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Original Article

Effect of Probiotic, Thyme, Garlic and Caraway Herbal Extracts on the Quality and Quantity of Eggs, Blood Parameters, Intestinal Bacterial Population and Histomorphology in Laying Hens

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Abstract

In the current Iranian poultry industry, antibiotics are the most frequently used additive in feeds to increase the productivity. Regarding the negative effects on human health due to consuming chicken whose feeds contain antibiotics, finding an appropriate alternative is of a great importance. This study aims to find an appropriate and harmless feed additive to increase the quality and quantity of poultry eggs. Total of 60 laying hens which had been in production for 85 weeks were allocated in a completely randomized design considering five treatments with four replicates and three birds in each. Group one received a layer basal diet with no supplementation which served as control. The second, third and fourth groups received basal diet with 1 mL of herbal extracts (garlic, thyme and caraway) / L drinking water, respectively. The fifth group fed the basal diet plus 1 g of probiotic / kg diet. The number and weight of produced eggs were measured in a daily manner, feed consumption in weekly manner and the egg quality, yolk cholesterol, intestinal bacterial population and effect of treatments on the morphology of the small intestine were measured at the end of experiment after 8 weeks. All treatments showed no overall effect on quality and quantity of produced eggs in comparison with controls; however, the thyme and garlic extracts reduced the cholesterol of serum and yolk relative to the control. The herbal extracts caused a significant decrease in the intestinal bacterial population and probiotic increased villus height in ileum ($P<0.05$).

Key words: Laying hens, Herbal extract, Probiotic, Blood parameters, Quality and quantity of eggs

Introduction

Currently, the use of antibiotics is a common way of preventing diseases and increasing the meat and egg production, as an essential tool for achieving higher yields in poultry systems. Nevertheless, continuous use of antibiotics in feeds leads to problems such as increasing drug resistance, remaining drug in chicken's body and loss of the natural balance of intestinal microflora [1]. Although antibiotics are less frequently used in egg-laying chickens' husbandry systems compared to meat type ones, the increase of pathogenic bacterial resistance to antibiotics in poultry and its transfer to human is a major concern. For this reason, researchers have focused on the use of additives which preserve the desired features and do not have negative effects on health and environment. Among the additives, herbal extracts and their products have a great importance. These products are harmless to human and animals with no undesired side effects. In addition, there is no

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drug resistance due to their consumption. It is clear that herbal plants and their extracts have various biological activities in poultry such as antibacterial properties, anti-parasitic, anti-viral and antioxidant [2-6]. It also determines that they have the ability to stimulate the immune system and endocrine glands [6]. Several clinical reports, including meta-analyses, have exposed a cholesterol lowering effect of garlic in human beings [7]. Allicin (the active compound built by garlic) may decrease the level of serum cholesterol, triglyceride and LDL [8]. Mottaghitalab and Taraz [9] inferred that diets including garlic powder have potential as feed additives, which may be useful in decreasing serum and egg cholesterol in hens. Khan *et al*. [10] also reported that feed expenditure, feed efficiency, egg mass and egg weight were not affected over 6 weeks when 0, 2, 6 and 8% dietary garlic powder was fed to the laying hens. Serum and egg yolk cholesterol condensation decreased with increasing levels of dietary garlic. Thyme (*Thymus vulgaris* L.) is an herbaceous perennial plant belonging to the Lamiaceae family. Thymol, a major component of thyme-essential oils, has been studied for its antimicrobial properties [11]. Carvacrol, an isomer of thymol, is detected in essential oils isolated from oregano and thyme. Like thymol, carvacrol also demonstrates antimicrobial activity [12]. Given their antimicrobial activity, it may be expected that thymol and carvacrol would have positive effects on growth performance in broilers. Such studies showed that thyme plant could be supposed as an alternative natural growth promoter for poultry instead of antibiotics [13]. Studies on Caraway (*Carum carvi* L.) against the pathogenesis of gastric lesions became important with the finding of its antioxidant activity. Some reports found about inhibit superoxide radicals, lipid peroxides and hydroxyl radicals. In addition terpenes including carvones and limonene are known to impel the detoxifying enzyme glutathione S-transferase in some mice target tissues [14]. Al-Kassi [15] reported a decrease in FCR and increase in BW, by using cumin in broiler chickens diets. As mentioned above it has become clear that there is a bite of benefits of caraway as a medical and nutritional resource to be utilized for poultry. The method of action of probiotics would act by producing antibiotic materials, preventing harmful bacteria, altering microbial metabolism, reduce intestine pH and simulating the immune system [16]. The aim of present study was to investigate the effects of probiotic and three herbal extracts additives on egg production and quality of laying hens**.**

Table 1 Nutrient composition of diets of laying hens

Calculated nutrient contents	
ME (kcal/kg)	2800
Crude protein (g)	16
DL-Methionine (mg)	340
L-Lysine (mg)	730
Calcium (g)	4.6
Available phosphorus (mg)	330
TSAA (mg)	600

ME: Metabolisable Energy
TSSA: Total Sulfur Amino Acids

Material and Methods

The study was performed in the Poultry Education Unit, Department of Poultry Science, Faculty of Agriculture, Tarbiat Modares University. Through a completely randomized design, 60 laying hens (Tetra-SL) in 85 weeks of age were divided in 5 treatments and 4 replications with 3 birds in each replication. The experiment carried out in eight weeks. Treatments included thyme, caraway and garlic extracts which were added to the birds' drinking water with the ratio of 1:1000, and the control group received a layer basal diet with no supplementation. The fifth group fed the basal diet plus 1g of probiotic/kg diet. Primalac® (Star-Labs/Forage Research, Inc. Clarksdale, MO, USA) contains a minimum of 1.0×10^8 CFU of *Lactobacillus* sp. organisms per gram. All groups had the same feed formulation. Ingredients and their amounts in this ration are presented in Table 1. Herbal extracts were purchased from Exir-e Gol-e Sorkh Co. (Mashhad, Iran). Brix of thyme, garlic and caraway extracts were 11, 13 and 8, respectively. The lighting program was 16 L/8 D, and the temperature of nests was kept 20°C during the experiment. The birds had free access to water and feed throughout the experiment.

Egg production was recorded daily, and the feed intake was measured at the end of each week by detracting the remained feed from the given feed. Weight of eggs was measured in a daily manner using a digital scale with precision of ±0.01 g. Egg mass was calculated by multiply hen day egg production in average egg weight.

Qualitative properties of eggs including weight, shell thickness, yolk color, shell strength, Haugh unit and yolk weight were measured in the last

week. Yolk of the eggs was separated and weighed, and the egg shells were washed, cleared and incubated in the room temperature for 24 hrs to be dried. Then, the shells were weighed using the digital scale with ±0.01 g precision. Shell thickness was gauged using a micrometer at three points in the center of shells, and the average of measured values was considered as the thicknesses of the shell [17]. The height of albumen was measured using the micrometer and the Haugh unit was calculated using the following formula:

$HU = Log [H + 7.57 - (1.7 \times W^{0.37})]$

Where H is the height of albumen and W is egg weight. The yolk color was assessed using the DSM index of the yolk color.

The shell strength was evaluated using the so-called Eggshell Force Gauge through the method of Er *et al*. [18]. At the end of experiment period, a bird was selected from each experimental unit and 1 mL blood was taken from its wing vein. Blood samples were centrifuged in the lab at 4000 RPM for 5 min. Concentrations of total protein, albumin, cholesterol, triglycerides and HDL in blood serum samples and egg yolk cholesterol were measured using commercial diagnostic kits (Zistshimi Co.) and a spectrophotometer (Jenway Genova MK3, UK).

For measurement of egg yolk cholesterol concentration 1 g of pooled yolks of each replication was added to 9 mL of 2% NaCl solution. Samples were shaken for 2 hrs by an electrical shaker. Then, 1 mL of the diluted yolk was re-diluted by 10 times. Ten µl of this sample was mixed with 100 µl of saline and 1 mL of the enzymatic agent. The same procedure was also implemented for the standard of cholesterol. As the blank sample, 10 µl of deionized water was used instead of sample or standard of cholesterol. The samples were incubated in water bath at 37 °C for 15 min and then the light absorbance at wavelength of 500 nm was read [19].

To assess the microbial population, a bird from each experimental unit was selected and slaughtered at the end of experiment. The contents of cecum were collected aseptically in petri dishes. Collected samples were immediately put on ice, transferred to the lab and prepared for microbial culture. To measure the microbial population, one gram of cecal contents were serially diluted and 10 µl of each dilution was spot on each plates count agar and Mac Conkey agar media to count total aerobes and *E. coli*, respectively.

After incubation, the bacteria were counted in petri dishes and the number of bacteria in the initial volume was calculated using the following formula: Number of bacteria = Number of colonies × (1/Dilution) × Cultured volume.

Then, the logarithms to base 10 of the obtained values were used in CFU/g for later analyses.

For the histomorphological examination after slaughtering the bird, small intestine was removed immediately and from the middle part of three sections (duodenum, jejunum, ileum) the fragments were separated by 1 to 2 cm. The separated segments were washed with PBS[1] and put on the plastic dishes involved with 10% neutral buffered formalin. Paraffin wax technique was used for preparing the thin tissue slides. A rotary type microtome[2] was used for cutting the paraffin sections. The sections cut at a thickness of 6 µm and their wrinkles were smoothed out with warm water (45 ºC) and put on slides. The slides after paraffin removal and dewatering were kept in a solution containing 5 g/L Periodic Acid-Schiff for 15 min [20] and stained with hematoxylin-eosin.

The morphometric variables measured included villi height, crypt depth, and villi width at the top and the base. To measure the villus height and villus width, a microscope of 40× magnification and for crypt depth 100 × magnifications were used and one of the ocular lenses of microscope was equipped with a graticule [21]. Finally, note values based on calibration were converted to mm by using millimeter slides. The mean from 15 villi per sample was used as the average value for further analysis.

The data obtained through the experiment were analyzed using the GLM procedure in SAS 9.1 software [22] and means of experiment groups were compared using Duncan's multiple-range test in 5% level of significance. The following statistical model was as used:

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

Where Y_{ij} is the mean of the *j*th observation of the *i*th treatment; µ is the sample mean; T_i is the effect of the *i*th treatment; and e_{ij} is the effect of error.

Table 2 The effects of herbal extracts and probiotic on the performance of laying hens including egg weight (g), egg mass production, feed conversion, daily feed intake (g) and production

Treatment	Egg weight (g)	Egg mass	FCR(feed intake/kg mass)	Feed intake (g hen^{-1} day^{-1})	Hen day egg production (%)
Control	64.13	33.65	3.55	115.905	52.67
Caraway	69.45	33.22	3.37	111.177	48.01
Garlic	67.01	34.86	3.35	116.419	52.18
Thyme	66.85	36.68	3.15	114.713	54.89
Probiotic	67.65	37.45	3.05	114.125	55.74
P value	ns	ns	ns	ns	ns
SEM	0.761	0.842	0.084	0.722	1.463

SEM= Standard Error of the Means

ns= Non Significant

Table 3 The effects of herbal extracts and probiotic on blood parameters and egg yolk cholesterol of laying hens

Treatment	Cholesterol (mg/dl)	Triglyceride (mg/dl)	HDL (mg/dl)	Albumin (g/dl)	Total protein (g/dl)	Yolk cholesterol (mg/g)
Control	183.55 a	1316 ab	49.49	1.31 b	4.65 b	13.03 a
Caraway	187.33 a	1481 a	54.49	2.44 a	5.99 a	13.13 a
Garlic	143.44 c	1163 b	50.00	1.66 b	4.87 b	11.72 c
Thyme	160.44 bc	1191 b	52.56	1.71 b	4.65 b	12.06 b
Probiotic	163.82 b	1206 b	50.39	1.66 b	4.86 b	12.16 ab
P value	**	*	ns	*	*	**
SEM	3.707	35.340	0.935	0.094	0.128	0.119

a,b,c Means within a column with no common superscript differ significantly ($^{*}P< 0.05$) ($^{**}P< 0.01$)

SEM= Standard Error of the Means

ns= Non Significant

Results

The effect of herbal extracts and probiotic on quantitative performance of laying hens, including productivity, daily feed intake (g), egg mass, feed conversion ratio and weight of eggs (g) has been reported in Table 2. The extracts showed no significant effect on measured quantitative indices in this experiment (P>0.05).

Effect of herbal extracts and probiotic on blood parameters and the yolk cholesterol have been presented in Table 3. As can be seen, the caraway extract significantly increased the total protein and albumin of the blood serum (P<0.05), while the garlic extract led to a significant decrease in cholesterol of hens' serum. The cholesterol decreased in yolks of the eggs supplemented garlic extract in diet (P<0.01).

As is observed in Table 4, the herbal extracts and probiotic have no significant effect on shell thickness, Haugh unit, yolk weight and shell strength (P>0.05).

In continue of Table 4, the effect of extracts and probiotic on populations of total aerobic bacteria and colibacilli is reported. The colony forming units of total aerobic bacteria and colibacilli in digesta of ileo-cecum in the herbal extract treatments showed a significantly lower number compared with control group. Thyme and caraway extracts have most effect on decrease of total aerobic bacteria (P<0.05) on comparison to garlic extract, but thyme extract shows the least rate of colibacilli grown in all of treatments (P<0.05), however, probiotic supplement could not decrease population of bacteria.

In table 5, the effect of probiotic and herbal extracts on intestine morphology in laying hens has been presented. Result of experiments on villus height, villus width and crypt depth in the duodenum and jejunum did not show significant differences between treatments (P>0.05), also crypt depth and villus width in the ileum have not significant differences (P>0.05), but the incremental effect of probiotic treatment on villus height in the ileum than other treatment is evident (P<0.05).

Discussion

As reported in Table 2, consuming probiotic and three herbal extracts do not influence on the performance of egg-laying hens. This is consistent with findings of Reddy *et al*. where feeding hens of strain Babkuk B-300 with 0.02% garlic essence for 8 weeks did not affect their egg production, egg mass, body weight and feed intake [23]. In addition, Yalçın *et al*. did not observe significant changes in body weight, feed intake when they added 0.5 and 10 gram trade garlic powder per kg of feed ration of laying hens [24].

In line with the study of Nahashon *et al*. [25] and Turtuero and Fernandez [26], administration of probiotic into the diet enhances egg production and egg quality. These results are in agreement with finding of Haddadin *et al*. who reported that egg production, size and qualities were improved by addition of probiotic [27]. In contrary, inclusion of the probiotic Bioplus in laying hens diet did not influence on egg production and egg weight [28].

The herbal essences are expected to decrease the level of cholesterol. It has been shown that such herbal essence compounds as broneol, citral, graniol, menthone, fenchone, fenchyl alcohol and beta-ionone reduce the activity of liver HMG-CoA reductase [29-31].

Several studies have revealed the reduction of cholesterol level by the garlic [7,32], which is in agreement with observations in our experiment (Table 3). Some studies, on the other hand, have reported that trade garlic essences, the garlic powder and trade garlic extract may not have decreasing effects on the level of cholesterol [33]. Although the reason for these contradictory results is unknown, it may be related to complementation methods, chemicals' stability and the period of experiments [34]. According to Sklan *et al*. 2% garlic feeding for 14 days decreases the hepatic cholesterol level [35]. Reduction of cholesterol level in plasma by the garlic is mediated by organosulfur compounds affecting the metabolism of cholesterol. These compounds block the secretion of hepatic enzymes involved in converting acetate to cholesterol, and thereby reduce the biosynthesis of cholesterol in the liver which in turn decreases the cholesterol concentration in the blood plasma [36]. Case *et al*. demonstrated that 150 ppm concentration of thymol and carvacrol (ingredients of thyme essence) decreases the serum cholesterol in leghorn hens [37]. This decreasing effect of garlic and thyme on serum and yolk cholesterol levels is consistent with reports in Table 3, with garlic treatment showing stronger effect than thyme. The exact reason for the reduction of cholesterol level by probiotic is not known. The *in vitro* experiments have shown various results that make difficult the general conclusions. However, several mechanisms have been proposed to reduce cholesterol that included absorption cholesterol by growing cells of intestine [38], cholesterol binding to cell surface [39], participation of cholesterol in cell membranes [40], deposition of cholesterol by conjugated bile salts [41]. However, some of these mechanisms depend on the strain bacteria and laboratory conditions [42]. It is reported that broilers feeding with probiotic containing (*Lactobacillus acidophilus*, *Bifidiobacter*, *Aspergillus oryzae*) have lower level of cholesterol compared with control group [43]. This observation is in agreement with our finding.

Feeding caraway has led to mild increasing level of triglycerides in blood serum compared to the control group. This result is inconsistent with Khajeali *et al*. who observed the decrease of triglycerides in serum of broiler fed with 1, 1.5 and 2% caraway concentrations [44]. This contradiction may be due to the biodiversity of medicinal herbs, difference in the effect of herbal alcoholic extracts and the direct use of caraway grains in that study. Use of concentrated extracts allows adding low amounts of them to the feed rations, while less-concentrated substances (such as the whole plant or the dried plants) should be added in higher amounts. To ensure the continuous quality of such products, posing stringent standards for active compounds is necessary, but this is a difficult task due to the diversity in origins of plants or their extracts. Reports in Table 3 indicate the significant increase of total protein and albumin of the blood serum in hens by the caraway extract. The same effect was observed in the cholesterol and HDL level of serum, but it was not statistically significant. The increase of serum fats has generally led to the increase of yolk weight and consequently the egg weight, this effect was not significant. This makes the necessity of more studies.

In Table 4, the effect of probiotic and herbal extracts on intestinal bacterial populations in laying hens has been presented. These results indicate decreasing population of microflora in the birds' gastrointestinal tracts. Multiple *in vitro* studies illustrated that essential oil consisting thymol, carvacrol, *etc*., represented antimicrobial activity

against intestinal microbes such as *C. perfringens*, *S. typhimurium* and *E. coli* [12,45]. Antimicrobial action of essential oil is interceded by lipophilic feature to pierce the bacterial membrane, which releases membrane components from the cells to the external environment [12]. Studies performed with broilers seem to affirm the *in vitro* findings. In a trial reviewed by Losa [46], it was seen that the inclusion of a mixture of plant extracts decreased in 70% the number of broilers infected with *C. perfringens*. A significant reduction of *C. perfringens* colonization was perceived in the intestine of broilers fed diets containing blends of thymol, eugenol, curcumin and piperin, or thymol, carvacrol, eugenol, curcumin and piperin [47] and it has been documented that garlic extracts exert a differential inhibition between beneficial intestinal microflora and potentially harmful enterobacteria [48].

The antibacterial effect of caraway essence against gram-negative bacteria such as *E. coli* and gram-positives such as *S. aureus* has been reported. This effect has been attributed to such compounds as carvene, limonene, carvacrol and linalool [49]. The same effect can be seen for our samples in Table 4. Another study aiming to investigate the antibacterial effect of some medicinal herbal essences against antibiotic-resistant microorganisms such as methicillin-resistant *S. aureus* and vancomycin-resistant *enterococci*, showed a stronger effect of thyme in comparison with other herbal essences [50]. These findings are in thorough consistency with our results of decreasing intestinal microflora of laying hens. It is important to consider that the *in vivo* antimicrobial property of essential oil in birds can be affected by environment conditions and basal diet. As shown in Table 5, result of experiments on villus height and villus width and crypt depth in the duodenum and jejunum did not show significant differences between treatments (P>0.05), also crypt depth and villus width in the ileum have not significant differences (P>0.05), but the incremental effect of probiotic treatment on villus height in the ileum than other treatment is evident (P<0.05).

Table 4 The effect of herbal extracts and probiotic on shell weight, shell thickness, shell strength, Haugh unit, yolk color, yolk weight and intestinal microbial flora in laying hens

Treatment	Shell weight (g)	Shell thickness (mm)	Shell strength (kg)	Haugh unit	Yolk color (DSM)	Yolk weight (g)	Colibacilli (log CFU/g)	Total count of aerobic bacteria (log CFU/g)
Control	5.67	0.286	1.91	63.93	4.75	17.63	6.72 a	7.06 a
Caraway	5.37	0.265	1.78	58.57	4.76	17.95	5.69 bc	5.46 c
Garlic	5.54	0.308	2.47	65.49	4.64	17.25	5.92 b	6.32 b
Thyme	5.43	0.324	1.93	53.91	4.72	17.44	5.09 c	5.45 c
Probiotic	5.62	0.299	2.20	69.98	4.73	17.36	6.13 ab	6.53 ab
P value	ns	ns	ns	ns	ns	ns	*	*
SEM	0.043	0.007	0.085	2.395	0.087	0.192	0.169	0.173

a,b,c Means within a column with no common superscript differ significantly ($^{*}P< 0.05$)
SEM= Standard Error of the Means
^{ns}= Non Significant

Table 5 The effect of three herbal extracts and probiotic on intestine morphology in laying hens

Treatments	Duodenum			Jejunum			Ileum		
	Villus Height (mm)	Villus Width (mm)	Crypt Depth (mm)	Villus Height (mm)	Villus Width (mm)	Crypt Depth (mm)	Villus Height (mm)	Villus Width (mm)	Crypt Depth (mm)
Control	0.976	0.281	0.0508	0.922	0.209	0.0546	0.748 b	0.182	0.0564
Caraway	1.023	0.261	0.0501	0.929	0.232	0.0518	0.717 b	0.176	0.0503
Garlic	1.079	0.249	0.0459	0.901	0.229	0.0496	0.745 b	0.188	0.0545
Thyme	0.993	0.248	0.0484	0.943	0.216	0.0520	0.722 b	0.209	0.0506
Probiotic	1.142	0.212	0.0494	0.936	0.176	0.0524	0.889 a	0.173	0.0546
P Value	ns	ns	ns	ns	ns	ns	*	ns	ns
SEM	0.036	0.011	0.001	0.038	0.008	0.002	0.016	0.011	0.001

a,b Means within a column with no common superscript differ significantly ($^{*}P< 0.05$)
^{ns} = Non Significant

Effect of *Bacillus subtilis* in broiler diets showed that the duodenum and ileum villus height at 28 days of age compared with the control group was significantly increased [51], which is consistent with some of our results. It is reported that villus height is correlated to mitotic cells [51] and higher villus cause more absorption capacity [21].

Garlic consumption seems to improve the quality of produced eggs, especially by reducing the yolk cholesterol. The herbal extracts used in this experiment decreased the intestinal population of bacteria which are competitors of the bird in consuming the feed nutrients. This can also to some extent improve the performance of egg-laying birds in terms of quantitative traits. Since medicinal herbs possess overlapping effects, essences of several plants are recommended to be simultaneously used in feeds of birds, with regard to the specific properties of their biological activities, in order to improve their effects in increasing the yield, the quality and the productivity. The best feed conversion ratio between treatments was related to probiotic (although this difference was not significant) and it appears the villus height would be effective in this event.

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