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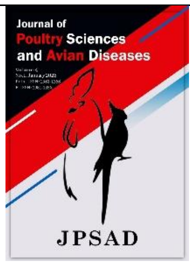

Investigating The Potential of Chasteberry as a Natural Phytobiotic Additive in Broilers

Abouzar Rahmani[1], Mohammad Houshmand[1*], Mokhtar Khajavi[1]

[1] Department of Animal Science, Faculty of Agriculture, Yasouj University, Yasouj, Iran

*** Corresponding author email address**: hooshmand@yu.ac.ir

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A B S T R A C T

To investigate the potential of the chasteberry *(Vitex agnus-castus)* leaf powder (CLP) as a new phytobiotic additive in broilers' diet, 600 one-day-old Cobb 500 broiler chicks were randomly distributed among five experimental groups with four replicates (30 birds/replicate). During the rearing period, one of the following additives was added to the diet of each experimental group: No additive as the control group, antibiotic (Erythromycin) at 0.025% of the diet, 0.3, 0.6, or 0.9% of CLP. The birds were kept under the same management and rearing conditions for 42 days. During the rearing period, the birds were fed starter, grower, and finisher diets from 1-12, 13-24, and 25-42 days of age, respectively. Performance traits, including feed intake (FI), body weight gain (BWG), and feed conversion ratio (FCR), were measured for the entire period (d 1-42) of the study. Also, the status of the immune system, some ileal bacterial count, and jejunum morphology were evaluated. The findings indicated that dietary inclusion of different levels of CLP had detrimental effects on broiler performance, such that FI and BWG decreased while FCR increased ($P<0.05$). No significant difference was observed between the control and CLP groups in view of Ig M, Ig Y, and Total Ig titers against SRBC ($P>0.05$). Dietary inclusion of 0.3 % CLP decreased ileal *E. coli* count compared to the control group ($P<0.05$). Jejunum morphology (villus height, crypt depth, villus height/crypt depth ratio) was not influenced by experimental treatments ($P>0.05$). In conclusion, dietary supplementation with 0.3, 0.6, and 0.9 % CLP had no significant effect on immunity and jejunum morphology but negatively influenced broilers' performance traits and thus could not be recommended as a phytobiotic additive. Investigation of the effects of lower levels of CLP is recommended for future studies.

Keywords: *broiler, chasteberry, ileal bacteria, immunity, performance, Vitex agnus-castus*

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1 Introduction

Public concerns related to the risk of continuous use of antibiotics in the livestock and poultry feed industry (the possibility of developing resistance against pathogenic bacteria and their residue in animal products) have led to restrictions on the use of antibiotic growth promoters in many parts of the world (1). Eliminating antibiotics can negatively influence poultry health and increase production costs (2). This condition has directed researchers to find natural, safe, and effective alternatives to antibiotics. Among the proposed replacements for antibiotic growth promoters, herbs and their products (phytobiotics) have attracted the attention of researchers, and are of particular importance (1). In addition to antimicrobial, anti-inflammatory, and antioxidant effects, they have other benefits, including growth-stimulating, immune system promotion, and hypolipidemic effects (3, 4).

One of the herbs is chasteberry (*Vitex agnus-castus*). Different parts (leaves, flowers, and fruits) of this plant contain various bioactive compounds, including tannins, iridoids, flavonoids, diterpenoids, volatile oils (limonene, pinene, and sabinene), phenolic acids and their derivatives, essential fatty acids (stearic, linolenic, oleic, and palmitic acids) (5-7). Due to the presence of these bioactive compounds, it has beneficial effects such as antifungal, antibacterial, antioxidant, anti-inflammatory, anticarcinogenic, antidiabetic, and hepatoprotective activity (8).

The effects of chsteberry or its products have been investigated on different parameters in layer hens (9-11) and Japanese quail (8). However, there is no report on the use of this plant or its derivative in broiler diets. Considering its bioactive compounds, it can be supposed that dietary inclusion of chasteberry can beneficially affect performance, gut bacterial population, immune function, and digestive tract morphology of broiler chickens. Therefore, the current study was designed and conducted to investigate the effect of different levels of CLP on such parameters in broiler chickens.

2 Materials and methods

This study was conducted in a commercial broiler farm in Semirom City, Isfahan Province, Iran. After preparing the rearing Unit, 600 one-day-old Cobb 500 chicks were purchased from a commercial hatchery and transferred to the experimental site. Immediately after arrival, the birds were distributed among five experimental groups (each with four replicates, 30 birds/replicate) in a completely randomized design. During the rearing period, each experimental group was fed with one of the following diets: basal diet without any additives (control), 2- basal diet + antibiotic (0.025 % Erythromycin), 3- basal diet + 0.3% CLP, 4- basal diet + 0.6% CLP, basal diet + 0.9% CLP.

The chasteberry plant was collected from the foothills of the Zagros Mountains located in Babameidan region, Fars province. After identification of the plant by the Botany Herbarium of Yasouj University, the leaves were separated, dried in a shaded area, then ground and added to the experimental diets in the required amount. As mentioned earlier, no report was found on the effect of chasteberry on broilers. Therefore, considering the dietary levels of various herbs in previous studies and as a starting point, these levels were chosen and used.

Table 1. Feed ingredient and nutrient composition of the experimental diets

Feed ingredient (%)	Starter (d 1-12)	Grower (d 13-24)	Finisher (d 25-42)
Corn	55.80	59.40	64.30
Soybean meal	39.00	36.00	31.50
Calcium carbonate	1.10	1.00	0.60
Commercial concentrate[1]	3.00	2.50	2.50
Vegetable oil	1.00	1.00	1.00
Common salt	0.10	0.10	0.10
Calculated nutrient composition			
ME (Kcal/kg)	2910	2950	3010
Crude protein (%)	22.20	21.20	19.60
Calcium (%)	0.95	0.86	0.75
Available phosphorus (%)	0.50	0.43	0.39
Sodium (%)	0.16	0.17	0.14
Digestible methionine (%)	1.05	0.47	0.42
Digestible lysine (%)	1.21	1.12	1.00

The commercial concentrate contained the following feed ingredients: soybean meal, DL-methionine, L-lysine hydrochloride, L-threonine, vitamin premix, mineral premix, dicalcium phosphate, calcium carbonate, and common salt.

The birds were fed with starter, grower, and finisher diets from 1 to 12, 13 to 24, and 25 to the end of the rearing period (42 d of age), respectively. The diets were prepared using the UFFDA software program and based on the minimum nutritional requirements recommended by the commercial strain. The feed ingredients and nutrient composition of the experimental diets are presented in Table 1. The birds had free access to water and feed throughout the experiment.

The chicks in each experimental unit were weighed in groups at the end of the rearing period, and the BWG was calculated. Feed intake was also measured for the overall period of the study. During the rearing period, chicks that were removed or died were recorded, and the FCR was corrected for those birds. All rearing conditions (temperature, light, ventilation, etc.) were the same for all groups during the 42-day rearing period.

To investigate the efficiency of the immune system, the hemagglutination method was used. In this method, the immune response is measured by injecting sheep red blood cells (SRBC) as an antigen. One milliliter of a 3% suspension of SRBC in phosphate-buffered saline was injected into the wing vein of the chicks. The injection was performed twice, at 28 and 35 days of age. Seven days after the last SRBC injection, blood was collected and serum was separated by centrifugation (3000 g for 10 minutes). The method of Shima et al. (12) was used to measure the titer of total antibodies, 2-mercaptoethanol-resistant antibodies (immunoglobulin Y), and 2-mercaptoethanol-sensitive antibodies (immunoglobulin M).

To determine the ileal bacterial count, samples were taken from its contents under sterile conditions at the time of slaughter (42 days of age). The tubes containing the samples were kept on ice and immediately transported to the Microbiology Laboratory. EMB and MRS media were used for *E. coli* and *Lactobacillus* cultivation, respectively, under anaerobic conditions. For bacterial culture, serial dilutions based on 1-10 were prepared in a sterile phosphate buffer. Next, 100 µl of each dilution was transferred in duplicate into EMB and MRS culture media and plated. EMB culture media were incubated at 37°C under aerobic conditions, and MRS culture media were incubated at 37°C under anaerobic conditions for 48 hours. A colony counter was used to count colonies. For measurement of jejunum morphology parameters, 2-cm segments from its middle part were removed, flushed with physiological saline, and kept in 10% buffered formalin solution until further laboratory processing.

Data analysis was performed using SAS software (13). Means were compared using Duncan's multiple range test (14) at the 5% level.

3 Results

3.1 *Performance traits*

Our results (Figures 1, 2, and 3) indicated that dietary supplementation with antibiotics did not affect performance traits (P>0.05). Thus, no significant difference was observed in BWG of the control (1917 ± 15 g) and antibiotic (1929 ± 6 g) groups. The amount of FI of birds in the control group (3370 ± 20 g) was significantly not different from the FI of the antibiotic group (3366 ± 11 g). Additionally, there was no significant difference in FCR between the control (1.758 ± 0.01) and antibiotic (1.746 ± 0.01) birds.

However, feeding birds with different levels of CLP impaired broiler performance traits. BWG values of birds in 0.3, 0.6, and 0.9 % CLP groups were 1755 ± 40, 1737 ± 26, and 1561 ± 20 g, respectively, which indicated significant decreases relative to the control and antibiotic groups (P<0.05). Moreover, our findings indicate that CLP negatively influenced the FI of birds. Birds in 0.3 %, 0.6, and 0.9 % CLP groups consumed 3215 ± 18 g, 3208 ± 25 g, and 3098 ± 18 g feed, respectively. These values were lower than those of the control and antibiotic groups (P<0.05). The same trend can be observed on the FCR. The birds in 0.9 % CLP had the worst FCR (1.983 ± 0.03), followed by 0.6% (1.848 ± 0.01) and 0.3 % (1.834 ± 0.02) groups (P<0.05), indicating the adverse consequences of CLP on the utilization of feed by broilers.

3.2 *Immunity response*

Results in Table 2 indicated that birds fed with antibiotics had higher levels of Total Ig and IgM than other groups (P<0.05). However, no significant difference was observed between the control and CLP groups (P>0.05).

Table 2. Effects of experimental diets on the antibody response to SRBC (Log2) of broilers at 42 d of age

Parameter	[1] Experimental diets					P value	SEM
	Control	Antibiotic	CLP 0.3%	CLP 0.6%	CLP 0.9%		
Total Ig	4.25[b]	5.80[a]	4.75[b]	5.25[b]	4.50[b]	0.003	0.45
IgY	2.50	2.25	2.00	2.25	2.25	0.68	0.24
IgM	1.75[b]	3.55[a]	2.50[b]	3.00[b]	2.25[b]	0.001	0.16

[1]Control: basal diet without any additives, antibiotic: diet containing 0.025% antibiotic (Erythromycin), CLP 0.3%, CLP 0.6%, CLP 0.9%: diet containing 0.3, 0.6, or 0.9 % chasteberry leaf powder, respectively.

3.3 *Population of ileal bacteria*

The effects of different experimental groups on bacterial count (Table 3) indicated that a lower count of *E. coli* was observed in birds fed with 0.3 % CLP than in the control group (P<0.05), indicating its antibacterial potential. The population of *Lactobacillus* bacteria remained unaffected; thus, no significant difference was noticed among different groups (P>0.05).

Table 3. Effects of experimental diets on ileal bacterial count (CFU/g) of broilers

Parameter	[1] Experimental diets					P value	SEM
	Control	Antibiotic	CLP 0.3%	CLP 0.6%	CLP 0.9%		
E. coli	8.58[a]	8.12[ab]	7.82[b]	8.19[ab]	8.12[ab]	0.02	0.25
Lactobacillus	8.15	7.70	7.65	8.14	8.13	0.21	0.21

[1]Control: basal diet without any additives, antibiotic: diet containing 0.025% antibiotic (Erythromycin), CLP 0.3%, CLP 0.6%, CLP 0.9%: diet containing 0.3, 0.6, or 0.9 % chasteberry leaf powder, respectively.

3.4 *Morphology of the jejunum*

We found (Table 4) that dietary supplementation with different additives, including various levels of CLP, did not change villus height, crypt depth, and villus height to crypt depth ratio in the jejunum (P>0.05).

Table 4. Effects of experimental diets on Jejunum villus height, crypt depth, and villus height to crypt depth ratio of broilers

Parameter	[1] Experimental diets					P value	SEM
	Control	Antibiotic	CLP 0.3%	CLP 0.6%	CLP 0.9%		
Villus height (µm)	1148	1047	1074	1049	1007	0.26	84
Crypt depth (µm)	226	228	196	194	186	0.31	11
Villus height/crypt depth ratio	5.08	4.59	5.48	5.41	5.40	0.20	0.5

[1]Control: basal diet without any additives, antibiotic: diet containing 0.025% antibiotic (Erythromycin), CLP 0.3%, CLP 0.6%, CLP 0.9%: diet containing 0.3, 0.6, or 0.9 % chasteberry leaf powder, respectively.

4 Discussion

The findings of the present study indicated that feeding birds with CLP had detrimental effects on their performance, and a dose-dependent response was observed in this regard, such that the highest level of CLP (0.9%) had the most detrimental effect.

No report was found in the literature regarding the effects of dietary inclusion of chasteberry in broilers. In a recent study, the effects of adding two levels (25 and 50 g/kg diet) of chsteberry seeds were investigated over 35 days in Japanese quail. The findings indicated that the addition of 25 g had no significant effect on performance traits, while the 50 g addition reduced their FI and BWG. The reduced performance was attributed to the presence of compounds such as polyphenols and oxalates in that additive. These compounds, which are known anti-nutritional factors, can reduce the efficiency of dietary nutrient utilization, particularly proteins, and also can inhibit the activity of digestive enzymes (8). However, more reports are available on the effects of chasteberry in laying hens. Nazari et al. (10)

found that dietary supplementation with 1 or 2 % chasteberry fruits did not influence the performance and egg quality of the laying hens. Additionally, in another study, adding different levels of chsteberry fruit powder (0, 0.5, 1, 1.5, and 2%) to the diets of laying hens in the second production period did not affect their performance characteristics (FI, egg production percentage, egg weight, egg mass, and FCR) as well as egg quality characteristics, but reduced serum cholesterol and triglyceride levels (15). Conversely, in another study, feeding laying hens with diets containing 2.5 or 5 g/kg chsteberry improved their performance (FCR and egg production) as well as egg quality traits (9).

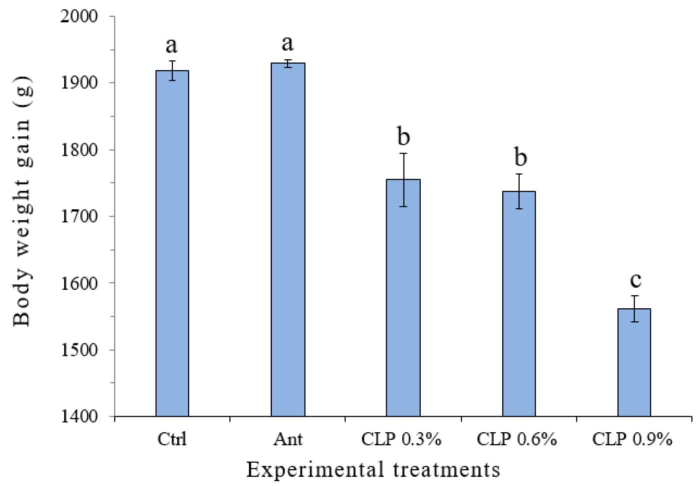

Figure 1. Effects of experimental treatments on overall (d 1-42) body weight gain (g) of broilers.

Ctrl: basal diet without any additives, Ant: diet containing 0.025% antibiotic (Erythromycin), CLP 0.3%, CLP 0.6%, CLP 0.9%: diet containing 0.3, 0.6 or 0.9 % chasteberry leaf powder, respectively.

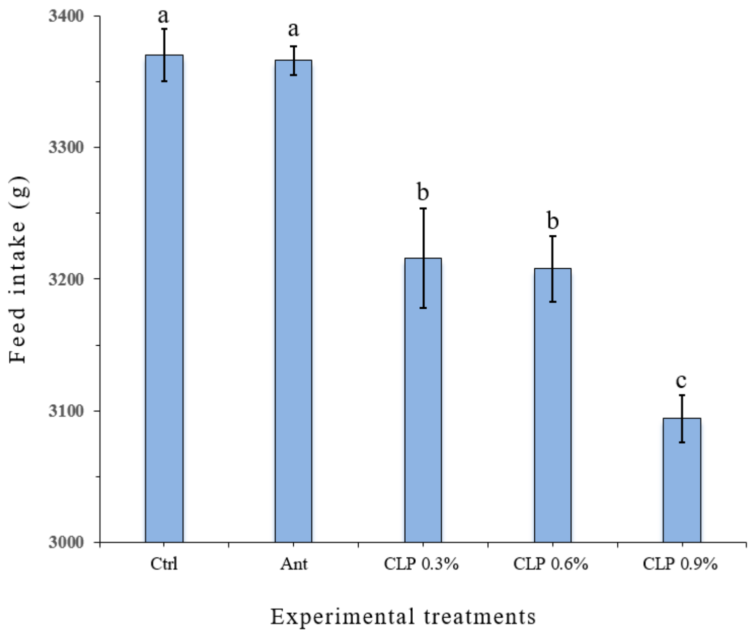

Figure 2. Effects of experimental treatments on overall (d 1-42) feed intake (g) of broilers.

Ctrl: basal diet without any additives, Ant: diet containing 0.025% antibiotic (Erythromycin), CLP 0.3%, CLP 0.6%, CLP 0.9%: diet containing 0.3, 0.6 or 0.9 % chasteberry leaf powder, respectively.

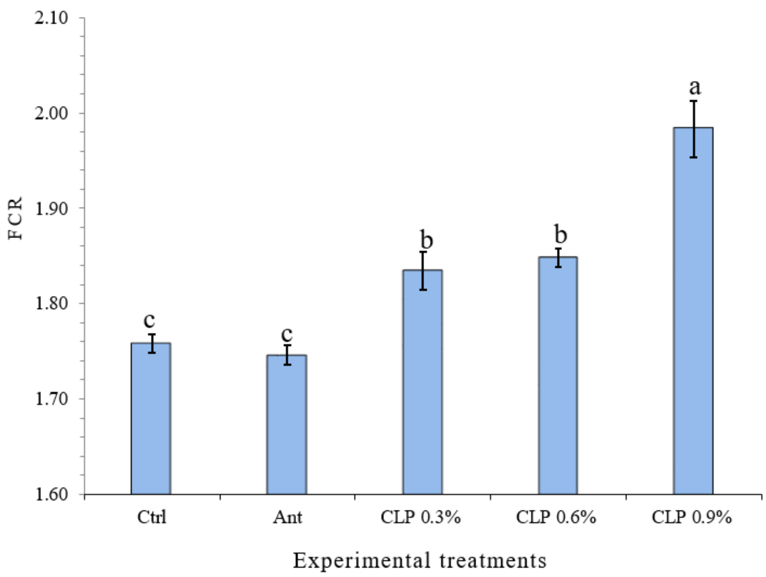

Figure 3. Effects of experimental treatments on overall (d 1-42) feed conversion ratio (FCR) of broilers.

Ctrl: basal diet without any additives, Ant: diet containing 0.025% antibiotic (Erythromycin), CLP 0.3%, CLP 0.6%, CLP 0.9%: diet containing 0.3, 0.6 or 0.9 % chasteberry leaf powder, respectively.

Probably, one of the important reasons for the reduced BWG of the birds fed with the CLP powder was their lower FI during the experimental period (Figure 2). Some herbs contain compounds that reduce the bird's feed intake and consequently growth rate (16). Also, some herbs can affect the gut passage rate, thereby resulting in less feed intake (17). Another important factor is the dietary level of CLP. It has been reported that the effects of phytobiotics are strongly influenced by their dietary dosage (18, 19). Despite the advantages and benefits of phytobiotics for broilers, these additives have the potential to interfere with the absorption of dietary nutrients and alter the gastrointestinal flora, which can lead to challenges. Therefore, determining the optimal dosage to maximize their benefits is of great importance (3). It seems that the dietary CLP levels in the present study were too high and therefore impaired the performance of broilers. Some previous findings have also indicated that the use of high levels of phytobiotics resulted in poor performance in broiler chickens (19-21).

No report was found on the effect of chasteberry on the immunity status of broilers. In line with our findings, antibody titer against sheep red blood cells in laying hens was not influenced by dietary inclusion of different levels (0, 0.5, 1, 1.5, and 2%) of chasteberry fruit powder. The possible reason for this effect could be related to the age of the birds (80-90 weeks old) or the insufficient levels of the additive (15). In another study, dietary supplementation with 1 or 2 % chasteberry fruit powder did not change immunity parameters (antibody titer against SRBC) in laying hens (10).

Herbs and their derivatives have beneficial effects on different functions of the immune system, including phagocytic activity, production of immunoglobulins and cytokines, histamine release, and lymphocyte expression (22). Considering these beneficial effects, it is expected that their dietary inclusion can improve the immune system, but no such effect was observed in the present study.

Intestinal microbial count is strongly influenced by host nutrition status. Herbal additives have a beneficial effect on the digestive tract flora by reducing pathogenic bacteria. This effect is considered the main mechanism of action for these additives. The antimicrobial activity of different phytobiotics against pathogenic bacteria has been indicated in several *in vitro and in Vivo* studies. Different factors, such as the level and physicochemical characteristics of active biochemical compounds and bacterial strains used, influence this property. Due to the presence of natural polyphenolic compounds or flavonoids in some herbs, including chasteberry, they have antimicrobial activity (4, 16). Herbal bioactive compounds can increase the permeability of the bacterial inner membrane, reduce bacterial DNA and RNA synthesis (by inhibiting DNA gyrase activity), denature bacterial cell proteins, reduce ATP production, and finally disrupt the normal functions of bacterial cells (23).

Many anatomical features, including the length of the digestive tract, the height and number of villi, as well as the villus height to crypt depth ratio, will affect the total absorptive surface area of the digestive tract and, consequently, the animal's absorptive ability (24). The morphological changes in villus height and crypt depth in different parts of the digestive tract, such as the duodenum, jejunum, and ileum, have beneficial consequences on the gut functions (25). No report was found on the effect of chasteberry on intestinal morphology, but dietary inclusion of different phytibiotics had inconsistent effects on the broilers' gut morphology (22).

Regarding the benefits and advantages of phytobiotics, it should be mentioned that in the literature, not all of these additives have been effective, and in many studies, their application did not affect the broilers' response (18). Attention should be paid that many factors such as type and additive dose, plant part, the form of herbal supplementation, level of active compounds, administration period, phytochemical action, environment condition, delivery method, gut microflora interactions, the infection, diet composition as well as physiological need, nutritional status and growth phase of the bird can influence broilers response to phytobiotic additives (3, 4, 18, 22). Additionally, climatic and geographical conditions of the plant's growing location, harvest season, maturity stage, and plant part used (roots, seeds, leaves, flowers, bark, buds) will affect the amount of bioactive compounds in the herb and, consequently, their effectiveness (25). Therefore, all of these factors can be considered as possible reasons for the inconsistency in the results.

5 Conclusion

Under the conditions of the current experiment, dietary supplementation with 0.3, 0.6, and 0.9 % CLP had no significant effect on the immunity status and jejunum morphology of broilers. Although a lower count of *E. coli* was observed in birds fed with a diet containing 0.3 % CLP, all levels of this phytobiotic negatively influenced the broilers' performance traits and thus cannot be recommended as a phytobiotic additive. Investigation of the effects of lower levels of CLP is proposed for future studies.

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Conflict of Interest

All authors declare that they have no conflicts of interest.

Author Contributions

M. H. and M. Kh. designed and directed the experiment, analyzed the data. A. R. and M. H. carried out the farm experiment, laboratory measurements and data collection. All authors participated in writing and reading the manuscript.

Data Availability Statement

The data that support the findings of this study are available from the corresponding author upon reasonable request.

Ethical Considerations

In conducting this study, ethical principles have been fully observed.

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