

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