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THE EFFECTS OF DIETARY SUPPLEMENTATION OF ENCAPSULATED THYME ESSENTIAL OIL ON GROWTH, PRO-INFLAMMATORY CYTOKINES, AND SERUM AMINO ACID PROFILES OF BROILER CHICKS CHALLENGED WITH *SALMONELLA TYPHIMURIUM*

Ali Olfati[1♦], Seyed Majid Hosseini[2]

[1]Young Researchers and Elites Club, Kermanshah Branch, Islamic Azad University, Kermanshah, Iran
[2]Animal Science Research Department, Khuzestan Agricultural and Natural Resources Research and Educational Center, AREEO, Ahwaz, Iran
♦Corresponding author: a.olfati65@gmail.com

Abstract

***Salmonella Typhimurium* (*S. Typhimurium*) causes inflammation and has adverse effects on the growth of broiler chicks. Meanwhile, plant derivations improve the growth performance and decrease inflammation, but they do not have enough stability. Encapsulation of essential oils is a new strategy for decreasing their instability. This study was conducted to investigate the effects of dietary supplementation of encapsulated thyme essential oil (ETEO) on growth, inflammatory cytokines, and amino acid profiles of broiler chicks challenged with *S. Typhimurium*. Three hundred one-d-old broiler chicks were assigned into 6 groups, and 5 replications per group. The broiler chicks (3 groups) were challenged with *S. Typhimurium* on day 21 and birds received basal diet (positive control), encapsulated thyme (P-ETEO), and non-capsulated thyme (P-NETEO), while other groups received the same diets, but under normal as a negative control, encapsulated thyme (N-ETEO) and non-capsulated thyme (N-NETEO). Growth performance, pro-inflammatory cytokines, and serum amino acid profiles were assessed on day 42. The challenged birds in positive control showed lower growth performance, higher concentration for inflammatory cytokines, and disturbed blood serum concentrations for amino acid profiles compared to the negative control ($P<0.05$). The result showed that dietary supplementation of the ETEO could improve growth performance and amino acid profiles, and also decrease inflammatory responses ($P<0.05$). In sum, *S. Typhimurium* had negative effects on growth, immunity, and inflammation, but dietary inclusion of the ETEO could decrease its negative effects.**

Key words: broiler chicks, encapsulation, pro-inflammatory cytokines, *Salmonella* infection, serum amino acids

Achieving profits in the poultry industry depends on used feed because it comprises a significant part of the costs (Olfati et al., 2018). Contamination of feed with

microbes and pathogens causes infection and economic losses. Feed ingredients are potential sources for contamination with *Salmonella* (Adhikari et al., 2020). *Salmonella* causes heavy economic losses by suppressing growth performance and increasing mortality rates in poultry industry (Abudabos et al., 2019). *Salmonella* infection stimulates immune responses and increases the concentration of pro-inflammatory cytokines (Fasina et al., 2008). Studies have also reported that infectious diseases disturb the amino acid balance in broiler chicks (Izadi Yazdanabadi et al., 2020). It is important to find alternative strategies for controlling pathogenic microbes and promoting the performance of broiler chicks. Antibiotics have been used for the treatment of infections induced by bacteria, but their uses are faced with limitations, such as bacterial resistance. The different strategies are used for alleviation of negative effects of *Salmonella* infection, such as nutrient strategies, medicinal plants for instance.

Medicinal plants and their derivations, such as essential oils, are commonly utilized for promoting broilers performance and health (Hafeez et al., 2016; Liu et al., 2017). Studies have reported antimicrobial properties and growth-promoting effects of the essential oils, and suggested them as natural replacements for antibiotics in broiler chickens feeds (Amerah et al., 2011, 2012). Encapsulated blends of essential oils and organic acids improve growth performance by modulation in gut microflora and improving intestinal absorptive function (Gao et al., 2019; Yang et al., 2019; Tabarraei et al., 2019; Yang et al., 2020). Essential oils are known to have antibacterial and anti-inflammatory properties (Giannenas et al., 2014). However, their uses are faced with limitations, because of their instability and volatility in storage, feed processing, and digestive tract (Gao et al., 2019). Encapsulation of the essential oils protects them from light, storage, loss during feed processing, and intestinal delivery (Yang et al., 2016). This method transfers substances into specific sites of the gastrointestinal tract and allows the slow release of effective materials in a specific moment or environment (Gao et al., 2019).

In the current study, we used thyme (*Thymus vulgaris* L.) essential oil as a medicinal plant for improving economic parameters in the broiler chicks challenged with *Salmonella Typhimurium* (*S. Typhimurium*). It is a medicinal plant that is mostly found in Mediterranean regions. Positive effects of thyme essential oil on growth performance and immune system of broiler chicks have previously been reported (Heydarian et al., 2020; Attia et al., 2017).

Seemingly, thyme essential oil can improve growth performance and inflammation, due to its effects on growth and immunity. We used capsulated form for the protection of essential oil from damage and compared its effects with non-capsulated form. Thus, the present study was conducted to investigate the effects of dietary supplementation of encapsulated thyme essential oil (ETEO) and non-encapsulated thyme essential oil (NETEO) on growth performance, pro-inflammatory cytokines, and serum amino acid profiles of broiler chicks challenged with *S. Typhimurium*.

Material and methods

The preparation of thyme essential oil

Thyme essential oil was purchased from Barij Essence Company (Kashan-Iran) and major compounds were thymol (50.20%), γ-terpinene (20.20%), ρ-cymene (12.02%), carvacrol (3.85%), α-terpinene (3.97%), myrcene (3.72%), α-pinene (2.98%), limonene (1.85%), and linalool (3.02%). Encapsulation was conducted as reported by Heydarian et al. (2020).

Table 1. Feed ingredients and nutrient composition of the prepared diets

Ingredients (%)	Starter	Grower	Finisher
Corn	58.58	62.22	66.14
Soybean meal	24.90	20.51	17.80
Corn gluten meal	6.92	7.25	7.28
Fish meal	5.00	4.38	3.00
DCP	1.64	1.41	1.33
Calcium carbonate	0.99	0.90	0.90
Vit. & Min.Premix[1]	0.50	0.50	0.50
Vegetable oil	0.48	1.27	2.18
L-lysine	0.33	0.29	0.31
Salt	0.22	0.21	0.23
L-Thr	0.14	0.09	0.08
Sodium bicarbonate	0.12	0.12	0.12
L-Arg	0.10	0.80	0.08
DL-methionine	0.08	0.054	0.051
Energy levels			
ME (Mcal/Kg)	3.00	3.1	3.21
Cp (g/Kg)	23.02	21.6	19.4
Ca (g/Kg)	9.65	8.60	7.81
K (g/Kg)	7.61	6.79	6.19
Available phosphorus (g/Kg)	4.92	4.29	3.91
Na (g/Kg)	1.73	1.72	1.69
Cl (g/Kg)	2.16	2.08	1.99
Met (g/Kg)	4.99	4.62	4.29
Met + Cys (g/Kg)	8.31	7.59	7.11
Lys (g/Kg)	13.02	11.41	10.32
Arg (g/Kg)	14.02	12.29	11.11
Thr (g/Kg)	8.71	7.69	6.91
Try (g/Kg)	2.10	1.79	1.70

[1]Minerals-vitamins premix provided the following per kilogram of diet: 99.2 mg manganese, 85 mg zinc, 50 mg iron, 10 mg copper, 0.2 mg selenium, 13 mg iodine, 44,000 IU vitamin A, 7,200 IU vitamin D_3, 440 IU vitamin E, 40 mg vitamin K, 70 mg cobalamine, 65 mg thiamine, 320 mg riboflavin, 290 mg pantothenic acid, 1220 mg niacin, 65 mg pyridoxine, 22 mg biotin and 270 mg of choline chloride.

Broiler chicks and rearing conditions

A total number of three hundred one-d-old broiler chicks (Ross 308) mixed with an initial weight of 43±3 g was prepared by Kimia Joojeh Amol Company (Mazandaran, Iran). A lighting program (23 h light: 1 h darkness) was considered

for all the periods and birds were reared in pens covered with fresh wood shavings. The broiler chicks had unlimited access to fresh water and pelleted feed. Temperature and ventilation were set based on recommendations of Ross Company. All the efforts were conducted to minimize the contamination in all the rearing periods. The diets were formulated based on the Ross catalog requirements (14) for starter (1–10 days), grower (11–24 days), and finisher (25–42 days) (Table 1) (Aviagen, 2014). The used ingredients were analyzed for proximate composition by standard methods suggested by AOAC (2000). The diets were prepared in mash form and formulated in iso-proteinous and iso-energetic forms.

Broiler chicks were assigned into 6 treatments with 5 replications and 10 birds per pen and fed diets supplemented with and diets lacking essential oils as negative and positive controls (2 groups), challenged and non-challenged broiler chicks fed with ETEO (200 mg/kg) as P-ETEO and N-ETEO, and also challenged and non-challenged broiler chicks fed with NETEO (200 mg/kg) as P-NETEO and N-NETEO. Broiler chicks that did not receive essential oil were considered as a positive control (infected with *S. Typhimurium*) and negative control (non-infected with *S. Typhimurium*). Non-challenged and challenged control were considered as negative control and positive control, respectively. The non-challenged broiler chicks were kept in a separate chamber from 1 to 42 days of age but in a rearing condition similar to challenged broiler chicks. On day 21, broiler chicks in challenged groups were orally administrated with a one-off dose of 0.5 mL *S. Typhimurium* (ATCC 14028) containing 1×10^6 CFU/mL of the bacteria that were prepared from the Iranian Research Organization for Science and Technology (Tehran, Iran) as reported by Jazi et al. (2019).

Growth performance

Growth performance was investigated at the end of periods (day 42). The broiler chicks were starved for 3 h and then weighed. Average daily gain (ADG) and average daily feed intake (ADFI) were daily recorded, and feed conversion ratio (FCR) was also calculated.

Pro-inflammatory cytokines and serum amino acid profiles

On day 42 of the trial, blood samples were collected from 3 birds per treatment and centrifuged in 2500 g for 15 min and 4°C. Sera samples were collected and stored at –20°C for analysis. Part of samples were analyzed for interleukin-1β (IL-1β), interleukin-2 (IL-2), interleukin-6 (IL-6), and tumor necrosis factor-α (TNF-α) concentrations by ELISA kits (eBioscience Company, USA) according to the manufacturer's instructions. Other parts of sera samples were analyzed for amino acid profiles using high-performance liquid chromatography by a lithium cation-exchange column (Model 0354100T, Pickering Laboratories, Inc., Mountain View, CA).

Data analysis

The data were analyzed by a completely randomized design in a factorial arrangement as a 2×3 framework with infection (non-challenge and challenge) and essential oils (none, ETEO and NETEO) as main factors and their interactions using SAS software (2005) with Proc ANOVA. The Duncan's test was performed for comparison of the means.

Table 2. The effects of dietary supplementation of encapsulated and non-capsulated thyme essential oil on growth performance of broiler chickens challenged with *Salmonella Typhimurium* in different periods

Groups	Starter			Grower			Finisher		
	ADG (g)	ADFI (g)	FCR	ADG (g)	ADFI (g)	FCR	ADG (g)	ADFI (g)	FCR
Negative control	18.18	29.32	1.63	57.12	89.00	1.59	86.61 a	163.21 a	1.93 d
Positive control	18.52	29.41	1.59	55.61	91.25	1.67	56.12 d	139.87 c	2.51 a
N-NETEO	18.15	29.16	1.62	56.21	92.13	1.65	86.43 a	165.41 a	1.91 d
N-ETEO	18.34	29.37	1.61	56.55	91.45	1.62	87.02 a	162.35 a	1.86 d
P-NETEO	18.73	29.18	1.57	58.14	92.16	1.60	68.41 c	150.61 b	2.21 b
P-ETEO	18.75	29.72	1.58	56.61	91.58	1.61	75.80 b	156.21 b	2.08 c
SEM	0.35	0.325	0.06	0.72	0.65	0.028	1.15	2.56	0.020
					P-values				
Infection	0.456	0.632	0.517	0.423	0.716	0.421	0.001	0.001	0.001
Essential oil	0.623	0.584	0.623	0.512	0.612	0.632	0.002	0.005	0.001
Interaction	0.518	0.632	0.581	0.514	0.518	0.514	0.039	0.012	0.001

SEM: standard error of means. The letters (a–d) show significant difference between groups.

Results

Growth performance

The results for growth performance in different periods are shown in Table 2. The results did not show a significant difference among groups in starter (P>0.05) and grower (P>0.05) periods for growth performance. It means that dietary supplementation of thyme essential oils in both forms did not have significant effects on growth performance in starter (P>0.05) and grower (P>0.05) periods. The results showed significant differences for the finisher period. The results for the effect of infection were significant (P<0.05) and comparing controls showed that challenge with *S. Typhimurium* decreased ADG (P<0.05) and ADFI (P<0.05), and increased FCR (P<0.05). The results for effects of essential oils were significant (P<0.05) and dietary supplementation of ETEO and NETEO under challenge condition increased ADG (P<0.05) and ADFI (P<0.05), and decreased FCR (P<0.05). The results showed that the encapsulation of thyme essential oil could efficiently improve growth performance in the finisher period (P<0.05). A significant interaction was only observed in the finisher period (P<0.05). Based on the results, the challenge with *S. Typhimurium* decreased growth performance in the finisher period, but adding essential oils, especially in encapsulated form improved it compared to positive control. Adding essential oils into diets did not have significant effects compared to the negative control. In sum, essential oils showed their effects under challenging conditions.

Table 3. The effects of dietary supplementation of encapsulated and non-capsulated thyme essential oil on serum concentrations of pro-inflammatory cytokines (pg/mL) of broiler chickens challenged with *Salmonella Typhimurium*

Groups	IL-1β	IL-2	IL-6	TNF-α
Negative control	321.30 d	108.41 d	16.32 d	83.61 d
Positive control	394.26 a	212.32 a	38.65 a	132.61 a
N-NETEO	315.63 d	111.76 d	17.76 d	85.61 d
N-ETEO	317.41 d	112.57 d	18.02 d	86.21 d
P-NETEO	361.25 b	196.21 b	30.61 b	116.95 b
P-ETEO	331.23 c	156.32 c	25.12 c	102.32 c
SEM	3.91	4.21	1.15	1.63
		P-values		
Infection	0.001	0.001	0.001	0.001
Essential oil	0.001	0.001	0.001	0.001
Interaction	0.001	0.001	0.001	0.001

SEM: standard error of means. The letters (a–d) show significant difference between groups.

Pro-inflammatory cytokines

The results for pro-inflammatory cytokines are presented in Table 3. The results showed that the main effect for infection was significant (P<0.05), and the chal-

lenge with *S. Typhimurium* increased the serum concentrations of pro-inflammatory cytokines ($P<0.05$), as controls were compared. However, dietary supplementation of the essential oil, especially in encapsulated form, decreased the serum concentrations of pro-inflammatory cytokines under challenge with *S. Typhimurium* ($P<0.05$). Adding the essential oils into the diet under normal conditions did not have significant effects on inflammatory responses, as compared with negative control ($P>0.05$). A significant interaction between infection and essential oil for all the inflammatory responses was observed ($P<0.05$). Adding essential oils into the diet, especially in capsulated form could decrease the inflammation in challenged broiler chicks, but not non-challenged broiler chicks.

Serum amino acid profiles

The results for the effects of dietary supplementation of encapsulated and non-capsulated thyme essential oil on the serum amino acid profiles of broiler chickens challenged with *S. Typhimurium* are shown in Table 4. The results showed that challenging birds with *S. Typhimurium* did not have significant effects on the serum concentrations of histidine ($P>0.05$), threonine ($P>0.05$), tryptophan ($P>0.05$), valine ($P>0.05$), leucine ($P>0.05$), and isoleucine ($P>0.05$). The main effect for infection was significant for other amino acids ($P<0.05$), so that the challenge with *S. Typhimurium* decreased the serum concentrations of methionine ($P<0.05$), arginine ($P<0.05$), glycine ($P<0.05$), glutamic acid ($P<0.05$) and aspartic acid ($P<0.05$), and increased the serum concentrations of lysine ($P<0.05$) and isoleucine ($P<0.05$), as negative control and positive control, were compared. The results for the main effect of essential oils were significant ($P<0.05$), the results showed that dietary inclusion of ETEO under challenge condition increased the serum concentrations of methionine ($P<0.05$), arginine ($P<0.05$), glycine ($P<0.05$), glutamic acid ($P<0.05$) and aspartic acid ($P<0.05$), and decreased the serum concentrations of lysine ($P<0.05$) and isoleucine ($P<0.05$) compared to positive control. Under challenge condition, dietary supplementation of non-coated essential oil did not have significant effects on amino acid profiles and had similar effects with positive control ($P>0.05$). In sum, the challenge with *S. Typhimurium* disturbed serum amino acid profiles, but coating essential oils could maintain the profile. A significant interaction between essential oil and challenge was observed for the serum concentrations of methionine ($P<0.05$), arginine ($P<0.05$), glycine ($P<0.05$), glutamic acid ($P<0.05$), aspartic acid ($P<0.05$), lysine ($P<0.05$), and isoleucine ($P<0.05$).

Table 4. The effects of dietary supplementation of encapsulated and non-capsulated thyme essential oil on serum amino acid profile of broiler chickens challenged with *Salmonella Typhimurium*

Groups	Met	Lys	Arg	His	Thr	Trp	Val	Leu	Ile	Gly	Glu	Asp
Negative control	201.40 a	258.33 b	533.33 a	120.33	1215.12	60.58	236.41	272.00	155.36 b	721.35 a	219.68 a	33.12 a
Positive control	165.26 c	282.14 a	498.66 b	118.71	1208.32	59.67	249.36	275.58	163.33 a	656.32 b	207.66 b	21.16 b
N-NETEO	205.16 a	257.32 b	536.12 a	121.15	1216.21	60.65	238.15	270.68	152.36 b	715.63 a	220.63 a	33.51 a
N-ETEO	207.19 a	259.16 b	539.14 a	119.71	1219.27	62.12	242.21	271.51	154.11 b	709.48 a	222.13 a	32.91 a
P-NETEO	172.10 c	279.00 a	492.32 b	123.12	1170.25	60.05	244.72	278.13	166.38 a	657.13 b	208.00 b	22.16 b
P-ETEO	189.31 b	262.11 b	528.33 a	119.41	1225.63	57.63	236.68	276.14	157.13 b	706.18 a	221.00 a	32.13 a
SEM	1.63	2.7966	3.65	0.65	7.06	0.356	0.59	0.73	0.76	6.13	1.04	0.93
							P-values					
Infection	0.001	0.023	0.033	0.71	0.761	0.129	0.357	0.851	0.025	0.014	0.032	0.021
Essential oil	0.023	0.019	0.015	0.16	0.751	0.135	0.412	0.815	0.032	0.021	0.025	0.019
Interaction	0.019	0.015	0.021	0.23	0.169	0.215	0.521	0.719	0.023	0.012	0.014	0.016

SEM: standard error of means. The letters (a–d) show significant difference between groups.

Discussion

This study was conducted to evaluate the effects of dietary supplementation of encapsulated thyme essential oil on growth, pro-inflammatory cytokines, and serum amino acid profiles of broiler chicks challenged with *S. Typhimurium*, and the results showed that challenge had negative effects on growth performance, inflammation, and amino acid profiles, but coating essential oils decreased the inflammation, growth deficiency and faulted amino acid balance induced by *S. Typhimurium*. The results did not show positive effects of essential oils on growth performance in starter and finisher periods, but dietary supplementation of the essential oils showed positive effects under challenge and normal conditions. Seemingly, essential oils and other plant derivations show their effects under abnormal conditions. However, the results showed negative effects of *S. Typhimurium* after challenge (day 42). Previous studies have reported the negative effects of *S. Typhimurium* on growth performance (Jazi et al., 2018, 2019). *Salmonella* has negative effects on growth performance by decreasing appetite and nutrient absorption due to damages induced in the intestine system and the use of nutrients for activation of the inflammatory system (Chalghoumi et al., 2009; Shao et al., 2016). Based on our findings, the inflammation was significantly higher and the amino acid profile was imbalanced in the positive control group. In addition, food consumption was significantly lower in positive control compared to the negative control. Seemingly, the challenge with *S. Typhimurium* decreases food consumption and absorption, increases food intake for production of cytokines, and consumes some amino acids for the production of cytokines. All the processes cause insufficient production of protein for growth and result in weak growth in challenged broiler chicks. Dietary supplementation of essential oils, especially in encapsulated form, improved growth performance. The improvement in performance might be attributed to phenolic compounds of thyme essential oil that have antibacterial and antioxidant properties and kill pathogens in the intestinal system, *Salmonella* for instance (Lee et al., 2004). They not only have pharmacological properties but also increase absorption of the amino acids in the intestinal system (Heydarian et al., 2020). Our findings completed previous reports. The results showed that the forms of capsulated and non-capsulated thyme essential oil decreased the inflammation and helped intestinal health. They also worked as appetizers and increased feed consumption. Torki et al. (2015) showed that dietary inclusion of thyme essential oil increased feed consumption in laying hens challenged with thermal stress. The results showed that encapsulated form had better efficiency compared to non-capsulated form that is due to more bioavailability of essential oil compounds in more time and blocking degradation. In sum, encapsulated essential oils provide compounds more times and the compounds increase feed consumption and decrease the inflammation that result in improved growth performance.

The results showed increased serum concentration of pro-inflammatory cytokines in positive control compared with negative control. The increase in the expression of cytokines in cecal tonsils during infection may show their roles in stimulating inflammatory response to *S. Typhimurium* in the chicken intestine. Previous studies have reported the increase in expression of the pro-inflammatory cytokines in

response to infection in broiler chickens (Hong et al., 2006; Chow et al., 2011). An exact mechanism is not presented for the anti-inflammatory properties of thyme essential oil. However, the anti-inflammatory properties of thyme essential oil were attributed to their compounds, thymol, carvacrol, and limonene (Ocaña and Reglero, 2012). They were found as major compounds in the essential oil used in the current study. Thus, active compounds of the essential oil decreased the inflammation and encapsulation protects more compounds from affecting inflammatory responses.

The results showed that infection disturbed amino acid balance. It was reported that environmental factors such as heat stress, microbial environment, and disease condition can influence the serum concentrations of amino acids (Corzo et al., 2007; Star et al., 2012). The profile implicates on the net effect of amino acid appearance from dietary absorption and tissue release and amino acid disappearance that can be due to participation in proteins, oxidation, metabolism, and excretion (Cynober, 2002). The results showed the increase in the concentration of lysine and isoleucine in infected broiler chicks that might be attributed to its use for participation in cellular response. Seemingly, lysine and isoleucine are metabolized from tissues and are utilized for immune response, the production of inflammatory cytokines for instance. The decrease in arginine might be attributed to its use for the production of nitric oxide (Izadi Yazdanabadi et al., 2020). The decrease in other amino acids might be attributed to their participation as fuel sources for inflammatory responses (Rochell et al., 2016). The results showed that encapsulated thyme essential oil improved profile in challenge condition that is due to its ability in killing bacteria and inflammation and increasing food consumption.

In sum, the challenge with *S. Typhimurium* had negative effects on growth performance and amino acid balance and increased inflammation, but encapsulation with thyme essential oil could alleviate its negative effects. Encapsulated and non-capsulated forms of the essential oil could not have significant effects under normal conditions. It could be suggested to apply encapsulated thyme essential oil for improving growth performance, decreasing inflammation, and balancing amino acids in broiler chicks challenged with *S. Typhimurium*.

Conflict of Interests

The authors report no conflicts of interest in this review paper.

Authors' contributions

Ali Olfati: writing, concept design and data collection. Seyed Majid Hosseini: concept design and data collection. All authors read and approved the final version of this paper.

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Ethical considerations

This article does not contain any studies involving human participants or animals performed by any of the authors.

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