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Synergetic effect between Thyme oil and betaine as growth promotor, performance enhancer and growth responsible gene stimulant in broiler chickens

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Abstract: The study aimed to evaluate the impact of different levels of betaine and thyme oil as growth promoters in broiler chickens. 600 broiler chickens (Cobb 500) a total number for experimental design at 1day of age were randomly chosen and distributed into 10 groups (60 broilers chickens / each group), that were fed different doses of betaine and thyme oil, except for the control group. broiler chickens of each group were nearly equal in averages body weight from one day of age. Each group sub-divided into three replicates, each of 20 broiler chickens. The experimental group was processed broiler chickens under similar hygienic and environmental conditions as the control group. Broiler chickens were housed in floor systems, and experimental treatments significantly improved growth performance and cecum properties comparison the control group, liver function, triglycerides, total cholesterol, hormones markers and immune and growth response genes). The highest live body weight of EG10 and EG9 groups were (2046.08 and 2009.75 g) as well as found in other experimental group when comparison the CG1 group (1789.5 g) in a row. Moreover, feed conversion ratio of EG10 and EG9 groups were (1.48 and 1.51) as well as found in other experimental group when comparison the CG1 group (1.70). While EG10, EG9 and other experimental group when comparison the CG1 group had a significant enrichment effect on the intestinal beneficial bacteria. The study concluded that the most effective interaction between betaine 500 mg and the experimental group ten had the most significant effect and thyme oil (50 mg) in liter as well as interaction between betaine 250 mg and thyme oil (50 mg) in liter of water has highly significant effect on live body weight and FCR. The study demonstrated a significant positive impact on blood physiology in experimental groups EG10, EG9, EG2, and other experimental groups respectively comparison the control group (CG1) without any significant side effects on broiler chickens.

Keywords: *Immunity, Betaine, Performance, Broiler Chickens, Thyme oil, Gene Expression.*

Introduction

The main advantage of natural growth promoters is to low risk regarding bacterial resistance or undesired residues in broiler chick's products such as meat, milk or eggs (Männer, 2011). Poultry is one of the most common food industries globally Chicken is the most popularly raised type, with over 90 billion tons of chicken meat produced each year (FAO, 2017). The European Union has banned the use of antibiotics at sub-therapeutic levels for disease prevention or growth promotion They've also prohibited antibiotics as growth promoters altogether (Delsol et al., 2005). Therefore, the search for alternative feed supplements has surged, with essential herbs being a top choice as a replacement for antibiotic growth promoters, potentially providing similar benefits. (Huyghebaert et al., 2011). The requirement for total sulfur amino acids can be met by either methionine alone or a suitable mixture of methionine and betaine. (Moon and Gatlin, 2002). In addition, choline oxidation may be a rate-limiting step in betaine synthesis, as pig diets with dietary betaine showed higher efficiency in addition to choline. (Siljander-Rasi et al., 2003). Betaine has the potential to reduce poultry's methionine requirement by 40-60%. (Harding et al., 2001). The study found that growth performance and feed efficiency in broiler chicks were linearly influenced by methionine and betaine supplementation, with betaine being twice as efficient as methionine. (Virtanen and Rosi, 1995). Multiple studies have shown that betaine and methionine cannot be substituted. (Schutte et al., 1997 and McDevitt et al., 2000). Feed additives are widely recognized as a significant alternative to antibiotics in animal production as feed additives. Losa and Kohler (2001) reported the study investigated the reduction of *Clostridium perfringens* in the intestine of poultry supplemented with a commercial preparation of essential oils in its diet. Marija et al. *(*2015) stated that essential oils obtained from plant extracts possess antimicrobial and antioxidant properties, derived from volatile molecules produced by secondary metabolism of aromatic and medicinal plants. The study suggests that it may disrupt the bacterial cell wall's function by affecting its lipopolysaccharide content, leading to increased cell membrane permeability. Faleiro (2011). Natural phytogenic feed additives like garlic, cinnamon, anise, coriander, oregano, pepper, chili, nettle, rosemary, and thyme can potentially enhance farm animal performance. (Živković et al., 2019 and Alloui et al., 2014). The majority of studies on the effects of various herbs on broiler performance have reported positive effects on gain, feed conversion and carcass quality (Puvača et al., 2014)

So, from the previously mentioned fact, the study aims to investigate the impact of different levels of Betaine supplementation and Thyme oil on broiler chickens' productivity, immune parameters, caecum properties, metabolic response, and growth-related gene expression. Results show positive effects on production performance, suggesting these additives can be a perfect feed for broiler chickens, maintain health, and improve poultry growth.

MATERIALS AND METHODS

Ethical approval

We were collected all samples as per standard procedure without giving any stress or harm to the animals and the study was conducted according to Cairo University at Institutional Animal Care and Use Committee (CU-IACUC) Veterinary Medical and Agricultural Sciences Sector, Egypt under approval code # CU/II/F/14/24#.

Experimental design

600 broiler chickens one-day old with average initial body weight of 45 ± 1.27 g strain Cobb 500, both genders, were randomly assigned to 10 similar groups (60 chickens individuals each) under identical environmental conditions; Control group (CG1) processed to feed on balanced diet without any additives in drinking water, Experimental group 2 (EG2) was processed a balanced diet containing 50 mg thyme oil per liter of water, Experimental group 3 (EG3) processed to fed balanced diet with the addition of 50 mg betaine per liter of water,

Experimental group 4 (EG4) was processed a balanced diet containing 100 mg betaine per liter of water, Experimental group 5 (EG5) was processed a balanced diet containing 250 mg betaine per liter of water, Experimental group 6 (EG6) was processed a balanced diet containing 500 mg betaine per liter of water, Experimental group 7 (EG7) was processed a balanced diet containing with a mixture of 50 mg betaine with 50 mg from thyme oil at liter of water, Experimental group 8 (EG8) was processed a balanced diet containing with a mixture of 100 mg betaine with 50 mg from thyme oil at liter of water, Experimental group 9 (EG9) was processed a balanced diet containing with a mixture of 250 mg betaine with 50 mg from thyme oil at liter of water and finally, Experimental group 10 (EG10) was processed a balanced diet containing with a mixture of 500 mg betaine with 50 mg from thyme oil at liter of water. The experiment concluded with the slaughtering of animals under tranquilization in accordance with Islamic guidelines, as reported by Awan and Muhammad Sohaib (2016).

Thyme oil have sourced from center of Al-Gabry for extract essential oil at Giza, Egypt. Betaine sourced from Regional Center for Food and Feed (RCFF), Giza governorate, Egypt. As well as product of Thyme underwent testing for its active ingredients at the Organic Pollutants department by Gas chromatography–mass spectrometry. Experimental diets were created to meet all the nutrient requirements of broiler chickens which calculated, The diet of Cobb 500 hybrid was used according to the manufacturer's ambient recommendations, as illustrated in the standard diet table (1) as mentioned to (Stanišić et al.,2023). Gas chromatography–mass spectrometry to analysis of thyme oil's phenolic compounds to revealed the highest active ingredients such as Carvacrol that showed retention times of compound at 9 minutes and also, Thymol that showed retention times of compound at 10 minutes., at the data presented in figure (1).

Table 1. Composition and calculated chemical analysis of experimental diet of broiler chickens

Ingredients (%)	**Starter diet (1– 21 day)**	**Finisher diet (22–35 day)**
Yellow corn	50.74	58.82
Soybean Meal (44%)	41.96	33.73
Corn oil	3.09	3.96
Di-calcium phosphate	1.72	1.35
Calcium carbonate	1.07	0.90
Sodium bicarbonate	0.15	0.16
L-Lysine (99%)	0.2	0.13
L-Threonine	0.09	0.03
DL-Methionine (99%)	0.23	0.18
Salt	0.25	0.24
Vitamin *	0.25	0.25
Mineral**	0.25	0.25
Total	100	100
Chemical composition* **		
Crude protein(CP) **%**	22.71	18.93
Crude fiber(CF)**%**	4.21	3.84
Ether extract(EE)**%**	5.01	5.65
Dry Matter(DM)%	89.40	89.30
Metabolism energy(ME) Kcal/Kg	2930	3100

* Vitamin following is provided per kg of diet: vitamin A, 9000 IU; vitamin D3, 2000 IU; vitamin E, 36 mg; vitamin K3, 2 mg; vitamin B1, 1.75 mg; vitamin B2, 6.6 mg; vitamin B6, 2.94 mg; vitamin B12, 0.015 mg; nicotinic acid, 29.7 mg; folic acid, 1 mg.

** Mineral following is provided per kg of diet: Calcium Pantothenate 9.8 mg; choline chloride, 250 mg; Mn, 99.2 mg; Zn, 84.7 mg; Cu, 10 mg; Fe, 50 mg; Se, 0.2 mg; I, 0.99 mg. *** The calculation was made based on (Stanišić et al.,2023).

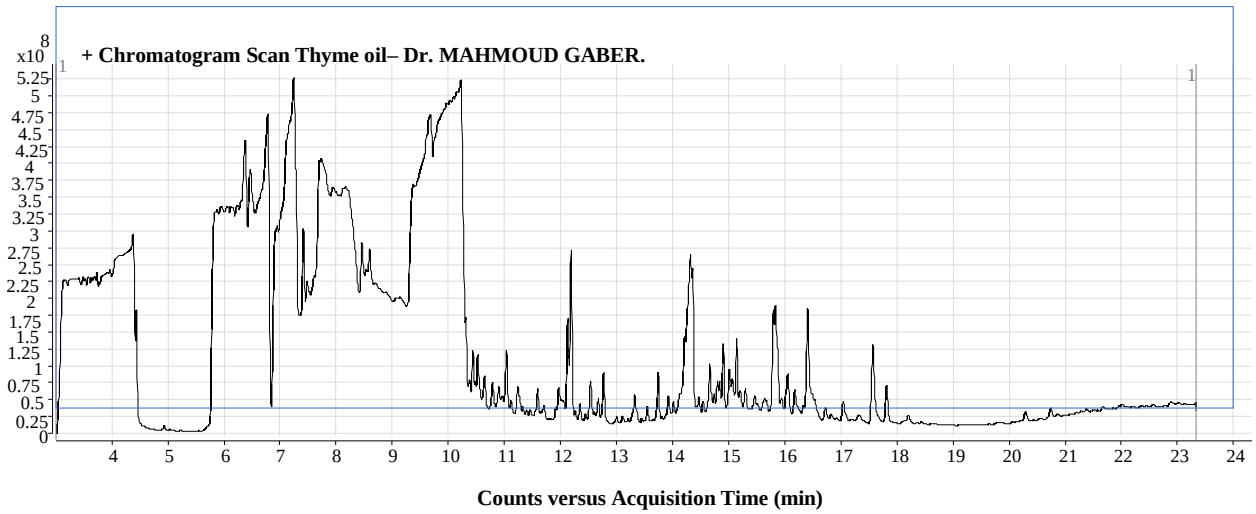

Figure 1. The GC-MS/MS instrument was used to analyze the chromatogram of Thyme oil.

Determination of thyme oil

Gas chromatography–mass spectrometry to analysis of thyme oil's phenolic compounds to revealed the highest active ingredients such as Carvacrol that showed a peak area % of compound at 23.22 % and also, Thymol that showed a peak area % of compound at 13.71 % as determined by Patricia et al. (2013) at the data presented in figure (1).The data from Figure (1) that confirms the main components of thyme, Thymol and Carvacrol, as per previous studies by Karousou et al. (2005), Ortega-Nieblas et al. (2011) and Amiri (2012)

Growth performance parameters

Broiler chickens in experimental and control groups were fed ad libitum were breeding under similar management conditions, diet and water, with measured to determine average live body weight at (Table 2) and actual feed conversion ratio at (Table 3). Broiler chickens were individually weighed weekly before being served in the morning meal, a process that continued for a period of five weeks. The feed conversion ratio (g feed/g body weight) and body weight values were recorded.

Caecum properties

The experiment involved killing nine broiler chickens, collecting their gastrointestinal tract, and estimating caecum count to determine cecal microflora for beneficial and pathogenic bacteria. Cecal appendix fluid was collected and the beneficial bacteria count *of Enterococcus species* and *Lactobacillus acidophilus* was measured without delay, a selective dehydrated media Slantz and Bartly agar; de Man Rogosa and Sharpe agar (MRS) from company (LabM a Neogen, United Kingdom), in a row. The pathogenic bacteria content was determined using Brilliant Green agar and Violet Red Bile agar, with *Salmonella Spp* count and *E. coli* count estimated through serial dilutions and inoculation into sterile petri dishes. Samples of caecum microbial species count were prepared, analyzed, agar was added, and the temperature required for incubating the bacteria was determined according to methods was according to Ahmed et al. (2020).

Serum biochemical parameters

Collecting blood samples has been from nine broiler chickens per treatment after slaughtering, also, plasma was collected and stored at (-20°C) to estimate total protein and also, aspartate aminotransferase (AST) also, alanine aminotransferase (ALT), total cholesterol, triglycerides. These constituents were measured using commercial kits from (DiaSya Diagnostic, Germany) according to manufacturer's instructions. the Analysis methods of blood samples previous as described by Ahmed et al. (2020). Radioimmunoassay (RIA) used to determined serum hormones (T3, T4 and TSH) according to Wartofsky and Burman (1982) and Bolognani et al. (2001).

Relative quantification of gene expression:

The GLM procedure was used to analyze the effects of treatments on gene expression, comparing each sample's relative expression to a control comparison the β-actin gene and calculated according to the delta-delta Ct guide "ΔΔCt" method as reported by Pfaffl. (2001) and (Yuan et al. (2006).

Statistical analysis

Final data analyzed by General Linear Models (GLM) procedure of the SAS program SAS (2004). Duncan's multiple range test was utilized to compare liver gene expression levels, revealing statistically significant treatments at ($P < 0.05$).

The statistical model used during the experimental period is as follows:

$Y_{ijk} = \mu + T_i + B_j + (TB)_{ij} + e_{ijK}$

Where:

Yijk = is the kth outcome in the i^{th} level of T and j^{th} level of B.

μ = is the overall mean,

T_i = the main effect of factor (T).

B_j = the main effect of factor (B).

$Ti*B_j$ =the effect of the interaction between factor T, B

e_{ijk} = is a random error.

The Duncan of multiple range test was used to identify significant differences among means (Duncan,1955).

RESULTS AND DISCUSSION

Growth performance

Data found in Table (2) show averages of live body weight was significant effect by using thyme oil extract, betaine and both as feed additives revealed that interaction between thyme and betaine concentration 500 mg, also 250 mg /L had significantly the highest value of live body weight ($P<0.05$) when comparison the control group followed by the interaction thyme and betaine (Table 2). These results were significantly ($P<0.05$) in agreement with those obtained with Placha et al. (2013) explained that Thyme oil, which improves intestinal health, was associated with the highest weight gain. In addition, these results agreed with those reported by Waldenstedt et al. (1999) who stated that betaine as a single supplement increase body weight. These results agreement with Attia et al. (2005) reported that in general betaine supplementation at medium or high dose improved body weight gain, betaine supplementation at 0.035 and 0.070% linearly increased body weight gain by 5.1 and 9.0% respectively.

The study concluded that thyme, betaine, and mixtures had better effects on performance properties than the control group, suggesting a synergistic effect between essential oil and betaine in poultry nutrition.

Table 2. The effect of The effect of Thyme, Betaine and interaction between them on average live body weight (g/chicken/d) of broiler chickens during the experimental periods aged 35 days

Treatments(mg/L)	Live Body Weight (g) at day			
	Initial weight	First period (1-13 d)	Second period (14-28 d)	Whole period (1-35 d)
Thyme(Thy)				
Thy0= Control	45.97±0.04	514.9±2.22^{b}	1465.5±6.49^{b}	1789.5±8.27^{b}
Thy1= 50	47.3±1.39	539.5±2.21^{a}	1496.2±5.87^{a}	1954.5±7.12^{a}
P-value	0.3313	<0.0001	0.0001	<0.0001
Betaine (Bet)				
Bet0=Control	46.09±0.07	529.20±4.41^{a}	1422.88±12.02^{c}	1765.43±16.23^{d}
Bet1=50	46.01±0.07	524.00±3.48^{a}	1496.61±7.76^{b}	1875.30±10.90^{b}
Bet2=100	45.86±0.07	525.87±3.27^{a}	1443.04±7.38^{c}	1803.89±10.83^{c}
Bet3=250	49.39±3.49	524.08±3.15^{a}	1510.17±8.52^{b}	1950.59±10.91^{a}
Bet4=500	45.88±0.06	532.83±3.92^{a}	1531.54±9.38^{a}	1967.06±12.41^{a}
P-value	0.4265	0.3104	<0.0001	<0.0001
Interaction				
Thy0 X Bet0	46.12±0.10	505.75±5.18^{d}	1387.41±16.51^{f}	1643.91±15.05^{h}
Thy0 X Bet1	46.05±0.10	519.00±5.37^{c}	1482.03±12.52^{c}	1805.86±12.76^{f}
Thy0 X Bet2	45.83±0.11	519.25±4.65^{c}	1437.33±10.28^{e}	1712.67±10.35^{g}
Thy0 X Bet3	45.94±0.10	514.16±4.29^{c}	1513.90±12.76^{b}	1889.40±13.91^{d}
Thy0 X Bet4	45.90±0.10	516.33±5.19^{c}	1505.50±13.65^{b}	1886.69±15.22^{de}
Thy1 X Bet0	46.05±0.10	552.66±5.75^{a}	1457.17±16.40^{d}	1876.83±18.60^{e}
Thy1 X Bet1	45.98±0.09	529.00±4.40^{b}	1511.19±8.87^{b}	1944.74±12.16^{c}
Thy1 X Bet2	45.90±0.09	532.50±4.48^{b}	1448.75±10.63^{d}	1893.56±9.05^{d}
Thy1 X Bet3	52.84±6.98	534.00±4.27^{b}	1506.50±11.39^{b}	2009.75±12.77^{b}
Thy1 X Bet4	45.86±0.09	549.33±5.09^{a}	1557.58±12.08^{a}	2046.08±13.18^{a}
P-value	0.4173	0.0007	0.0201	0.0005

$^{a-h}$ Mean± SME in same row with different superscripts are significantly (P<0.05) different, Significance (P<0.05); Non-Significant (P>0.05).

The study presented in Table (3) displays the FCR. that EG10 and EG9 (1.48 and 1.51) had the lowest FCR groups and betaine followed by the other experimental groups, Thyme and mixture both had significant better effects on growth performance properties respectively if comparison the control group. indicating a synergistic effect between essential oil and betaine in poultry nutrition., While the value obtained in the control group(CG1) was the highest indicated that, interaction between thyme and betaine had the best effect on FCR followed. This result was confirmed by those obtained by El-Husseiny et al. (2007) showed that The study found that the feed consumption, conversion, and efficiency increased with an increase in betaine levels up to 0.75 g/kg diet. The findings contradict Waldenstedt et al.'s (1999) findings, which suggested that betaine supplementation in broilers did not significantly impact feed intake and conversion ratio. In addition, Hassan et al. (2005) reported that the addition of betaine to the diet improved feed conversion in broiler.

Table 3. The effect of Thyme, Betaine and interaction between them on Feed Conversion Ratio (FCR) of broiler chickens during the experimental periods aged 35 days

Treatments(mg/L)	Feed Conversion Ratio (feed g: gain g) at day		
	First period(1-13 d)	Second period(14-28 d)	Whole period(1-35 d)
Thyme(Thy)			
Thy0= Control	1.05±0.01^{a}	1.40±0.01^{a}	1.70±0.01^{a}
Thy1= 50	0.97±0.01^{b}	1.36±0.01^{b}	1.55±0.01^{b}

P-value	<0.0001	<0.0001	<0.0001
Betaine (Bet)			
Bet0=Control	1.05±0.01^{a}	1.45±0.01^{a}	1.73±0.01^{a}
Bet1=50	1.02±0.01^{b}	1.37±0.01^{c}	1.62±0.01^{c}
Bet2=100	1.01±0.01b	1.41±0.01^{b}	1.68±0.01^{b}
Bet3=250	1.00±0.01^{b}	1.35±0.01^{cd}	1.56±0.01^{d}
Bet4=500	0.97±0.01^{c}	1.33±0.01^{d}	1.54±0.01^{d}
P-value	<0.0001	<0.0001	<0.0001
Interaction			
Thy0 X Bet0	1.15±0.01^{a}	1.50±0.01^{a}	1.85±0.01^{a}
Thy0 X Bet1	1.04±0.01^{b}	1.39±0.01^{c}	1.68±0.01^{c}
Thy0 X Bet2	1.02±0.01^{c}	1.42±0.01^{b}	1.77±0.01^{b}
Thy0 X Bet3	1.03±0.01^{c}	1.35±0.01^{d}	1.61±0.01^{d}
Thy0 X Bet4	1.01±0.01^{d}	1.36±0.01^{d}	1.61±0.01^{d}
Thy1 X Bet0	0.95±0.01^{f}	1.40±0.01^{c}	1.62±0.01^{d}
Thy1 X Bet1	1.00±0.01^{d}	1.35±0.01^{d}	1.56±0.01^{e}
Thy1 X Bet2	0.99±0.01^{de}	1.41±0.01^{b}	1.60±0.01^{d}
Thy1 X Bet3	0.98±0.01^{e}	1.35±0.01^{d}	1.51±0.01^{f}
Thy1 X Bet4	0.93±0.01^{g}	1.30±0.01^{e}	1.48±0.01^{g}
P-value	<0.0001	0.0004	<0.0001

$^{a-g}$ Mean± SME in same row with different superscripts are significantly (P<0.05) different. Significance (P<0.05); Non-Significant (P>0.05).

Cecum properties

Table (4) demonstrates that essential oils and betaine positively affect the caecum properties of poultry, improving, controlling harmful bacteria, and stimulating beneficial bacteria growth, comparison the control group. This finding was confirmed by those reported by Bölükbaşi and Erhan (2007) discovered that thyme, at concentrations of 0.1% and 0.5%, significantly reduced *E.coli* levels in laying hen feces comparison the control group., Also Cross et al. (2002) found that thyme oil reduced count of coliforms. Essential oils exhibit antimicrobial activity linked to phenolic compounds like Thymol and Carvacrol, the information provided aligns with the statements made by other authors. (Karaman et al., 2001; Rota et al., 2008).

Essential oils and probiotics improved intestinal gram-positive microflora growth, reduced salmonella and E.coli colonization, and positively impacted *Lactobacillus* and *Enterococcus* count, according to Mookiah et al. (2014) 's study. (The highest number of colony forming units of the helpful bacteria was recorded in experimental treatment groups which were treated with thyme oil, betaine and Mixture of Thyme and betaine). All treatments had significantly higher counts if comparison the count recorded in the control group. The study found that groups treated with Thyme and betaine showed a significant bear effect on *salmonella* and *E.coli*, with the added mixture also showing similar effects comparison the control group. All treatments had a significant positive effect on the count of harmful bacteria if comparison the control group and this result agreement with Bölükbasi and Erhan (2007) emphasized that essential oil stimulates beneficial bacteria growth, reducing harmful microbes' growth and multiplication in the intestines, thus enhancing the life and existence of the intestines. Also, *in vitro* studies have demonstrated that essential oils possess antibacterial properties against *Escherichia coli* and *Salmonella typhimurium*, as reported by Cosention et al. (1999).

Table 4. The effect of Thyme, Betaine and interaction between them on cecum properties of broiler chickens during the experimental periods aged 35 days

Treatments(mg/L)	**Cecum bacterial count at cfu/g**			
	***Lactobacillus acidophilus* count as (log_{10} cfu/g)**	***Enterococcus species* count as (log_{10} cfu/g)**	***E. coli* count as (log_{10} cfu/g)**	***Salmonella Count* as (log10 cfu/g)**

Thyme(Thy)				
Thy0= Control	6.49±0.11^{b}	6.04±0.18^{b}	3.08±0.28^{a}	1.02±0.37^{a}
Thy1= 50	7.17±0.07^{a}	6.87±0.07^{a}	1.39±0.08^{b}	0^{b}
P-value	<0.0001	<0.0001	<0.0001	<0.0001
Betaine (Bet)				
Bet0=Control	6.20±0.21^{d}	5.43±0.37^{d}	3.78±0.51^{a}	2.55±0.76^{a}
Bet1=50	6.90±0.07^{b}	6.67±0.07^{b}	2.75±0.45^{b}	0^{b}
Bet2=100	6.61±0.16^{c}	6.29±0.12^{c}	1.94±0.16^{c}	0^{b}
Bet3=250	7.10±0.10^{ab}	6.81±0.12^{b}	1.53±0.08^{d}	0^{b}
Bet4=500	7.33±0.12^{a}	7.09±0.10^{a}	1.19±0.11^{e}	0^{b}
P-value	<0.0001	<0.0001	<0.0001	<0.0001
Interaction				
Thy0 X Bet0	5.54±0.04^{h}	4.21±0.11^{i}	5.47±0.11^{a}	5.1±0.03^{a}
Thy0 X Bet1	6.87±0.04^{e}	6.79±0.06^{d}	4.25±0.09^{b}	0^{b}
Thy0 X Bet2	6.21±0.21^{g}	5.94±0.13^{h}	2.48±0.07^{c}	0^{b}
Thy0 X Bet3	6.80±0.10^{f}	6.45±0.11^{g}	1.81±0.03^{e}	0^{b}
Thy0 X Bet4	7.01±0.12^{c}	6.83±0.07^{c}	1.40±0.05^{f}	0^{b}
Thy1 X Bet0	6.87±0.16^{e}	6.65±0.15^{e}	2.10±0.12^{d}	0^{b}
Thy1 X Bet1	6.94±0.14^{d}	6.55±0.13^{f}	1.25±0.09^{g}	0^{b}
Thy1 X Bet2	7.01±0.07^{c}	6.63±0.08^{e}	1.40±0.04^{f}	0^{b}
Thy1 X Bet3	7.41±0.06^{b}	7.17±0.08^{b}	1.25±0.04^{g}	0^{b}
Thy1 X Bet4	7.65±0.11^{a}	7.35±0.13^{a}	0.98±0.19^{h}	0^{b}
P-value	0.0002	<0.0001	<0.0001	<0.0001

$^{a\text{-}i}$ Mean± SME in same row with different superscripts are significantly (P<0.05) different.($\log_{10}$ cfu/g) the count of microbial colony-forming units (CFU) has expressed as logarithmic ($\log_{10}$) transformation per gram of cecum content. Significance (P<0.05); Non-Significant (P>0.05).

Blood Parameters

Data presented in table (5) that the study revealed that all treatments significantly increased TSH, T3 and T4 levels compared to the control group at 35 days of age. This finding was similar to that obtained by Beisel. (1982) who reported that Feed additives, significantly improved T3, T4, and TSH values in broiler feed, highlighting the important of nutrition in immune system development and function. that reported, nutrition plays an important role in the development and function of the immune system. However, feed additives can increase thyroxin (T4) levels in broiler, suggesting that incorporating living organisms can enhance TSH effects as reported that Aluwong et al. (2012).

Table 5. The effect of Thyme, Betaine and interaction between them on hormones indices in broiler chickens during the experimental periods aged 35 days

	Indices

Treatments(mg/L)	**T3 (ng/dl)**	**T4 (ug/dl)**	**TSH (ulU/mL)**
Thyme(Thy)			
Thy0= Control	221.23±6.01^{b}	0.81±0.04^{b}	0.04±0.001^{b}
Thy1= 50	259.93±5.73^{a}	1.18±0.06^{a}	0.06±0.001^{a}
P-value	<0.0001	<0.0001	<0.0001
Betaine (Bet)			
Bet0=Control	210.5±10.26^{e}	0.76±0.04^{d}	0.02±0.001^{e}
Bet1=50	224.42±6.76^{d}	0.80±0.05^{d}	0.03±0.001^{d}
Bet2=100	235.12±5.71^{c}	0.95±0.07^{c}	0.05±0.001^{c}
Bet3=250	250.55±6.38^{b}	1.05±0.07^{b}	0.07±0.001^{b}
Bet4=500	282.31±8.82^{a}	1.40±0.10^{a}	0.08±0.001^{a}
P-value	<0.0001	<0.0001	<0.0001
Interaction			
Thy0 X Bet0	184.10±2.98^{i}	0.64±0.01^{h}	0.01±0.001^{f}
Thy0 X Bet1	206.77±2.03^{h}	0.66±0.02^{h}	0.01±0.001^{f}
Thy0 X Bet2	220.22±0.64^{g}	0.75±0.01^{g}	0.02±0.001^{e}
Thy0 X Bet3	234.45±4.10^{f}	0.85±0.01^{f}	0.07±0.001^{b}
Thy0 X Bet4	260.60±3.44^{c}	1.13±0.04^{c}	0.08±0.001^{ab}
Thy1 X Bet0	236.90±4.30^{f}	0.88±0.01^{e}	0.03±0.001^{d}
Thy1 X Bet1	242.07±1.31^{e}	0.94±0.03^{d}	0.06±0.001^{c}
Thy1 X Bet2	250.02±2.04^{d}	1.16±0.04^{c}	0.07±0.001^{b}
Thy1 X Bet3	266.65±0.81^{b}	1.25±0.02^{b}	0.07±0.001^{b}
Thy1 X Bet4	304.02±6.10^{a}	1.67±0.01^{a}	0.09±0.001^{a}
P-value	0.0078	<0.0001	<0.0001

$^{a\text{-}i}$ Mean± SME in the same row with different superscripts are significantly (P<0.05) different. Significance (P<0.05); Non-Significant (P>0.05).

Table 6. The effect of Thyme, Betaine and interaction between them on some blood parameters of the broiler chickens during the experimental periods aged 35 days

Treatments(mg/L)	**Parameters**				
	AST (U/L)	**ALT (U/L)**	**Total protein (g/dl)**	**Total cholesterol (mg/dl)**	**Triglycerides (mg/dl)**
Thyme(Thy)					

Thy0= Control	254.46±10.65^{a}	17.13±0.84^{a}	4.26±0.13^{a}	145.43±5.40^{b}	91.56±2.89^{b}
Thy1= 50	279.60±9.16^{b}	19.96±0.90^{b}	4.96±0.07^{b}	109.50±2.30^{a}	70.66±1.82^{a}
P-value	<0.0001	<0.0001	<0.0001	<0.0001	<0.0001
Betaine (Bet)					
Bet0=Control	247.33±22.78^{c}	15.58±1.42^{c}	3.84±0.28^{d}	140.58±11.54^{ab}	89.33±6.91^{a}
Bet1=50	227.00±2.48^{d}	15.41±0.46^{c}	4.39±0.06^{c}	147.58±7.76^{a}	88.16±5.14^{a}
Bet2=100	228.91±5.86^{d}	16.00±0.38^{c}	4.54±0.07^{c}	134.08±5.08^{b}	86.50±2.70^{a}
Bet3=250	305.50±5.16^{b}	19.75±0.44^{b}	4.97±0.07^{b}	112.25±3.84^{c}	75.41±2.43^{b}
Bet4=500	326.41±4.27^{a}	26.00±0.99^{a}	5.3±0.1^{a}	102.83±2.73^{d}	66.16±1.62^{c}
P-value	<0.0001	<0.0001	<0.0001	<0.0001	<0.0001
Interaction					
Thy0 X Bet0	179.66±21.17^{g}	11.33±0.76^{g}	2.95±0.19^{i}	177.50±4.73^{a}	110.33±3.37^{a}
Thy0 X Bet1	227.00±3.05^{e}	14.83±0.70^{f}	4.22±0.09^{h}	170.50±6.52^{b}	103.16±2.84^{b}
Thy0 X Bet2	239.50±9.75^{d}	16.33±0.61^{e}	4.38±0.10^{g}	149.83±3.28^{c}	92.83±2.07^{c}
Thy0 X Bet3	306.66±2.27^{c}	19.50±0.71^{d}	4.75±0.06^{d}	122.00±4.76^{d}	82.00±1.86^{d}
Thy0 X Bet4	319.50±5.20^{b}	23.66±1.08^{b}	4.98±0.02^{c}	107.33±3.73^{e}	69.50±1.33^{ef}
Thy1 X Bet0	315.00±2.16^{b}	19.83±1.07^{c}	4.73±0.04^{e}	103.66±4.37^{f}	68.33±4.75^{f}
Thy1 X Bet1	227.00±4.23^{e}	16.00±0.57^{e}	4.56±0.04^{f}	124.66±3.56^{d}	73.16±4.30^{e}
Thy1 X Bet2	218.33±3.39^{f}	15.66±0.49^{e}	4.70±0.06^{e}	118.33±1.99^{e}	80.16±3.43^{d}
Thy1 X Bet3	304.33±10.56^{c}	20.00±0.57^{c}	5.20±0.05^{b}	102.50±2.06^{f}	68.83±2.30^{f}
Thy1 X Bet4	333.33±5.85^{a}	28.33±0.98^{a}	5.62±0.05^{a}	98.33±3.29^{g}	62.83±2.32^{g}
P-value	<0.0001	<0.0001	<0.0001	<0.0001	<0.0001

$^{a-i}$ Mean± SME in the same row with different superscripts are significantly (P<0.05) different. Significance (P<0.05); Non-Significant (P>0.05).

The table (6) showed that, all treatments caused increase in some measured parameters which indicated harmful positive effect on liver enzymes (ALT and AST), Total protein. These results were agreed with those obtained with Attia et al. (2005) reported that there was significant effect of methionine level on AST and ALT. Broiler chicks fed diet containing 0.37 and 0.42% methionine showed lower AST and ALT than those fed the basal diet. They also added that, betaine supplementation had no significant effect on most of serum constituents except for ALT where a decrease was shown of birds fed diet containing 0.07% betaine. As general, a progressive decrease in serum ALT with increasing betaine supplementation to groups fed diets containing 0.32 and 0.37% methionine level.

In addition, result in table (6) that agree with reported Su et al. (2009) reported that betaine treatment for broiler was significantly increased AST and ALT (P<0.05) comparison the control diet. At another study for blood parameters, it is clear from Similar table (6) that, all treatments comparison the control group (G1) had significant highest by reducing effect of Triglycerides and Total cholesterol as an indication of positive effect on the physiology of the experimental animals. These results were similar to the report of Shabaan (2012) who stated that broiler chicks fed the low energy diets supplemented with a mixture of 0.15% thyme and 0.15% cumin gave recorded lower values of plasma total protein as comparison the control group. So, it is perceived

both Thyme oil and betaine had harmful effect on liver function and blood protein. These data are in agreement with Lee et al. (2003) reported that, Thymol and Carvacrol have effects on growth performance and triglyceride metabolism in broiler chickens and reducing plasma triglyceride concentrations.

Gene expression

Table (7) demonstrates that essential oils and betaine positively affect Mucins$_2$ play an essential role in the prevention of infection by pathogens. through the results showed MUC$_2$ has perfect effect on EG10,9,2,6 beaters than EG7,5,3,8,4, that highly significant comparted to as well as control (CG1) Mucin is a major constituent of the mucosal layer. Mucin$_2$ (MUC2) is the primary mucin produced and secreted by goblet cells in intestinal epithelial tissue under normal physiological conditions. When the expression of mucins in the intestinal epithelium declines, mucosal barrier function may be weakened, leading to increased susceptibility to pathogenic agents (Kunikata et al., 2002)

AT the similar table showed that IL-6 has proinflammatory cytokines that increase with inflammation, indicating the activation of microglial cells. through the results showed IL-6 has beater effect on EG2 and EG10 that highly significant comparted to the other treatment group as well as control CG1.

Table 7. The effect of Thyme, Betaine and interaction between them on some on immune and growth performance gene response markers (Interleukin6 (IL6) and Mucin2) of the broiler chickens during the experimental periods aged 35 days

	immune response and growth genes markers	
Treatment(mg/L)	**IL6**	**MUC2**
CG1=Control	1.02^{b}± 0.13	3.16^{g}±1.28
EG2= Thyme 50	5.29^{a}±0.53	7.20^{b}±0.77
EG3= Betaine 50	1.41^{b}±0.41	3.25^{g}±1.74
EG4= Betaine 100	1.38^{b}±0.39	4.12^{f}±0.67
EG5= Betaine 250	1.77^{b}±0.59	6.11^{e}±1.75
EG6= Betaine 500	1.06^{b}±0.16	6.75^{c}±1.73
EG7=Thyme 50* Betaine 50	1.50^{b}±0.25	4.06^{f}±0.73
EG8=Thyme 50* Betaine 100	2.76^{b}±0.28	6.60^{d}±1.93
EG9=Thyme 50* Betaine 250	2.40^{b}±0.54	7.33^{b}±1.54
EG10=Thyme 50* Betaine 500	5.60^{a}±1.85	8.72^{a}±1.85

$^{a:g}$ Means with different superscript in the same column are significantly different at P<0.05. Significance (P<0.05); Non-Significant (P>0.05).

CONCLUSION

The study suggests that thyme oil and betaine can be used as feed additives for broiler chickens, as they promote growth, improve performance, and stimulate immunity without significant side effects. We were recommended that mixture of Betaine 500 mg and thyme oil (50 mg) at liter of water has high significantly positive effect on live body weight as well as feed conversion ratio, based on the above observation it was concluded that, a good positive effect on the blood physiology significantly for immune response and growth genes of the experimental broilers comparison the control group.

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Competing interests

The authors declare that they have no conflict of interest with respect to the research, authorship, and/or publications of this article. The authors declare that they have no competing interests.

Author's contribution

Mahmoud G.Y. Abdel-Mageed writing in field study, collected data, laboratory analyses, statistical analysis, manuscript writing Ahmed M. El-kaiaty and Gihan M. El-Moghazy they designed the experiment. Ahmed M. El-kaiaty designed article helped, revision and approval, Gihan M. El-Moghazy designed tabulation of experimental data, manuscript writing, commenting and approval; while, Mohamed A.F. El-Manylawi helped in tabulation of experimental data and article revision. All authors have read and approved the final manuscript.

References

Alloui, M.N.; Agabou, A.; Alloui, M. (2014). Application of Herbs and Phytogenic Feed Additives in Poultry production - A Review. Glob. J. Anim. Sci. Res., 3, 234–243. Available online: http://archives.gjasr.com/index.php/GJASR/article/view/57 (accessed on 16 January 2023).

Aluwong T, Raji MA, Hassan BF, Kawu MU, Kobo PI and Ayo JO (2012). Effect of different levels of supplemental Yeast on performance indices and serum biochemistry of broiler chickens. The Open Conference Proceedings Journal, 3 (Suppl 1-M7): 41-45. DOI: http://dx.doi.org/10.2174/1876326X01203020041

Amiri H (2012). Essential oils composition and antioxidant properties of three thymus species. Evidence-Based Complementary and Alternative Medicine. DOI: https://doi.org/10.1155/2012/728065

Attia, Y. A.; Hassan , R. A.; Shehatta, M. H. and Abd-El-Hady,S. B. (2005). Growth, carcass quality and serum constituents of slow growing chicks as affected by betaine addition to diets containing 2. different levels of methionine. Int. J. Poult. Sci.4:856-865.

Awan, J. A., & Muhammad Sohaib, M. S. (2016). Halal and humane slaughter; comparison between Islamic teachings and modern methods. Available online:http://ijtihadnet.com/wp-content/uploads/Halal-and-humane-slaughter-Comparison-between-Islamic-teachings-and-modern-methods.pdf

Beisel W R (1982). Single nutrients and immunity. American Journal of Clinical Nutrition, 35:417-468. DOI: https://doi.org/10.1093/ajcn/35.2.417.

Bolognani F, Goya RG and Ronderos JR (2001). Thyroid-stimulating hormone and growth hormone release alterations induced by mosquito larvae proteins on pituitary cells, Cell Biology International, 25(9): 885-892. DOI: https://doi.org/10.1006/cbir.2001.0716

Bölükbaşi ŞC and Erhan MK (2007). Effect of dietary thyme (Thymus vulgaris) on Laying Hens performance and Escherichia coli (E. coli) concentration in feces. International Journal of Natural and Engineering Sciences. 1(2):55–58. Available at: http://agris.fao.org/agris-search/search.do?recordID=TR2007000095

Cosentino S, Tuberoso CIG, Pisano B, Satta M, Mascia V, Arzedi E and Palmas F (1999). In vitro antimicrobial activity and composition of Sardinian Thymus essential oils. Letters in Applied Microbiology, 29:130-135. DOI: https://doi.org/10.1046/j.1472-765x.1999.00605.x

Cross DE, Svoboda K, Hillman K, Mcdevitt R and Acamovic T (2002). Effects of Thymus vulgaris L. Essential oil as an in vivo dietary supplement on chicken intestinal microflora. Proceedings of the 33rd International Symposium on Essential Oils, Lisbon, Portugal, 3-7th Sept.

Delsol AA, Randall L, Cooles S, Woodward MJ, Sunderland J and Roe JM (2005). Effect of the growth promoter avilamycin on emergence and persistence of antimicrobial resistance in enteric bacteria in the pig. Journal of Applied Microbiology, 98: 564–571. DOI: https://doi.org/10.1111/j.1365-2672.2004.02461.x

Duncan DB (1955). Multiple range and multiple F-tests. Biometrics, 11:1-42. Available at: https://www.jstor.org/stable/3001478

El-Husseiny, O.M.; Abo-El-Ella, M.A.; Abd-Elsamee, M.O. and Magda, M. A. (2007). Response of broilers performance to dietary betaine and folic acid at different methionine levels. International Journal of Poult. Sci., 6 (7): 515-523.

El-Kaiaty, A. M., El-Moghazy, G. M., El-Manylawi, M. A. F., & Abdel-Mageed, M. G. Y. (2020). Impact of thyme oil and Lactobacillus acidophilus as natural growth promoters on performance, blood parameters and immune status in growing rabbits. World's Veterinary Journal, (1), 1-11.

Faleiro ML (2011). The mode of antibacterial action of essential oils. Science against microbial pathogens: communicating current research and technological advances A.Méndez-Vilas (Ed).p.1143-1156. Available at: https://www.academia.edu/download/37147183/EO.pdf

Food and Agricultural Organization. FAO Publications Catalogue (2017). United Nations: Food and Agricultural Organization. Retrieved from http://www.fao.org/3/b-i6407e.pdf on 14th April, 2018

Harding , D.E.; Allen, O.W. and Wilson, R.P. (2001). Sulfur amino acid requirement of poultry: L-methionine and L-cystine. J. Nutr. 107, 2031-2035.

Hassan, R.A.; Attia, Y.A. and El-Ganzory, E.H. (2005). Growth, carcass quality and serum constituents of slow growing chicks as affected by betaine addition to diets containing 1. Different levels of choline. Inte. J. Poult. Sci. 4: 840-850.

Huyghebaert G, Ducatelle R and Immerseel F van (2011). An update on alternatives to antimicrobial growth promoters for broilers. The Veterinary Journal, 187: 182-188. DOI: https://doi.org/10.1016/j.tvjl.2010.03.003

Karaman S, Digrak M, Ravid U and Ilcim A (2001). Antibacterial and antifungal activity of the essential oils of Thymus revolutus Celak from Turkey. Journal of Ethnopharmacology, 76(2):183–186. DOI: https://doi.org/10.1016/S0378-8741(01)00238-0

Karousou R, Koureas DN and Kokkini S (2005). Essential oil composition is related to the natural habitats: Coridothymus capitatus and Satureja thymbra in NATURA 2000 sites of Crete. Phytochemistry, 66:2668–2673. DOI: https://doi.org/10.1016/j.phytochem.2005.09.020

Kunikata, T., Tanaka, A., Miyazawa, T., Kato, S. and Takeuchi, K. (2002). Dimethyl prostaglandin E2 inhibits indomethacin-induced small intestinal lesions through EP3 and EP4 receptors. DigestionDiseases Science, 47, 894–904.

Lee KW, Everts H, Kappert HJ, Yeom, KH. and Beynen AC (2003). Dietary carvacrol lowers body weight gain but improves feed conversion in female broiler chickens. Journal of Applied Poultry Research, 12: 394–399. DOI: https://doi.org/10.1093/japr/12.4.394

Losa R and Kohler B (2001). Prevention of colonisation of Clostridium perfringens in broilers intestine by essential oils. In: Proc. 13Th Eur. Symp. Poult. Nutr., Blankenberge, Belgium, p. 133.

Männer, K.(2011). Effects of a phytogenic feed additive on growth performance and ileal nutrient digestibility in broiler chickens". Poult Sci. 90 (12): 2811–6.

Marija B, Nemanja Z, Jelena I, Jelena J, Jasna D, Marija S and Milan Z (2015). Antimicrobial activity of Thyme (Thymus vulgaris) and Oregano (Origanum vulgare) essential oils against some food-borne microorganisms. Journal of Procedia Food Science, 5:18-21. DOI: https://doi.org/10.1016/j.profoo.2015.09.005

McDevitt, R.M.; Mack, S. and Wallis, I.R. (2000). Can betaine partially replace or enhance the effect of methionine by improving broiler growth and carcass characteristics. British Poult. Sci., 41, 473–480.

Mookiah S, Sieo CC, Ramasamy K, Abdullah N and Ho YW (2014). Effects of dietary prebiotics, probiotic and synbiotics on performance, caecal bacterial populations and caecal fermentation concentrations of broiler chickens. Journal of the Science of Food and Agriculture, 94: 341-348. DOI: https://doi.org/10.1002/jsfa.6365

Moon, H. Y. and Gatlin, D. M. (2002). Total sulfur amino acid requirement of juvenile red drum, Sciaenops ocellatus. Aquaculture 95, 97-106.

Ortega-Nieblas MM, Robles-Burgueño MR, Acedo-Félix E, González-León A, Morales-Trejo A and Vázquez-Moreno L (2011). Chemical composition and antimicrobial activity of oregano (Lippia palmeri S.WATS) essential oil. Revista Fitotecnia Mexicana, 34 (1): 11 – 17. Available at: https://www.redalyc.org/pdf/610/61017038002.pdf

Patricia MS, Migdalia M, Juan AP, Mario S, VÍctor H and Esther P (2013). Gas Chromatography- Mass Spectrometry Study from the Leaves Fractions Obtained of Vernonanthura patens (Kunth) H. Rob. International Journal of Organic Chemistry, 3: 105-109. DOI: http://dx.doi.org/10.4236/ijoc.2013.32011

Pfaffl, M. W. (2001). A new mathematical model for relative quantification in real-time RT–PCR. Nucleic acids research, 29(9), e45-e45.

Placha I, Chrastinova L, Laukova A, Cobanova K, Takacova J, Strompfova V, Formelova Z and Faix S (2013). Effect of thyme oil on small intestine integrity and antioxidant status, phagocytic activity and gastrointestinal microbiota in rabbits. Acta Veterinaria Hungarica, 61(2): 197-208. DOI: https://doi.org/10.1556/AVet.2013.012

Puvača, N., Kostadinović, L., Ljubojević, D., Lukač, D., Popović, S., Dokmanovć, B., & Stanaćev, V. S. (2014). Effects of dietary garlic addition on productive performance and blood lipid profile of broiler chickens. Biotechnology in Animal Husbandry, 30(4), 669-676. Available online: http://doi.org/10.2298/BAH1404669P .

Rota MC, Herrera A, Martinez RM, Sotomayor JA and Jordan MJ (2008). Antimicrobial activity and chemical composition of Thymus vulgaris, Thymus zygis and Thymus hyemalis essential oils. Food Control, 19(7): 681–687. DOI: https://doi.org/10.1016/j.foodcont.2007.07.007

SAS (2004). Statistical analysis system User's Guide: Statistics. Version 9.1. SAS Inst. Inc., Cary, North Carolina, USA.

Schutte , J. B. ; Dejong , J. ; Smink, W. and Pack M. (1997). Replacement value of betaine for dl-methionine in male broiler chicks. Poultry Science 76:321–325.

Shabaan M (2012). Effect of using thyme (Thymus vulgaris L) and cumin (Cuminum cyminum L) seeds for improving the utilization of low energy broiler diet. Egyptian Poultry Science Journal, 32 (ш): 579-591. Available at: https://pdfs.semanticscholar.org/5d95/dc0af95bc1cd484b07846f549cd4cf4fc0b3.pdf

Siljander-Rasi , H. ; Peuranen , S. ; Tiihonen , K. ; Virtanen , E. ;Kettunen , H. ; Alaviuhkola, T. and Simmins , P.H. (2003). Effect of equi-molar dietary betaine and choline addition on performance, carcass quality and physiological parameters of pigs. Animal Science 76, 55–62.

Stanišić, N., Škrbić, Z., Petričević, V., Milenković, D., Petričević, M., Gogić, M., & Lukić, M. (2023). The fatty acid and mineral composition of Cobb 500 broiler meat influenced by the Nettle (Urtica dioica) dietary supplementation, broiler gender and muscle portion. Agriculture, 13(4), 799. Available online: https://doi.org/10.3390/agriculture13040799

Su , S.Y.; Dodson, M.V. ; Li , X.B. ; Li , Q.F. ; Wang , H.W. and Xie, Z. (2009). The effects of dietary betaine supplementation on liver performance, serum parameters, histological changes and methylation status in broiler. Comparative Biochemistry and Physiology, Part A 154 (2009) 308–314.

Virtanen , E. I. and Rosi , L. (1995). Effects of betaine on methionine requirement of broilers under various environmental conditions. Pages 88–92 in: Proceedings of the Australian Poultry Science Symposium. Adelaide, Australia.

Waldenstedt , L. ; Elwinger, K. ; Thebo , P. and Uggla, A. (1999). Effect of betaine supplement on broiler performance during an experimental coccidial infection. Poult. Sci. 78:182-189.

Wartofsky L and Burman KD (1982). Alteration in thyroid function in patients with systemic illness: The 'Euthyroid sick syndrome'. Endocrine Reviews, 3(2): 164-217. DOI: https://doi.org/10.1210/edrv-3-2-164

Yuan JS, Reed A, Chen F, Stewart CN. (2006). Statistical analysis of real-time PCR data. BMC Bioinformatics 7, 85.

Živković, V., Stanković, B., Radović, Č., Gogić, M., Stanojković, A., Obradović, S., & Stojiljković, N. (2019). Garlic as alternative for antibiotics in diet for growing pigs. Biotechnology in Animal Husbandry, 35(3), 281-287. Available online: http://doi.org/10.2298/BAH1903281Z .