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Original Article

Evaluation of Microencapsulated Essential Oils in Broilers Challenged with Salmonella Enteritidis: A Focus on the Body's Antioxidant Status, Gut Microbiology, and Morphology

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Abstract

Restrictions on antibiotic use encourage researchers to seek natural substitutes with the same effects without adverse end effects resulting from antibiotic use. Savory and black pepper have been challenged against *Salmonella enteritidis* (*S. enteritidis*) bacterium using the spray dryer method to evaluate growth performance, antioxidant status, immune response, and intestinal health parameters in broilers. In this study, thyme essential oil (50%), savory (25%), peppermint (12.5%), and black pepper seeds (12.5%) were mixed to form essential oil-loaded spherical microcapsules with the particle size of 323 nm and encapsulation efficiency of 96.2%. The main bioactive compounds used in the core of microcapsules included thymol, carvacrol, p-cymene, γ-terpinene, and menthol. Moreover, modified starch (25%) and maltodextrin (55%) were used for the preparation of spherical microcapsules for the enclosed wall with 20% whey protein concentrate. The dietary addition of microcapsules containing essential oil significantly reduced the *S. enteritidis* population in both ileum and cecum ($P<0.05$). The results revealed that the dietary inclusion of essential oil-loaded microcapsules significantly ($P<0.05$) increased the villus height, villus width, V: C ratio, and the number of goblet cells and decreased the crypt depth. Microcapsules have antioxidant and antibacterial activity and their dietary use as feed additive at 0.5, 1, and 2 kg/t concentrations in broilers has been challenged and showed that the final weight, total feed intake, and FCR improved the body's antioxidant status, structure, and inflammation in the ileum tissue.

Keywords: Antibiotic alternative, Broilers, Essential oils, Natural antibiotic, Plant bioactive compounds

1. Introduction

Morbidity caused by Salmonella, as the most common foodborne pathogenic bacteria, has been increased notably worldwide in the last few years (1). These bacteria enter the human food chain from poultry sources and result in carcass contamination from infected fecal matter or eggs (1). Currently, changes in different serotypes of this bacteria have been reported in poultry and diverse geographical regions, particularly in association with the spread of certain well-adapted clones (2). Therefore, it is necessary to supervise and identify the spread of Salmonella serotypes in poultry and especially in humans and provide a control program for the area (1). Commonly, antibiotics are used to inhibit the growth of pathogenic bacteria. Antibiotics that are used in poultry production cause antibiotic resistance in birds, while the remains that are passed on to humans can lead to public health

hazards. Furthermore, antibiotics also cause misbalance in the intestinal normal flora of birds (3). The use of antibiotics against pathogens in broilers can lead to the appearance of antibiotic-resistant microbial species, and their residues in the products cause adverse effects (4). The limitation posed on the consumption of antibiotics persuaded scientists to find substitutes with the antibiotic impact that are non-toxic and natural and do not have any side effects and residue in poultry meat.

Phytochemicals have recently gained much attention from the research community since studies have consistently shown several benefits from the consumption of these natural phytochemical compounds. Based on the evidence, various bioactive compounds contained in plants include phenolic compounds with approved antimicrobial action. In general, these bioactive compounds target the microbial membranes and generate their action through binding to the receptor on the membranes and interacting with microbial enzymes by fitting sterically into a binding pocket (5). Nowadays, the addition of these natural compounds to the feed had gained great attention since it improves shelf life and food safety and can be an alternative to antibiotic therapy. Furthermore, several studies have illustrated the fundamental role of these compounds as antimicrobial agents which modulate the gut microbiota and improve host metabolism (6). The antimicrobial potential of plant bioactive compounds has been widely investigated in poultry. In this regard, the essential oil of thyme, rosemary, garlic, ginger, black cumin, and cinnamon appear to be antibiotic growth promoters that can improve animal health and performance. Other studies have confirmed that the blend of thymol and cinnamaldehyde enhance body weight gain in broilers, while others like thymol and essential oil of star anise improve the feed efficiency (7). The essential oil loaded microcapsules can be considered as a promising phytobiotic against *C. perfringens* infection in broiler chickens (8).

Encapsulation technology has recently attracted much attention from the pharmaceutical and food industries. In this process, tiny particles are surrounded by a coating wall to make small capsules and a barrier is built between the core and wall to keep the material away from chemical and physical reactions and maintain the biological, functional, and physicochemical properties of the material in the core. The main benefit of the microencapsulation of natural bioactive compounds is to protect bioactive phytochemicals from oxidative, degradation, and volatilization reactions (9). This study aimed to synthesize the microcapsules that preserve the mixture of thyme, peppermint, savory, and black pepper essential oils together using spray drying technique and to evaluate the growth performance, antioxidant status, immune response, and gut health parameters in broiler chickens challenged with *salmonella enteritidis*.

2. Materials and Methods

The dried leaves of thyme (*Thymus vulgaris*), savory (*Satureja hortensis*), peppermint (*Mentha piperita*), and black pepper seeds (*Piper nigrum*) were purchased from the herbal medicine market in Mashhad, Iran. Moreover, the modified starch (MS) (HI-CAP® 100, Ingredion, Humberg, Germany), whey protein concentrate (WPC) (Milk product, Auckland, New Zealand), and maltodextrin (MD) (DE=18-20; Foodchem, China) were used in encapsulation as wall materials. The *S. enteritidis* bacteria (RTCC 1621), known as poultry and the food-borne pathogen and isolated from a poultry farm, was obtained from the microbial culture collection of Razi Vaccine and Serum Research Institute, Karaj, Iran. The 2,2-diphenyl-1-picrylhydrazyl (DPPH) and acid washed glass bead (G8772) were purchased from Sigma (Germany). All other solvents and chemicals not mentioned here were purchased from Merck, Germany.

2.1. Essential oils Extraction and Encapsulation

The dried plant materials were subjected to hydrodistillation process in a Clevenger-type apparatus for 4 h following the recommendation from the European Pharmacopoeia 6.0, 2008 (10). The essential oil was collected and dried over anhydrous sodium

sulfate (Na_2SO_4) and used for encapsulation. The essential oils obtained from thyme, savory, peppermint, and black pepper were mixed at the ratio of 50%, 25%, 12.5%, and 12.5%, respectively. A pilot plant scale spray dryer with counter-current airflow was applied to encapsulate the mixed essential oils as described earlier by Sablania et al. (2018) (11). Briefly, different percentages of wall materials including maltodextrin, modified starch, and whey protein concentrate was dissolved in 150 ml distilled water and stirred for 24 h at room temperature (Table 1). Subsequently, mixed essential oils (7.5 g) and tween 80 (1%) were gradually added to the wall material solution and stirred at 9,000 rpm for 5 min using Rotor-Stator high speed stirring. The solution was then homogenized using Ultra Turrax high-speed homogenizer (Ika, Staufen, Germany) before spry drying at 10,000 rpm for 5 min. The spray dryer temperature, pressure, and flow rate were 80°C-130°C, 0.4 kg/cm^2, and 8 mL/min, respectively. Eventually, the dried powder obtained from different treatments was stored for further analysis.

Table 1. Ratio of different wall materials for the encapsulation process

Treatments	Whey protein concentrate (%)	Modified starch (%)	Maltodextrin (%)
T1	10	65	25
T2	10	25	65
T3	12.5	53.75	33.75
T4	12.5	33.75	53.75
T5	17.25	35.3	48.73
T6	17.25	48.73	35.3
T7	20	25	55
T8	20	55	25

2.2. Physicochemical Characterization of Essential Oil-Loaded Microcapsules

The microcapsules that were loaded with essential oil and developed using different compositions of wall materials were characterized for their physicochemical traits, including surface oil, retention oil, Brix, emulsion quality, particle size, and encapsulation efficiency. The Brix, emulsion quality, and surface oil content of microcapsules were determined according to the study conducted by Jafari et al. in 2007-2008 (12, 13). The total oil content of the microcapsules was determined by hydrodistillation using the Clevenger apparatus, as described earlier (14). The retention oil, defined as the proportion of the core material that remained in the powder, was obtained from the below formula (14).

$$\text{Retention oil (\%)} = \frac{\text{Total oil}}{\text{Initial oil}} \times 100$$

The size of the microcapsules in each treatment was determined by dynamic light scattering using Zetasizer Nano ZS (Malvern Instruments, UK) (13). The encapsulation efficiency (EE) is the amount of core material capsulated inside the powder and calculated based on the below formula (14). In this formula, total oil is defined as the internal surface oil content of the powders, and the surface oil presents the unencapsulated oil at the surface of particles.

$$\text{EE (\%)} = \frac{\text{Total oil} - \text{Surface oil}}{\text{Total oil}} \times 100$$

The morphology and microstructure properties of microcapsules were determined using scanning electron microscopy (SEM) (15). The results of these evaluations indicated that the best wall material composition resulted in the highest encapsulation efficiency with proper particle size.

2.3. Phytochemical, Antioxidant, and Antibacterial Analysis of Essential Oil-Loaded Microcapsules

The best wall material composition was selected for the analysis which result in higher encapsulation efficiency with proper particle size. Afterward, 5 g of essential oil-loaded microcapsule was mixed with 0.5 g of the acid-washed glass bead, crushed in the pestle and mortar, and extracted for 1 min using 30 ml of hexane as a solvent. The extraction was repeated using fresh hexane, and the solvent was filtered using Whatman filter paper No.1. The solvent was evaporated and the obtained extract was used to determine the bioactive phytochemicals using a gas chromatography-mass spectrometry (GC-MS) (Shimadzu QP2010PLUS

system, Japan) equipped with a capillary column (30 m, 0.25 mm i.d., 0.25 μm film thickness). The carrier gas was helium at the flow rate of 1 mL min^{-1}. The inlet and the detector temperature were 250°C and 340 °C, respectively. The solvent delay was adjusted at 4 min. The bioactive volatile compounds were identified based on the computer marching of the mass spectra (16). The antibacterial activity of the extract was assessed through the determination of the minimum inhibitory and minimum bactericidal concentrations of essential oil-loaded microcapsules against *S. enteritidis*, according to the resazurin-based 96-well plate microdilution method (17). The antioxidant activity of the extract was measured using DPPH scavenging activity (18) and nitric oxide scavenging activity assays (19).

2.4. Culture and Preparation of *S. enteritidis* Inoculant

The *S. enteritidis* was subcultured to a fresh Luria-Bertoni broth for three consecutive days and then 10-fold dilutions of the bacterial suspension were prepared and cultured on brilliant green agar with 200 ppm nalidixic acid to confirm the number of colony-forming units (cfu) per milliliter of broth. Subsequently, the bacteria were diluted with peptone water (0.1%) and then used in a chicken trial for infection through oral gavage on day 3 by 1 ml of peptone water containing 1×10^9 cfu of *S. enteritidis*.

2.5. Experimental Design and Animal Trial

All the methods used in this study were approved by the Animal Care Committee of Agricultural Research, Education, and Extension Organization (AREEO; IR.AREEO.14365.125.264). A total of 350 one-day-old male broiler chickens (Ross 308) were purchased from Fariman Broiler Breeder Company, Fariman, Iran, and reared in a pen system under strict hygienic conditions in a ventilated and temperature-controlled area. The lighting, humidity, and temperature were set according to Aviagen guidelines (2018). The chickens were randomly divided into seven treatment groups (Table 2) with five replicates and each replicate involved 10 chickens (Table 2). Diet (Starter) and water were provided *ad libitum*. The experimental diets (starter) are presented in table 3. The best wall material composition resulting in higher encapsulation efficiency of essential oils with proper particle size was selected as a developed phytobiotic for the chicken trial. Moreover, in the current study, the Digestarom P.E.P. (Biomin GmbH, Herzogenburg, Austria) was used as reference controls. Digestarom P.E.P. is a commercial natural product based on a matrix-encapsulated mixture of natural extracts from aromatic plants containing thymol, carvacrol, anethol, limonene, and oxytetracycline (Razak, Tehran, Iran) used as a common synthetic antibiotic growth promoter. The chickens were infected through oral gavage on day 3 using 10^9 cfu of *S. enteritidis*. The uninfected group was orally gavaged with 1 ml of sterile peptone water on the same date. The experiment lasted for 10 days. The final weight, total feed intake, and feed conversion ratio (FCR) were determined subsequently. On day 10, the chickens were euthanized using cervical dislocation, and blood samples were collected to determine the immunoglobulin G (IgG), immunoglobulin A (IgA), and liver enzymes, including alanine aminotransferase and aspartate aminotransferase. The lipid peroxidation was analyzed in the breast meat and serum by measuring the malondialdehyde content (20), while the free radicals of DPPH were used to determine the antioxidant activity of the breast meat and serum (21). The total phenolic content in the breast meat was determined using the Folin-Ciocalteu method (22). All the results for lipid peroxidation, antioxidant activity, and total phenolic content of breast meat and serum were reported as percentage changes, relative to the control group. The ileum tissues were collected and fixed in neutral buffered formalin (10%) and used for histopathological analysis following standard histopathological protocols (23). The morpho-structural characteristics of ileum, including villus height, villus width, crypt depth, and goblet cells count were determined as well. The pH of ileum and cecum were measured, and the ileum digesta was collected and kept at -20 $^{C^\circ}$ for molecular quantification of lactic acid bacteria and *S. enteritidis*.

Table 2. Experimental treatments

Treatments	Diet	Status
T1	Normal diet	Uninfected
T2	Normal diet	Infected
T3	Normal diet+0.5 kg/ton microencapsulated essential oil	Infected
T4	Normal diet+1 kg/ton microencapsulated essential oil	Infected
T5	Normal diet+2 kg/ton microencapsulated essential oil	Infected
T6	Normal diet+1 kg/ton Digestarom P.E.P.	Infected
T7	Normal diet+0.4 g/ton oxytetracycline	Infected

Digestarom P.E.P.: commercial natural product based on a matrix-encapsulated mixture of natural extracts from aromatic plants, including thymol, carvacrol, anethol, and limonene
Oxytetracycline: a common synthetic antibiotic growth promoter inducted by oral gavage of 109 cfu

Table 3. Diet composition used in the current study

Ingredients	Starter (0-10)
Corn (%)	52.5
Soybean meal (%)	40.0
Soybean oil (%)	3.2
Dicalcium phosphate (%)	1.5
Calcium carbonate (%)	1.3
DL-methionine (%)	0.38
L-lysin (%)	0.25
Salt (%)	0.20
Vitamin supplement[1] (%)	0.25
Mineral supplement[2] (%)	0.25
Sodium bicarbonate (%)	0.12
Choline chloride (%)	0.07
Calculated nutrient levels[3]	
Metabolizable energy (Kcal/kg)	2950
Crude Protein (%)	24.0
Calcium (%)	1.0
Available phosphorous (%)	0.5
Lysin (%)	1.0
Methionine (%)	0.5

[1]Vitamin supplement provided per Kg of diet: vitamin A (retinylacetate, 9500 IU; vitamin E (DL-α-tocopherol acetate, 30 IU; vitamin K3 (menadione sodium bisulfate), 2.65 mg; vitamin D3 (cholecalciferol), 2500 IU; vitamin B12 (cyanocobalamin), 0.025 mg; biotin, 0.30 mg; nicotinic acid, 50 mg; folic acid, 1.25 mg; d-pantothenic acid, 12 mg; riboflavin, 6.5 mg; pyridoxine hydrochloride, 6.0 mg; thiamine mononitrate, 3 mg.
[2]Mineral supplement provided per Kg of diet: zinc, 80 mg; manganese, 100 mg; copper 8 mg; iron, 80 mg; iodine, 0.35 mg; selenium, 0.15 mg.
[3]Calculated values according to the analyzed data of experimental diets

2.6. Statistical Analysis

All the data were analyzed using one-way analysis of variance following the GLM procedure of SAS software (Version 9.2; SAS Institute Inc., Cary, NC). The significant differences among treatments were determined by Duncan's multiple test range. A *P*-value less than 0.05 ($P<0.05$) was considered statistically significant. The results were reported as mean±SEM.

3. Results

3.1. Physicochemical Analysis

In the present study, the essential oil was

microencapsulated using the spry drying technique through the application of maltodextrin, modified starch, and whey protein concentrate as wall materials (Table 1). The observation of microcapsules via scanning electron microscope demonstrated the individual spherical particles and confirmed the smooth surface of microcapsules (Figure 1).

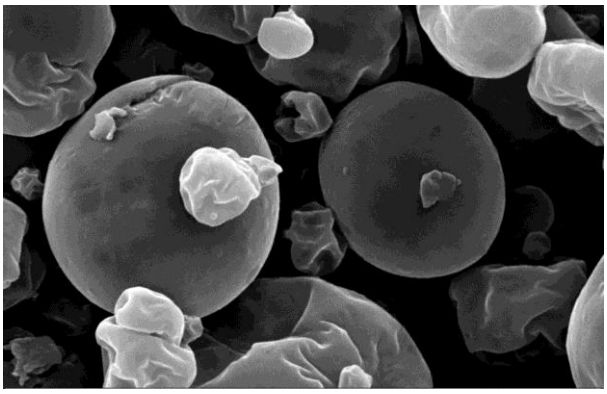

Figure 1. Scanning electron microscopy image of the selected microcapsule

3.2. Phytochemical, Antioxidant, and Antibacterial Activities

The GC-MS analysis of microcapsules obtained from the previous section resulted in the identification of more than 12 major compounds (Table 4). The main volatile compounds included thymol (35.20%), carvacrol (20.58%), p-Cymene (9.89%), γ-terpinene (8.48%), and menthol (5.12%). These results indicated the presence of phytochemicals in the core material of microcapsules, and early studies confirmed the presence of such bioactive phenolics as thyme (*Thymus vulgaris*), savory (*Satureja hortensis*), peppermint (*Mentha piperita*), and black pepper seeds (*Piper nigrum*) in the plant (5). Table 5 indicate the emulsion quality, surface oil, retention oil, particle size, and encapsulation efficiency of essential oil-loaded capsules.

Table 6 presents the results of *in vitro* evaluation of antibacterial and antioxidant activities of the extract obtained from microcapsules. The microcapsules extract showed antibacterial activity against *S. enteritis*, as a poultry pathogen with the MIC value of 1.87 mg/ml.

3.3. Animal Trial

The results of final weight, total feed intake, FCR, together with the pH of ileum and cecum are presented in table 7. The results indicated that the chickens challenged by *S. enteritis* (T2) showed a significant reduction in the final weight, total feed intake, and an increase in the FCR (P<0.05), compared to the unchallenged chickens (T1). Taken together, dietary inclusion of essential oil-loaded microcapsules at the concentrations of 0.5, 1, and 2 kg/ton significantly (P<0.05) improved the final weight, total feed intake, and FCR values in a dose-dependent manner (T3, T4, and T5). The results of liver enzymes (ALT, AST) accompanied with immunoglobulins (IgG, IgA) are summarized in table 8. Overall, the infection caused by *S. enteritidis* in the chickens (T2) significantly (P<0.05) elevated the liver enzymes (ALT, AST) and immunoglobulin (IgG, IgA) values, compared to the group of uninfected chickens (T1).

Table 4. Phytochemical analysis of essential oils extracted from microcapsules

Compounds	Percentage of total oil
Thymol	35.20
Carvacrol	20.58
p-Cymene	9.89
γ-terpinene	8.48
Menthol	5.12
Caryophyllene	2.79
Linalool	2.46
Menthone	1.89
Limonene	1.42
Camphene	1.23
1,8-Cineole	1.19
β-pinene	1.17
Total	91.47

Table 5. The Brix, emulsion quality, surface oil, retention oil, particle size, and encapsulation efficiency of essential oil-loaded capsules

Treatment	Brix (%)	Emulsion quality*	Surface oil (%)	Retention oil (%)	Particle size (nm)	Encapsulation efficiency (%)
T1	21.56	Poor	9.4^{a}	nd	1022 ^{b}	74.1^{d}
T2	21.44	Poor	6.4^{b}	nd	747^{d}	78.8^{cd}
T3	20.47	Weak	2.1^{e}	nd	1259^{a}	91.3^{ab}
T4	19.32	Good	5.2^{bc}	nd	630^{e}	81.5^{c}
T5	22.40	Good	3.6^{cd}	nd	360^{f}	88.3^{b}
T6	22.07	Weak	3.1^{d}	nd	366^{c}	91.5^{ab}
T7	22.87	Good	2.1^{de}	80.9^{a}	323^{f}	96.2^{a}
T8	22.34	Good	s1.4^{e}	75.2^{b}	406de	95.3^{a}
SEM	1.68	-	0.46	2.18	37.8	3.42

*The dispersity, stability, size, and shape uniformity of oil droplets in water emulsion was analyzed by an inverted microscope.
Means with different letters in the same column are significantly different (P<0.05).
Each experiment was performed in triplicate.

Table 6. Antibacterial and antioxidant activities of essential oil-loaded microcapsules

Antibacterial activity	*S. enteritis*		SEM
	Microcapsules extract	Oxytetracycline	
MIC (mg/ml)[1]	1.87^{a}	0.62^{b}	0.32
MBC (mg/ml)[2]	5.0	5.0	0.00
Antioxidant Activity	**Microcapsules extract[3]**	**Vitamin C**	**SEM**
DPPH scavenging IC_{50} (µg/ml)	89.7^{a}	16.2^{b}	3.42
NO scavenging IC_{50} (µg/ml)	106.7^{a}	43.7^{b}	5.08

[1]Minimum inhibitory concentration.
[2]Minimum bactericidal concentration.
[3]The concentration corresponds to inhibition of 50% of DPPH or nitric oxide free radicals.
Different letters in the same column indicate a significant difference (P<0.05).
The analysis was performed in triplicates.

Table 7. The final weight, total feed intake, FCR, pH of ileum, and cecum of chickens receiving different treatments

Treatments	Final weight (g)	Total feed intake (g)	FCR	Ileum pH	Cecum pH
T1	294.6^{b}	325.2^{a}	1.10^{bc}	6.2	7.7
T2	263.4^{d}	318.7^{b}	1.21^{a}	6.7	7.8
T3	287.0^{c}	319.2^{ab}	1.11^{bc}	6.4	7.7
T4	296.8^{ab}	317.2^{b}	1.07^{bcd}	6.0	6.9
T5	301.3^{a}	312.1^{c}	1.04^{d}	6.0	6.9
T6	299.1^{a}	312.8^{c}	1.05^{cd}	6.2	6.9
T7	286.7^{c}	321.6^{a}	1.12^{b}	6.1	7.2
SEM	2.67	1.47	0.031	0.28	0.37

T1: Uninfected; T2: Infected; T3: Infected+0.5 kg/ton; T4: Infected+1 kg/ton; T5: Infected+2 kg/ton; T6: Infected+DPEP; T7: Infected+Oxy 0.4 kg/ton
FCR: Feed conversion ratio.
The experiment was conducted for 10 days.
Different letters in the same column indicate significant differences (P<0.05).
The analysis was performed in triplicates.

Table 8. Liver enzymes and immunoglobulins content of the serum

Treatments	Liver enzymes		Immunoglobulins	
	ALT(U/L)	AST(U/L)	IgG(ng/ml)	IgA(ng/ml)
T1	17.3^{bc}	206.6^{d}	57.0^{c}	35.6^{c}
T2	33.3^{a}	255.3^{a}	81.6^{a}	52.6^{a}
T3	28.6^{a}	252.6^{a}	65.6^{bc}	41.6^{abc}
T4	21.6^{b}	243.6^{ab}	75.3^{ab}	42.0^{abc}
T5	12.3^{c}	231.6^{bc}	71.0^{abc}	48.6^{ab}
T6	17.3^{bc}	214.6^{cd}	38.6^{d}	23.0^{d}
T7	15.6^{c}	223.0^{cd}	70.6^{abc}	40.6^{bc}
SEM	1.80	5.73	4.28	3.46

T1: Un-infected; T2: Infected; T3: Infected+0.5 kg/ton; T4: Infected+1 kg/ton; T5: Infected+2 kg/ton; T6: Infected+DPEP; T7: Infected 0.4 kg/ton.
Different letters in the same column indicate a significant difference (P<0.05).
The analysis was performed in triplicates.

3.4. Lipid Peroxidation and Antioxidant Activity

The lipid peroxidation, antioxidant activity of the serum and meat, and the total phenolic content of the meat are presented in table 9. The results revealed that *S. enteritidis* challenge in chickens increased the lipid peroxidation and significantly impaired the antioxidant activity of the breast meat and serum (P<0.05), compared to that in the unchallenged chickens (T2 vs T1).

3.5. Morphometric Analysis

The results of the morphometric analysis of the ileum are presented in table 10. The infection caused by *S. enteritidis* in chickens reduced the villus height, villus width, V: C ratio (villus height to crypt depth ratio), and the number of goblet cells; however, it increased the crypt depth significantly (P<0.05) (Figure 2).

3.6. Microbial Population Analysis

The ileum and cecum microbial population analysis is presented in table 11. Compared to the uninfected chickens, the population of *S. enteritidis* in the ileum and cecum of infected chickens increased significantly (P<0.05) by 5.8 and 2.6 folds, respectively.

In the current study, the essential oils obtained from thyme, summer savory, peppermint, and black pepper seed were encapsulated to prepare the protecting wall materials and form essential oil-loaded microcapsules. The antibacterial activity of essential oil-loaded microcapsules turns them into phytogenic feed additives known as phytobiotics.

Table 9. Antioxidant activity and total phenolic content of the breast meat and serum

Treatments	MDA (%)*		DPPH scavenging activity (%)		Breast meat total phenolic (%)*
	Serum	Breast meat	Serum	Breast meat	
T1	100^{d}	100^{d}	54.0^{cd}	15.1^{e}	100.0^{b}
T2	174.4^{a}	161.1^{a}	39.7^{f}	11.1^{e}	98.5^{b}
T3	150.2^{b}	150.1^{b}	55.5^{c}	29.4^{c}	102.7^{b}
T4	129.5^{c}	144.7^{b}	58.0^{b}	47.5^{b}	117.8^{a}
T5	102.0^{d}	135.8^{c}	60.6^{a}	52.10^{b}	117.3^{a}
T6	101.2^{d}	130.4^{c}	45.4^{e}	60.4^{a}	114.8^{a}
T7	153.7^{b}	86.7^{e}	51.8^{d}	21.2^{d}	99.1^{b}
SEM	4.50	2.22	0.81	1.88	1.91

T1: Uninfected; T2: Infected; T3: Infected+0.5 kg/ton; T4: Infected+1 kg/ton; T5: Infected+2 kg/ton; T6: Infected+DPEP; T7: Infected+Oxy 0.4 kg/ton
*relative to control
Different letters in the same column indicate significant differences (P<0.05).
The analysis was performed in triplicates.

Table 10. Ileum morphology and goblet cell count in chickens receiving different treatments

Treatments	Villus Height (µm)	Villus Width (µm)	Crypt Depth (µm)	V: C ratio	Goblet Cells (n)
T1	430.6^{d}	142.0^{bc}	107.0^{c}	4.1^{a}	4.9^{bc}
T2	409.0^{e}	101.0^{d}	140.0^{a}	2.9^{c}	7.9^{a}
T3	441.6^{c}	124.2^{bcd}	114.0^{c}	3.9^{ab}	6.9^{b}
T4	471.0^{b}	149.3^{b}	114.0^{c}	4.1^{a}	4.7^{bc}
T5	501.0^{a}	216.7^{a}	120.0^{bc}	4.2^{a}	3.8c
T6	504.0^{a}	111.4^{cd}	120.0^{bc}	4.2^{a}	3.8^{c}
T7	476.0^{b}	130.1^{bcd}	136.0^{ab}	3.5^{b}	4.7^{bc}
SEM	3.08	11.31	5.53	0.18	0.52

n=20 per treatment; Villus Height: Crypt Depth (V:C); (n/100 µm villus height)
T1: Uninfected; T2: Infected; T3: Infected+0.5 kg/ton; T4: Infected+1 kg/ton; T5: Infected+2 kg/ton; T6: Infected+DPEP; T7: Infected 0.4 kg/ton
Different letters in the same column indicate significant differences (P<0.05).
The analysis was performed in triplicates.

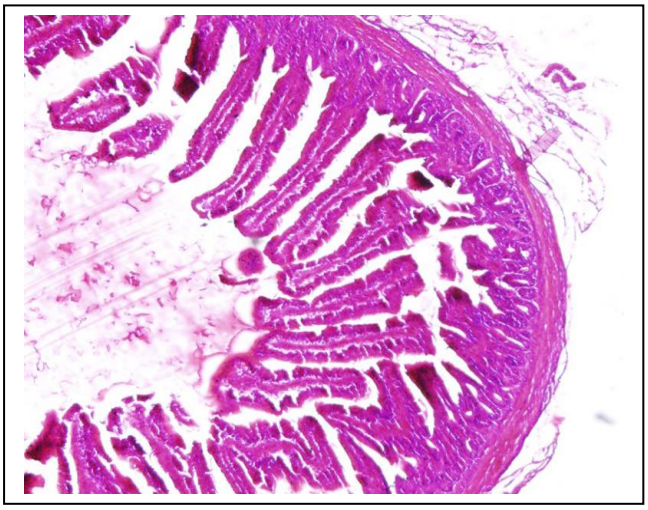

Figure 2. Ileum morphology in chickens receiving treatment five

Table 11. Microbial population analysis of ileum and cecum

Treatments	Ileum		Cecum	
	LABs	*S. enteritidis*	LABs	*S. enteritidis*
T1	1.0^{b}	1.0^{e}	1.0^{b}	1.0^{d}
T2	0.38^{c}	5.8^{a}	0.65^{bc}	2.64^{a}
T3	0.39^{c}	5.0^{b}	0.69^{bc}	2.49^{a}
T4	0.54^{c}	3.4^{c}	0.74^{bc}	1.96^{b}
T5	0.69^{bc}	1.6^{d}	1.0^{b}	1.62^{bc}
T6	3.5^{a}	1.8^{d}	1.6^{a}	1.54^{c}
T7	0.60^{c}	0.3^{f}	0.35^{c}	0.57^{e}
SEM	0.10	0.11	0.11	0.11

T1: Uninfected; T2: Infected; T3: Infected+0.5 kg/ton; T4: Infected+1 kg/ton; T5: Infected+2 kg/ton; T6: Infected+DPEP; T7: Infected 0.4 kg/ton.
Different letters in the same column indicate significant differences (P<0.05).
The analysis was performed in triplicates.

Authors' Contribution

Study concept and design: E. O.
Acquisition of data: M. M.
Analysis and interpretation of data: R. V., E. O. and G. R.
Drafting of the manuscript: M. M.
Critical revision of the manuscript for important intellectual content: R. V.
Statistical analysis: M. M.
Administrative, technical, and material support: R. V., E. O. and G.R.
Study supervision: R.V.

Conflict of Interest

The authors declare that they have no conflict of interest.

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