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Effects of Normal and Nano-Capsulated Thyme and Peppermint Essential Oils on Intestinal Morphology and Microbial Population of Broilers Fed on Standard and Low Crude Protein Diets

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ABSTRACT

This study was conducted to investigate the effects of normal and nano-capsulated forms of thyme and peppermint essential oils on intestinal morphology and microbial population of broilers fed on normal and low crude protein diets. The study was carried out with a total of 320 Ross-308 broiler chickens as a 2×2×2 (2 medicinal plant essential oils, 2 forms of oils and 2 levels of diet crude protein) factorial arrangements with 32 groups (10 chicks per replicate) according to a completely random design. This experiment was done in three periods included: Starter (1-10 days), grower (11-24 days) and finisher (25-42 days). The experimental diets had significant effects on intestinal morphology ($P<0.05$), so that the use of the usual form of essential oils while reducing the depth of the crypt, increased the ratio of the length of the villa to the depth of the crypt. In use of peppermint essential oil with standard protein, crypt depth increased, and the ratio of villi height to crypt depth was minimal ($P<0.05$). A decrease in dietary crude protein level reduced the population of lactobacilli in the intestine ($P<0.05$). However, the level of crude protein in the diet and the form of use of the essential oil, as well as the type of essential oil and its form, did not have significant effects on the intestinal microbial population ($P>0.05$). Based on the results of this experiment, it can be stated that in broilers, the use of the encapsulated form of thyme essential oil in diets with standard crude protein levels, improved the intestinal morphology and intestinal non-pathogenic microbial population of broilers. Ten percent reduction in dietary crude protein level had adverse effects on measured traits and is not recommended, and in case of reduction of dietary protein, it is necessary to use peppermint essential oils supplements.

Keywords: Crude protein levels, Essential oil, Intestinal morphology, Microbial population, Nano-capsulation

1. Introduction

Recently, the use of medicinal plants in poultry diets has been on the rise (Darabighane et al. 2017; Gadde et al. 2017). Medicinal plants are added to poultry diets for a variety of reasons. These include antimicrobial, antioxidant, growth enhancement, reducing drug use and chemical additives, improving the taste and marketability of poultry products, and improving blood parameters and immunity (Habibi & Ghahtan 2019; Moryani et al. 2020). Among the high-quality and widely used plants in this field, we can mention the medicinal plants of thyme and mint (Hassan 2019; Nouri 2019). Different types of thyme and mint essential oil are special importance due to their abundant contains of active ingredients such as menthol, carvone, limonene, β-pinene, menthone, α- pinene, geraniol, and effective drug combinations (Rabiei et al. 2011; Morsy 2017; Tariq et al. 2019; Aydın & Barbas 2020). In order to save on essential oil consumption and make it more effective, the use of encapsulated forms of various medicinal plants, including thyme, has become common, and it show promising results in the field of working with medicinal plants (Hosseini & Banabazi 2020; Maty & Hassan 2020).

Since protein is an expensive part of the diet and its excretion into the environment causes many environmental problems, in recent years special attention has been paid to reducing the crude protein content of animal rations by responsible institutions and breeders. Since reducing the level of crude protein in poultry rations has adverse effects such as reduces growth, reduces production, weakens the immune system, so this reduction should be in accordance with the criteria and the use of instructions that while achieving the above, animal performance should not be reduced (Shazali et al. 2019; UlAbiden et al. 2019).

Nanotechnology is a general term that refers to all advanced technologies in the field of nanoscale work. Nanocapsules, are capsules that have a nanometer diameter and can be inserted and encapsulated (Ji et al. 2019; Elbaz et al. 2020). Nano-capsuling of aromatic substances can improves the healing properties and facilitates their access. Because of their small size, these substances increase the mechanism of cell uptake and increase their efficiency (Bayramzadeh et al. 2019). Present experiment carried out to evaluate the effects of using normal and nano-capsulated thyme and peppermint essential oils on intestinal morphology and microbial population of broiler with standard and low crude protein diets.

2. Material and Methods

A total of 320 male Ross-308 broilers (one-day-old) were sexed and weighed before starting the trial, then were divided into 2×2×2 factorial experiments consisting of thyme essential oils (0, 0.2 mL normal and capsulated), peppermint essential oils (0, 0.2 mL normal and capsulated) and crude protein (standard and 10% lower) with 32 units. For copulating, the loading capacity of essential oil at a concentration of 0.5–2% sodium alginate was first investigated, then was encapsulated with biocompatible calcium alginate hydrogel (Dima et al. 2013). Experiment was conducted in a completely randomized design with three growth periods including starter (1 to 10 days), growing (11 to 24 days) and finisher (25 to 42 days). Diets were formulated using the user-friendly feed formulation (UFFDA) program according to the Ross-308 broiler nutrition specification guidelines.

Table 1- Calculated nutrient contents of diets used in broilers

Feed ingredients	*Feeding periods*					
	Starter (1 to 10 days)		*Grower (11 to 24 days)*		*Finisher (25 to 42 days)*	
	Standard CP	*10% Lower*	*Standard CP*	*10% Lower*	*Standard CP*	*10% Lower*
Corn	58.0	63.3	61.7	60.6	65.5	69.0
Soybean meal	37.1	31.8	32.8	28.0	28.0	24.0
Canola oil	1.4	1.4	2.1	2.5	3.0	3.5
Dicalcium phosphate	2.3	2.3	2.3	2.3	2.3	2.3
Salt	0.3	0.3	0.3	0.3	0.3	0.3
Vitamin suppliment[1]	0.25	0.25	0.2	0.25	0.25	0.25
Mineral supplement[2]	0.25	0.25	0.2	0.25	0.25	0.25
L-Lysine hydrochloride	0.1	0.1	0.1	0.1	0.1	0.1
DL- Methionine	0.3	0.3	0.3	0.3	0.3	0.3
Calculated nutrients (%)						
Metabolizable energy (Kilocalorie per Kg)	3000	3000	3100	3100	3200	3200
Crude protein (%)	23.2	20.7	21.5	19.35	19.5	17.55
Calcium (%)	0.81	0.74	0.45	0.55	0.56	0.54
Available phosphor(%)	0.5	0.5	0.54	0.54	0.53	0.63
Sodium (%)	0.19	0.19	0.18	0.17	0.18	0.16
Available Lysine(%)	1.17	1.24	1.05	1.05	0.93	0.93
AvailableMethionine + Cystine(%)	0.63	0.63	0.59	0.59	0.53	0.49

[1]Composition of the supplement of used vitamins per Kg including: Vitamin A (IU) 22500, Vitamin D_3 (IU) 5000, Vitamin E (IU)45, Vitamin K (mg) 5, Vitamin B1 (mg) 4.3, Vitamin B_2 (mg) 16.5, Vitamin B12 (mg) 0.04, Acid Pentatonic (mg) 24.5, Acid Folic (mg) 2.5, Niacin (mg) 74, Pyridoxine (mg) 7.3, Biotin (mg) 0.04. [2]Composition of the supplement of used minerals per Kg including: Manganese sulfate (mg) 248, ferrous sulfate (mg) 125, zinc oxide (mg) 211, copper sulfate (mg) 25, iodate Calcium (mg) 25, Selenium (mg) 0.5, Colin (mg) 625, anti-oxidation (mg) 2.5

At the end of the experiment (42 days old), after 6 hours of starvation, 2 birds (10 birds in each replicate) were selected from each cage that close to the mean weight of the cage and after slaughter, intestinal morphology and microbial population were evaluated.

Data were subjected to statistical analysis according to a completely randomized design as a factorial arrangement of 2×2×2 using the general linear model procedure of SAS (9.2). Data were log-transformed before analysing in case of unequal variances (Hosseinpour et al., 2018). Means were compared using Tukey's tests at 5% probability, according to the following model:

$$Y_{ijkl} = \mu + A_i + B_j + C_k + AB_{ij} + AC_{ik} + BC_{jk} + ABC_{ijk} + \varepsilon_{ijkl}$$

Where; Y_{ijkl}: dependent variable; μ: overall mean; A_i: the effect of thyme essential oils; B_j: the effect of peppermint essential oils; C_k: the effect of crude protein levels; AB_{ij}: Interaction of Factors A×B; AC_{ik}: Interaction of Factors A×C; BC_{jk}: Interactivity of Factors B×C; ABC_{ijk}: Interaction of Factors A×B×C and ε_{ijkl} = the random error.

3. Results and Discussion

3.1. Intestinal morphology

The effects of experimental treatments on intestinal morphology of broilers are presented in Table 2. Experimental treatments had no significant effects on villi and goblet cells of chickens, but resulted in changing the crypt and villi/crypt parameters, and the difference between means was statistically significant ($P<0.05$). Capsulation in contrast to non-capsulation, significantly increased the depth of crypts and reduced the ratio of villi to crypt ($P>0.05$). In interaction between diet crude protein and medicinal plant, using standard protein with thyme had the best result about crypt depth and villi height. About interaction between diet protein level and capsulation, the best result about crypt depth and villi height was observed with normal protein level and no capsulated form of oils. In interaction between medicinal plant essential oil and form of use, the lowest crypt depth was resulted in group with normal crude protein level and non-capsulated. Thyme with non-capsulated form of oil had the lowest crypt depth (P>0.05). In interaction between diet crude protein level, medicinal plant essential oil and form of application, the best result was obtained with normal level of crude protein, thyme essential oil and non-capsulated forms of them (P.0.05). Among the crypt and villi/crypt parameters related to interactions (protein levels and essential oil type), the use of peppermint essential oil with normal protein, crypt depth increased and the ratio of villi height to crypt depth was minimal (P<0.05).

In an experiment was conducted by using thyme and oregano extracts on intestinal morphology, observed that the mixture of these herbal extracts effectively increased the heights of villi cells and reduced the numbers of goblets (Manafi et al. 2018). The antimicrobial properties of thyme were reported by many researchers.

Table 2- Effect of experimental treatments on intestinal morphology (µm)

Effects	*Villi*	*Crypt*	*Villi/Crypt*	*Goblet Cells*
Protein				
Abnormal	1117.8	173.2	6.4	42.1
Normal	1124.1	176.2	6.7	45.8
SEM	26.39	2.65	0.15	2.10
P-value	0.867	0.425	0.395	0.223
Medicinal plant				
Peppermint	1144.8	183.8	6.6	46.1
Thyme	1097.2	165.7	6.6	41.7
SEM	26.39	2.64	0.15	2.08
P-value	0.215	<0.0001	0.969	0.156
Capsulation				
Capsulated	1103.0	181.6^{a}	6.1^{b}	44.9
non-capsulated	1138.9	167.8^{b}	6.9^{a}	42.9
SEM	26.39	2.64	0.15	2.08
P-value	0.345	0.001	0.0009	0.517
Protein× Medicinalplant				
Abnormal×Peppermint	1128.1	184.2^{a}	6.2^{b}	44.6
Abnormal×Thyme	1107.5	162.2^{c}	6.7^{ab}	39.5
Normal×Peppermint	1161.4	183.3^{ab}	6.9^{a}	47.5
Normal×Thyme	1086.8	169.1^{bc}	6.4^{ab}	44.0
SEM	37.32	3.74	0.22	2.94
P-value	0.477	0.031	0.016	0.785
Protein×Capsulation				
Abnormal×Capsulated	1092.6	179.2^{ab}	6.0^{b}	44.4
Abnormal×non-capsulated	1143.0	167.2^{b}	6.9^{a}	39.7
Normal× Capsulated	1113.3	184.0^{a}	6.2^{ab}	45.4
Normal×non-capsulated	1134.9	168.5^{b}	7.0^{a}	46.1
SEM	37.32	3.74	0.22	2.95
P-value	0.703	0.050	0.043	0.371
Medicinal plant×Capsulation				
Peppermint×Capsulated	1111.2	186.8^{a}	6.1	44.1
Peppermint×non-capsulated	1178.2	180.7^{a}	6.9	48.0
Thyme× Capsulated	1094.7	176.4^{a}	6.1	45.6
Thyme×non-capsulated	1099.6	154.9^{b}	6.9	37.9
SEM	37.32	3.74	0.22	2.95
P-value	0.414	0.050	0.948	0.060
Protein×Medicinal plant×Capsulation				
Abnormal×Peppermint×Capsulated	1046.0	182.5^{ab}	5.8	44.5
Abnormal×Peppermint×non-capsulated	1210.2	185.8^{ab}	6.6	44.7
Abnormal×Thyme×Capsulated	1139.2	175.9^{ab}	6.3	44.2
Abnormal×Thyme×non-capsulated	1075.7	148.5^{c}	7.2	34.7
Normal×Peppermint×Capsulated	1176.5	191.0^{a}	6.5	43.7
Normal×Peppermint×non-capsulated	1146.2	175.6^{ab}	7.3	51.2
Normal×Thyme×Capsulated	1050.2	176.9^{ab}	5.9	47.0
Normal×Thyme×non-capsulated	1123.5	161.3^{bc}	6.7	41.0
SEM	52.78	5.26	0.31	4.17
P-value	0.362	0.048	0.828	0.753

a, b, c: Values within a row with different superscripts differ significantly at P<0.05

Since the villi of the gastrointestinal tract are the first place to communicate with nutrients, they play a very important role in digestion and absorption of the small intestine (Sohel et al. 2019; Reynolds et al. 2020). An increase in villi height means an increase in intestinal absorption, and a decrease in crypt depth indicates a decrease in replacement of enterocytes, and a decrease in tissue changes. In other words, increasing the height of the villi and the depth of the crypt are directly related to the increase in renewal in epithelial cells (Rubin & Levin 2016; Martin et al. 2017; Khan et al. 2020).

3.2. Microbial characteristics of the intestine

The effects of experimental treatments on intestinal microbiology are presented in Table 3. In relation to the effects of experimental diets on the beneficial microbial population of small intestine in chickens, a decrease in the level of crude protein in diets reduced the population of lactobacilli in the intestine ($P<0.05$). The type and form of essential oils did not have significant effects on the microbial population of small intestine ($P>0.05$). Regarding the interactional effects of experimental materials on the microbial population of small intestine of chickens, the use of normal protein levels along with thyme essential oil increased the population of lactobacilli and the overall form in the intestines of chickens ($P<0.05$). However, the level of crude protein in the diet and the form of use of the essential oil, as well as the type of essential oil and its form did not have significant effects on the microbial population of the intestine ($P<0.05$). In the study of the three-way effects between the level of crude protein, the type and form of essential oil, the use of normal protein level with the encapsulated form of thyme essential oil, the total microbial population of form and lactobacilli was at its maximum ($P<0.05$).

Table 3- Effect of experimental treatments on intestinal microbiology (CFU/gr)

Effects	*Lactobacylus*	*Choliform*
Protein		
Abnormal	3.78^{b}	5.35
Normal	4.95^{a}	5.53
SEM	0.311	0.365
P-value	0.013	0.713
Medicinal plant		
Peppermint	4.22	5.32
Thyme	4.51	5.56
SEM	0.311	0.365
P-value	0.514	0.654
Capsulation		
Capsulated	4.00	5.64
non-capsulated	4.72	5.24
SEM	0.311	0.365
P-value	0.112	0.437
Protein × Medicinal plant		
Abnormal × Peppermint	4.00^{ab}	5.83^{b}
Abnormal × Thyme	3.55^{b}	4.85^{b}
Normal × Peppermint	4.43^{ab}	4.81^{b}
Normal × Thyme	5.46^{a}	6.26^{a}
SEM	0.439	0.516
P-value	0.032	0.027
Protein × Capsulation		
Abnormal × Capsulated	3.25	5.86
Abnormal × non-capsulated	4.30	4.83
Normal × Capsulated	4.75	5.43
Normal × non-capsulated	5.14	5.64
SEM	0.439	0.516
P-value	0.459	0.240
Medicinal plant × Capsulation		
Peppermint × Capsulated	3.75	5.51
Peppermint × non-capsulated	4.69	5.13
Thyme × Capsulated	4.25	5.77
Thyme × non-capsulated	4.762	5.34
SEM	0.439	0.516
P-value	0.629	0.965
Protein × Medicinal plant × Capsulation		
Abnormal × Peppermint × Capsulated	3.71^{ab}	6.99^{a}
Abnormal × Peppermint × non-capsulated	4.29^{ab}	4.67^{ab}
Abnormal × Thyme × Capsulated	2.78^{b}	4.72^{ab}
Abnormal × Thyme × non-capsulated	4.32^{ab}	4.99^{ab}
Normal × Peppermint × Capsulated	3.78^{ab}	4.03^{b}
Normal × Peppermint × non-capsulated	5.08^{ab}	5.59^{ab}
Normal × Thyme × Capsulated	5.72^{a}	6.82^{a}
Normal × Thyme × non-capsulated	5.21^{ab}	5.69^{ab}
SEM	0.621	0.729
P-value	0.013	0.017

a, b, c: Values within a row with different superscripts differ significantly at $P<0.05$

Numerous studies have shown the antimicrobial properties of plant extracts that can improve the flora and intestinal health of the bird's digestive tract by reducing the number of pathogenic bacteria (Ebrahimi et al. 2016; Yadav et al. 2016; Jin et al. 2020). Disadvantages include the presence of harmful microbes in the gastrointestinal tract, increased protein and amino acid degradation due to the secretion of substances such as urease enzymes by microbes (Sharma et al. 2019; Thapa et al. 2019). On the base of Hajipour et al. (2015) study, using thyme powder and essential oils in broilers diets significantly increased the lactobacilli population and reduced the numbers of E coli, also there have beneficial effect on intestinal cells morphology, So the highest villi and the lowest depth of crypt cells blogged to thyme treatments. Using capsulated form of oregano essential oil in contrast to normal form can improve the intestine microbial population and health status of broilers (Ghasemloo et al. 2017). Recently on the base of Mahdavi & Nobakht (2018) study, the mixture of thyme and ziziphora extracts and increase the population of lactobacillus and reduced of E coli numbers in broiler digestive tract. As a result, the use of medicinal plants essential oils was reduced the gram-negative microbial population of the gastrointestinal tract, so the rate of proteins and amino acids degradation were decreased and more of them are absorbed and stored in the body.

4. Conclusions

Based on the results of this experiment, it can be stated that in broilers, the capsulated form of dietary thyme essential oil with standard crude protein level improved intestinal morphology and changed microbial population on the behalf of Lactobacillus. Also, a 10 percent reduction in the level of crude protein in the diet had adverse effects on measured traits and should be not recommended.

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