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Effect of microencapsulated *Minthostachys verticillata* essential oil as an antibiotic substitute on broiler performance

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Abstract

This study aimed to evaluate the effects of replacing antibiotics with microencapsulated *Minthostachys verticillata* essential oil (M-Mv-EO) as a phytogenic feed additive (PFA) on growth performance, liver and intestinal histological changes, serum biochemical parameters, genotoxic activity, and cecal microorganisms in broiler chickens. The Mv-EO was microencapsulated using spray-dried wall material (WM) consisting of Gum Arabic, Maltodextrin, and Whey Protein Concentrate (1:1:1, w/w/w). Forty-eight Cobb broilers aging one-day-old were randomly assigned to four dietary treatments (three replicates per treatment) and fed with a basal diet (CON), a diet supplemented with antibiotics (Bacitracin zinc salt, 7.5 mg/kg) (BZS), a diet supplemented with M-Mv-EO at a concentration of 100 mg/kg, or a diet supplemented with WM only (CON-WM). The results showed that the microcapsules obtained by spray drying remained stable without modifying the composition of Mv-EO. Dietary supplementation with M-Mv-EO increased weight gain and feed intake and improved the feed conversion ratio (FCR; $P<0.05$) compared to the CON, CON-WM, and BZS groups. Furthermore, M-Mv-EO supplementation did not cause histological changes in the small intestine or the liver. M-Mv-EO supplementation reduced total cholesterol levels and demonstrated non-cytogenotoxic effects in a bone marrow micronucleus test. Moreover, supplementation with M-Mv-EO promoted the growth of Lactobacillus spp., Enterobacteria, and total aerobic bacterial proliferation compared to the BZS group ($P<0.05$). These findings suggest that M-Mv-EO as a PFA, at a concentration of 100 mg/kg, could replace antibiotics in broiler chicken diets with comparable growth performance and health parameters.

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Introduction

For decades, antibiotics have been widely used in animal production to improve growth performance and prevent bacterial infections. However, overuse of antibiotics, particularly synthetic ones, has led to the emergence of antibiotic-resistant bacteria, posing a significant threat to public health (1,2). Therefore, there is a growing need to identify alternative strategies to promote growth performance and maintain animal health without antibiotics (3-6). One promising approach uses phytogenic feed additives (PFAs), plant-derived compounds with antimicrobial, immunomodulatory, and growth-promoting properties (6-12). *Minthostachys verticillata*, also known as peperina, is a plant species native to South America that has been used in folk medicine for the treatment of various

diseases, including gastroenteric, carminative, antispasmodic, and antirheumatic disorders (13,14). The essential oil of *Minthostachys verticillata* (Mv-EO) is composed mainly of monoterpenes, such as menthone, pulegone, menthol, and limonene. Scientific studies have demonstrated that Mv-EO possesses antimicrobial, anti-inflammatory, and antioxidant properties (15-21). Previous studies by our group have confirmed that Mv-EO is neither cytotoxic in vitro nor cyto-genotoxic in vivo, both at low and high concentrations in acute and subchronic studies (22,23). Recently, Montironi *et al.* (24) reported that oral administration of Mv-EO modulated inflammatory and oxidative parameters and favored the development of beneficial microflora, maintaining an anti-inflammatory gut microenvironment in weaned piglets. Therefore, Mv-EO represents a promising phytogenic additive that could potentially replace in-feed antibiotics in animal production. However, their volatility and instability limit the use of essential oils in animal feed. Efforts are being made to develop more stable formulations of essential oil that can be safely used in animal feed (25). Microencapsulation, which protects and controls the release of bioactive compounds such as essential oils (26-28), is being explored to enhance stability and efficacy.

This study evaluated the effects of replacing antibiotics with microencapsulated Mv-EO as a PFA on growth performance, histopathological parameters, blood biochemical parameters, cytogenotoxic activity, and cecal microorganisms in broiler chickens.

Materials and methods

Plant material, essential oil extraction, and chemical characterization

Thin stems and fresh leaves from *M. verticillata* were used to obtain essential oil (Mv-EO). The plant material was purchased from a local herb store, and it was identified and authenticated by a botanist at the Universidad Nacional de Río Cuarto. The Mv-EO was extracted by the steam distillation procedure for 1 h in Figmay Semi-Laboratory-Scale extractor equipment (Figmay, Argentine). The extracted oil was then dehydrated using anhydrous sodium sulfate, and the purified Mv-EO was kept at 4 °C in the dark for further use. The Mv-EO was characterized by gas chromatography-mass spectrometry using a Clarus 580-SQ8 equipped (serial number 648N7021501, PerkinElmer®, USA) with an Elite 5ms column (30 m, 0.25 mm ID, 0.25 µm film thickness). The analysis was performed as a service by the Instituto Multidisciplinario de Biología Vegetal (IMIV-Conicet) (Cátedra de Química Orgánica, Facultad de Ciencias Exactas, Físicas y Naturales, Universidad Nacional de Córdoba). The sample was prepared by dissolving 1 µL of the original sample in 500 µL of analytical-grade hexane (PA hexane) according to the method described by Escobar *et al.* (13). The injection volume was 1 µL in split mode. The initial temperature was set at 60°C for 5 min, then ramped at 5°C/min to 240°C for 10 min. The chromatogram was obtained in scan mode from m/z = 50 to m/z = 350 (scan time: 0.2 s, inter-delay: 5 min). The chemical constituents were identified by comparing their mass spectra with Spectra Libraries V2.4.

Microencapsulation of Mv-EO as a phytogenic feed additive

The phytogenic feed additive used in this study was prepared by microencapsulating *M. verticillata* essential oil (Mv-EO) using a spray-drying technique according to Ozdemir *et al.* (29) with some modifications. Briefly, solutions of three different wall materials, Gum Arabic (GA), maltodextrin (MD), and whey protein concentrate (WPC) with a ratio of 1:1:1 (w/w/w), were left standing overnight at room temperature to allow the polymer molecules to fully hydrate. Emulsions were obtained by mixing the Mv-EO and the wall material solution using a rotor-stator blender (PRO Scientific PRO250 Laboratory Homogenizer, Germany) at 15,000 rpm. The ratio of the total solids (oil and wall material) to water in the emulsions was 30:70 (w/w), with the solids comprising 6% oil and 24% wall material. The emulsion was spray-dried using a Buchi Mini Sprayer Dryer B-290 (Büchi, Switzerland) equipped with a 0.7 mm diameter nozzle to produce Mv-EO microcapsules. The spray-drying parameters were set as follows: inlet temperature of 180°C, feed rate of 3 mL/min, drying air flow rate of 601 L/h, and aspiration rate of 35 m^3/h. The resulting encapsulated powder was stored in a sealed bottle at −18°C until further use when incorporated into broiler chicken feed (29).

Characterization of Mv-EO microcapsules

The yield of Mv-EO microcapsules was determined by the mass of total solids in powder in ratio to total solids in the initial emulsion (29). To obtain the solid content weight of the emulsion, approximately 1 mL of the emulsion was placed in the Unibloc MOC-63U Moisture Analyzer until a constant weight was achieved. The powder recovery was calculated using the following equation (Equation I): Powder recovery (%) = (mass of microcapsules/ mass of emulsion solid content) x 100. A modified version of the method described by Ozdemir *et al.* (29) was used to determine the total Mv-EO content in the microcapsules and encapsulation efficiency. Briefly, 0.1 g of the microcapsule powder was dissolved in 10 mL of distilled water and centrifuged at 5000 rpm for 1 min. The resulting mixture was then transferred to a 50 mL falcon tube, and 10 mL of hexane was added before stirring the solution for 30 min at room temperature. The sample was subsequently centrifuged at 9000 rpm for 10 min to separate the hexane from the aqueous phase, and the absorbance of the hexane layer was measured at 245 nm using a UV-Vis spectrophotometer (Hitachi, U-2800A, Tokyo, Japan) to determine the total Mv-EO content.

The Integral Center for Electron Microscopy (CIME) at the National University of Tucumán (Conicet-UNT) performed the microcapsules' particle morphology and size distribution analysis. The samples were mounted on an aluminum support stub with conductive double-sided adhesive carbon tape, followed by gold coating using the sputtering technique in a Jeol model JFC-1100 ion sputter equipment. The morphology and size distribution of the microcapsules were then examined using a Zeiss model supra 55 VP scanning electron microscope (Carl Zeiss AG, Germany).

Experimental animals and management

Forty-eight one-day-old broiler chickens (commercial line Cobb) vaccinated against Marek's disease were obtained from a commercial hatchery (INDACOR SA, Córdoba, Argentina). Animals were provided with feed and water *ad libitum* during the experiment. The light was continued for the first week, while a 23 h light:1 h dark cycle was used for the remaining trial period. Chickens were acclimated on place and ration for 7 days, then on the 8th day, distributed randomly into four groups (4 chickens/ replicate). The broilers were housed in metal cages (90×100×39 cm) equipped with drinking fountains and feeders, which were distributed to ensure homogenous live weight in each experimental unit. Heating, ventilation, and air-conditioning devices were used to maintain a comfortable thermal environment for birds at 28±2 °C. The experiment lasted for 42 days, during which the broiler chickens were fed a diet corresponding to each treatment in a mash form. A standard maize-soybean meal starter (d 0–21) and grower (d 22-42) commercial basal diet, which complies with the rules and regulations of the National Research Council for broilers (30), was used to formulate the basal diet (Table 1). The experimental diets were formulated by mixing the basal diet with a phytogenic feed additive (PFA) using a food mixing machine (130 L) equipped with a WEG electric motor (WEG, Argentina). The experimental diets were formulated as follows: treatment 1 (T1) was the control group (CON), consisting of the basal diet (BD); treatment 2 (T2) was the antibiotic group (BZS), which included the BD plus bacitracin zinc salt (7.5 mg/kg); treatment 3 (T3) was the basal diet with wall materials (BD-WM), but without M-Mv-EO; and treatment 4 (T4) was the *M. verticillata* essential oil group (M-Mv-EO), which included the BD plus M-Mv-EO (100 mg/kg). Broiler chickens were weighed at the start, weekly, and end of the study. Animals were monitored daily for signs of morbidity and mortality. This study was approved by the Research Ethics Committee of the National University of Río Cuarto (Research project PICT 2019-3427, Protocol Code 428/22).

Growth performance

The birds' body weight was measured at the start of the study and every seven days until the end of the 42-day trial. Feed consumption was measured by subtracting the weight of the residual feed from the total feed offered, and the cumulative feed consumption for each replicate was calculated every 7 days. These measurements were used to calculate final body weight (FBW), body weight gain (BWG), average feed intake (FI), and feed conversion ratio (FCR).

Table 1: Components of the basal diet (%, as fed basis)

Item	Diets starter	Finisher
Yellow corn	62.90	67.20
Soybean flour	22.60	19.00
Heat treated soybeans	5.50	5.00
Meat meal 40%	6.90	7.00
Vitamins	0.15	0.15
NaCl	0.20	0.20
Calcite 38%	0.35	0.30
sunflower oil	1.0	1.00
DL-Methionine	0.16	0.10
L-Lysine	0.10	—
Monensin	0.05	0.05
Total	100	100
Proximal Composition (%)		
Crude protein	20.33	18.90
crude fat	5.47	5.53
Crude fiber	3.34	3.08
Calcium	0.97	0.95
Total phosphorus	0.59	0.57
Lysine	1.14	0.93
Methionine	0.50	0.42
Tryptophan	0.24	0.22
ME, kcal.kg	3047	3062

The premix contained the following per kg of powder: calcium 10.2%, starch 0.016%, crude fiber 0.012%, vitamin A 1,600,000 IU, vitamin D3 320,000 IU, vitamin E 4,800 IU, vitamin B1 320 mg, vitamin B2 800 mg, vitamin B6 640 mg, vitamin B12 3,200µg, vitamin K3 320 mg, pantothenic acid 1,600 mg, niacin 6,400 mg, biotin 24,000µg, folic acid 160 mg, choline chloride 24,000 mg, iron 6,400 mg, iodine 160 mg, copper 1,600 mg, manganese 12,800 mg, zinc 9,600 mg, selenium 24 mg.

Pathological examination and histomorphometry

At the end of the 42-day feeding trial, a random selection of two broiler chickens from each replicate of every treatment was slaughtered and used for necropsy examination. Small liver and small intestine pieces were preserved in 10% neutral buffered formalin. The fixed tissues were trimmed, paraffin-embedded, and stained with hematoxylin and eosin for histopathological evaluation under optical microscopy (OM). Segments of the small intestine, from the gizzard to the pancreatic and bile ducts, were sampled from the midpoint of the duodenum to the intestine. Tissue samples of approximately 6 mm^2 were fixed

in 4% buffered saline formaldehyde (pH 7.2 to 7.4 at 4 °C), dehydrated through a graded ethanol series (30, 50, 70, 80, 90, 95, and 100%), and xylene solutions. Subsequently, they were embedded in paraffin and cut into approximately 4 µm histological serial sections. For microscopic analysis, hematoxylin-eosin (HE) staining was performed on the histological sections. For morphometric measurements of intestinal variables, two slides per animal/intestine, two sections per slide, and five fields per section were used (31). Morphometric assessments included villus length and width as well as intestinal crypt depth. Digital images were captured using a microscope with a high-resolution PowerShot G6 7.1 megapixels digital camera (Canon, Tokyo, Japan). Image analysis and morphometric measurements were conducted using the AxioVision AxioVs40 V4.6.3.0 software (Carl Zeiss, Göttingen, Germany). Subsequently, the apparent absorptive surface area (μm^2) was calculated using the following surface area formula proposed by Sakamoto *et al.* (32): surface area = π × (villus width/2) × (villus height).

Serum biochemistry

At the end of the feeding trial, the birds were euthanized by exsanguination, and 4 mL of blood was collected via the subclavian vein using 5 ml tubes without anticoagulant. The serum levels of total protein (TP), cholesterol (CHOL), albumin (ALB), globulin (GLB), and albumin-to-globulin ratio (A: G) were determined using standard laboratory methods.

Genotoxicity assay

The genotoxic effect of the microencapsulated *M. verticillata* essential oil (100 mg/kg) in broiler chicken erythrocytes was evaluated through the bone marrow micronuclei assay (33). Femurs were removed, and bone marrow cells were harvested using fetal bovine serum. Cells were centrifuged twice at 112 × g for 5 minutes at room temperature, and the supernatant was discarded, leaving 0.2 ml to resuspend the cell pellets. Slides were prepared from the cell suspensions, allowed to dry, heat-fixed, and stained consecutively with 0.4% May Grunwald and 5% Giemsa stains. Micronucleated erythrocytes per thousand erythrocytes (MNE‰) were determined by scoring 2000 erythrocytes per animal.

Bacteria quantification

To evaluate the effects of the microencapsulated *M. verticillata* essential oil (100 mg/kg) on the gut microflora of broiler chickens, bacterial quantification by drop plate technique was carried out (34). Digesta samples from the cecum of each bird, 6 broilers per treatment, were serially diluted (w/v) in a sterile physiology solution and plated on corresponding media. Nutrient Agar was used to enumerate aerobic bacteria, MacConkey Agar for Enterobacteria, both incubated at 37 °C for 24 h, and Rose Bengal Agar was used to quantify *Lactobacillus* spp and incubated at 37°C in a 5% CO_2 atmosphere for 48 h. Bacterial counts were obtained and expressed as colony-forming units per gram of cecal content (CFU/g) for each plate.

Statistical analysis

Values were expressed as the mean±standard deviation (SD) and analyzed by complete factorial ANOVA using GraphPad Prism version 8.0.1. The mean values were compared using Fisher's protected least significant test ($P<0.05$). For the micronuclei data, a one-way ANOVA followed by Tukey's multiple comparison test was performed using GraphPad Prism software. Statistical significance was established at $P<0.05$.

Results

Plant material, essential oil extraction, and chemical characterization

The essential oil of *Minthostachys verticillata* was extracted by steam distillation, yielding an average of 3.88% (**δ**: 0.961 gr/mL). The oil was mainly composed of pulegone 58.7% and menthone 33.6%, with smaller amounts of spathulenol 1.51%, piperitenone 1,25%, and limonene 1.21%. The remaining compounds (isomenthone, isopulegone, and piperitone) were present in trace amounts, and the sum of the identified compounds accounted for 98.2% of the oil. The oil was found to contain unidentified compounds.

Characterization of Mv-EO microcapsules

Microcapsules were thoroughly characterized using various methods to ensure their quality and stability. Scanning electron microscopy (SEM) was used to evaluate the morphology and size of the microcapsules. As shown in figure 1, the microcapsules exhibited a collapsed, spherical shape with smooth surfaces, typical of spray-dried powders. The microcapsules showed a wide variability in size, ranging from 1.16 µm to 16.85 µm, with an average size of 3.11±1.82 µm. The powder recovery value of the Mv-EO microcapsules was 72.27±3.4%, and the encapsulation efficiency, representing the total oil content, was 52.28±2.2%. Furthermore, chemical characterization analysis of the Mv-EO contained in the microcapsules using SEM did not reveal any significant changes in the quality and quantity of the identified compounds compared to the unencapsulated essential oil.

Growth performance

The growth performance data is summarized in table 2. The results are reported for different periods: days 1-21, days 22-42, and days 1-42. During the initial period (days 1-21), there was no difference in average feed intake (FI), average body weight (BW) gain, or feed conversion ratio (FCR) among all groups. Nonetheless, from days 22 to 42, the M-

Mv-EO group showed significantly (P<0.05) improved BW gain compared to the other treatments. Similarly, the FI and FCR were also significantly improved (P<0.05) in the M-Mv-EO group compared to the basal diet (CON) and BZS groups starting from the fourth week of the treatment. Over the entire experimental period (days 1-42), the M-Mv-EO treatment resulted in higher FI, BW gain, and improved FCR compared to the CON and BZS groups. These differences were statistically significant (P<0.05).

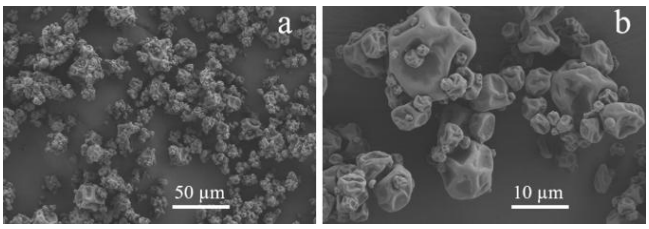

Figure 1: SEM micrographs of the microcapsules containing *Minthostachys verticillata* essential oil produced under the optimized spray-drying conditions: (a) scale bar 50 µm and (b) scale bar 10 µm.

Pathological examination and histomorphometry

Figure 2 shows photomicrographs of hematoxylin and eosin-stained liver sections from chickens subjected to various dietary treatments. Histological examination of the liver tissue revealed no signs of damage or abnormalities in any of the treatment groups. The absence of observable histological alterations suggests that including M-Mv-EO in broiler chicken diets did not induce adverse effects on liver morphology. Furthermore, morphometric analysis of the gut showed no significant differences in any of the measured parameters among the treatment groups. These parameters included the villus length, width, intestinal crypt depth, and apparent absorptive surface area (Table 3). These consistent findings indicate that dietary supplementation with microencapsulated *M. verticillata* essential oil did not exert substantial effects on the histomorphometry of the small intestine.

Table 2: Effect of replacing in-feed antibiotics with microencapsulated *Minthostachys verticillata* essential oil growth performance of broiler chickens

		CON	BZS	WM	M-Mv-EO
d 1-21	FI (kg)	1.31±0.32^{a}	1.22±0.29^{a}	1.25±0.85^{a}	1.36±0.74^{a}
	BW (kg)	1.11±0.45^{a}	1.12±0.33^{a}	1.21±0.85^{a}	1.26±0.47^{a}
	FCR	1.09±0.63^{a}	1.16±0.65^{a}	1.03±0.85^{a}	1.07±0.61^{a}
d 22-42	FI (kg)	2.98±0.54^{b}	2.96±0.39^{b}	3.28±0.88^{a}	3.38±0.43^{a}
	BW (kg)	1.06±0.48^{a}	1.24±0.35^{b}	1.38±0.85^{b}	1.53±0.73^{a}
	FCR	2.78±0.64^{a}	2.38±0.52^{a}	2.36±0.85^{a}	2.19±0.39^{a}
d 1-42	FI (kg)	4.28±0.44^{b}	4.19±0.42^{b}	4.52±0.90^{a}	4.74±0.57^{a}
	BW (kg)	2.37±0.21^{b}	2.18±0.31^{b}	2.66±0.42^{a}	2.80±0.30^{a}
	FCR	1.81±0.16^{b}	1.93±0.12^{b}	1.70±0.09^{a}	1.69±0.05^{a}

CON: basal diet (BD) without microencapsulated *Minthostachys verticillata* essential oil (M-Mv-EO); BZS: BD plus bacitracin zinc salt (7.5 mg/kg); WM: BD plus wall materials, but without M-Mv-EO; and M-Mv-EO: BD plus with M-Mv-EO (100 mg/kg). $^{\gamma}n = 12$ broilers per treatment. BW: Average body weight gain; FI: Average feed intake; FCR: feed conversion ratio (kg of feed intake/kg of BW gain, kg/kg). $^{a-b}$ Means in the same column without common superscripts differ significantly (P<0.05).

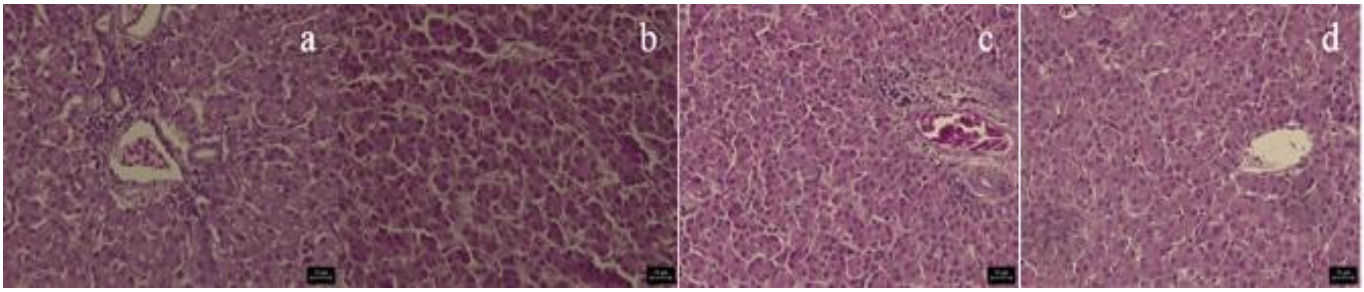

Figure 2: Photomicrographs of hematoxylin and eosin-stained chicken liver sections. (a) CON: basal diet (BD) without microencapsulated *Minthostachys verticillata* essential oil (M-Mv-EO); (b) BZS: BD plus bacitracin zinc salt (7.5 mg/kg); (c) WM: BD plus wall materials, but without M-Mv-EO; and (d) M-Mv-EO: BD plus with M-Mv-EO (100 mg/kg). Bar equals 75 µm.

Serum biochemistry

Table 4 presents the serum biochemistry results for the different treatment groups. It is important to note that all measured parameters, including total protein, albumin, globulin, and the A/G ratio, were within the normal range for all groups. Notably, the dietary supplementation of M-Mv-EO showed a trend of decreased cholesterol levels compared to the other treatment groups. Although this difference in cholesterol levels did not reach statistical significance, it is worth highlighting as it indicates a potential positive effect of the M-Mv-EO in modulating cholesterol metabolis.

Genotoxicity assay

A genotoxicity assay was performed to assess the potential cytotoxic effects of the treatments on the bone marrow erythrocytes. The results, summarized in table 5, indicate that the micronucleated erythrocyte per thousand erythrocytes (MNE‰) values were within the normal range for all treatment groups. No significant differences were observed among the treatments, suggesting that the microencapsulated *Minthostachys verticillata* essential oil did not induce genotoxic effects under the tested conditions.

Table 3: Effect of replacing in-feed antibiotics with microencapsulated *Minthostachys verticillata* essential oil on gut morphology of broiler chickens

	CON	BZS	WM	M-Mv-EO
Intestinal villus height (µm)±SD	599.6±176.8	686.7±78.9	674.1±70.9	698.6±98.5
Intestinal villus width (µm)±SD	133.6±52.9	151.5±60.3	113.1±22.5	122.6±29.1
Crypt depth (µm)±SD	92.1±17.1	62.2±28.9	77.9±11.5	91.5±20.9
Apparent absorption area ($\times 10^4$ µm^2)±SD	26.6±15.3	32.9±10.9	23.9±5.49	27.1±8.12

CON: basal diet (BD) without microencapsulated *Minthostachys verticillata* essential oil (M-Mv-EO); BZS: BD plus bacitracin zinc salt (7.5 mg/kg); WM: BD plus wall materials, but without M-Mv-EO; and M-Mv-EO: BD plus with M-Mv-EO (100 mg/kg). $^{\gamma}n = 6$ broilers per treatment. Columns without stats do not show statistical differences among treatments.

Table 4: Effect of replacing in-feed antibiotics with microencapsulated *Minthostachys verticillata* essential oil on serum biochemistry of broiler chickens

	CON	BZS	WM	M-Mv-EO
Total protein (g/dl) (mean±SD)	3.75±0.85	3.58±0.36	3.97±0.52	3.61±0.43
Albumin (g/dl) (mean±SD)	1.56±0.14	1.55±0.22	1.67±0.22	1.51±0.10
Globulin (g/dl) (mean±SD)	2.19±0.85	2.03±0.15	2.30±0.59	2.03±0.28
Ratio A/G (mean±SD)	0.62±0.36	0.75±0.07	0.72±0.24	0.78±0.11
Cholesterol (mg/dl) (mean±SD)	148.38±32.2	145.09±26.9	177.59±12.2	128.70±9.89

CON: basal diet (BD) without microencapsulated *Minthostachys verticillata* essential oil (M-Mv-EO); BZS: BD plus bacitracin zinc salt (7.5 mg/kg); WM: BD plus wall materials, but without M-Mv-EO; and M-Mv-EO: BD plus with M-Mv-EO (100 mg/kg). $^{\gamma}n = 6$ broilers per treatment. Columns without stats do not show statistical differences among treatments.

Table 5: Effect of replacing in-feed antibiotics with microencapsulated *Minthostachys verticillata* essential oil on bone marrow erythrocytes of broiler chickens.

Treatment	‰ MNE (mean±SD)
CON	2.65±0.65
BZS	2.47±0.53
WM	2.75±0.78
M-Mv-EO	2.44±0.72

CON: basal diet (BD) without microencapsulated *Minthostachys verticillata* essential oil (M-Mv-EO); BZS: BD plus bacitracin zinc salt (7.5 mg/kg); WM: BD plus wall materials, but without M-Mv-EO; and M-Mv-EO: BD plus with M-Mv-EO (100 mg/kg). $^{\gamma}n = 6$ broilers per treatment. Columns without stats do not show statistical differences among treatments.

Bacteria quantification

The quantification of total aerobic bacteria, enterobacteria, and lactobacilli in cecum samples using the drop plate technique is presented in figure 3. The results revealed significant differences in bacterial counts among the treatment groups, whereas the basal diet (CON) exhibited the highest bacterial counts. Moreover, the M-Mv-EO treatment group showed significantly higher counts for all three bacterial determinations than the BZS treatment group ($P<0.05$). Specifically, the M-Mv-EO group had counts of 6.2 x 10^8 CFU/g of total aerobic bacteria, 5.63 x 10^9 CFU/g of lactobacilli, and 2.2 x 10^8 CFU/g of enterobacteria. In contrast, the BZS group had counts of 2.05 x 10^8 CFU/g for total aerobic bacteria, 1.1 x 10^9 CFU/g for lactobacilli, and 1.15 x 10^8 CFU/g for enterobacteria. These findings suggest that dietary supplementation with microencapsulated *Minthostachys verticillata* essential oil significantly impacted broiler chickens' abundance of gut bacteria. The

higher bacterial counts observed in the M-Mv-EO group highlight their potential to influence the composition of the gut microflora. Further investigation is warranted to elucidate the specific mechanisms underlying these effects.

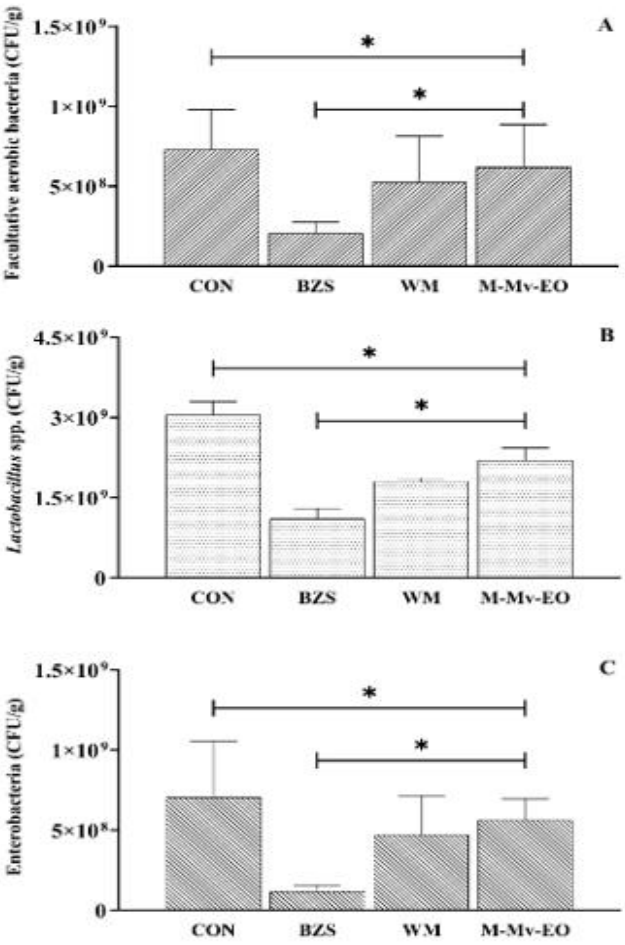

Figure 3: Effect of replacing in-feed antibiotics with microencapsulated *Minthostachys verticillata* essential oil on quantification of viable gut bacteria in broiler chickens. A) Aerobic bacteria, B) Lactobacillus sp. C) Enterobacteria. Data are expressed as mean±SD. CON: basal diet (BD) without microencapsulated *Minthostachys verticillata* essential oil (M-Mv-EO); BZS: BD plus bacitracin zinc salt (7.5 mg/kg); WM: BD plus wall materials, but without M-Mv-EO; and M-Mv-EO: BD plus with M-Mv-EO (100 mg/kg). $^{\gamma}n = 6$ broilers per treatment. * Indicates $P<0.05$.

Discussion

Modern poultry production faces challenges regarding antibiotic use and the need for sustainable alternatives. Consequently, interest in phytogenic feed additives has increased. The use of essential oils as viable replacements for antibiotics in animal agriculture has gained significant attention because of their health-promoting properties (35,36). Interest in plant-derived feed supplements stems from their potential to enhance animal growth, nutrient absorption, and overall health. In this context, *Minthostachys verticillata* essential oil has been noted for its diverse biological properties and demonstrated safety both in vitro and in vivo, even at high concentrations (15,17,19,37,38). This study contributes to the growing evidence supporting phytogenic feed additives, specifically *M. verticillata* essential oil, in broiler diets.

Microencapsulation is a key approach for addressing difficulties related to the effective use of essential oils, providing innovative solutions, and boosting their applicability in various domains, including the poultry industry (39,40). The Mv-EO was nano-emulsified and then dehydrated using spray drying. The formulation combined three biopolymers (GA, MA, and WPC) to stabilize the nanoemulsion systems and form the encapsulating wall. The powder recovery of 72.27±3.4%, average particle size of 3.11±1.82 µm, and encapsulation efficiency of 52.28±2.2% of the Mv-EO microcapsules were similar to those reported in previous studies (41-43). Furthermore, the characterization of the components in the microcapsules showed no discernible changes compared to the essential oil without encapsulation. These findings confirmed the success of the microencapsulation process in producing stable, high-quality microcapsules that effectively retained the active compounds of the Mv-EO.

Enhancement of growth performance is pivotal in broiler production and directly affects economic gain. In the present study, dietary supplementation with M-Mv-EO significantly enhanced the productive parameters of broilers from days 1 to 42 compared to the control group. Moreover, broilers receiving M-Mv-EO exhibited superior growth performance compared to those administered antibiotics, suggesting that M-Mv-EO could serve as a viable and superior alternative to antibiotics in poultry diets. This study marks the pioneering use of M-Mv-EO as a phytogenic additive in animal production. Our findings align with other studies that have demonstrated the beneficial effects of different essential oils on body weight gain, feed index, and FCR in broilers (44-46).

The gastrointestinal mucosa and liver are pivotal tissues that directly interact with dietary components and serve as vital indicators for assessing potential damage induced by these substances. In this study, histopathological examination of the liver and intestine of all animals did not show any detectable abnormalities compared with the control group, indicating that M-Mv-EO did not induce organ damage. These findings agree with previous studies showing that treating Mv-EO did not cause organ damage in rats (23). However, while M-Mv-EO did not induce damage to the intestinal mucosa of broiler chickens, no significant differences were observed in the histomorphometry analysis of the intestine compared to the control group. These

findings contrast our previous results, where treatment with Mv-EO significantly increased villus height and improved overall intestinal morphology in rats, indicating potential benefits for intestinal health (23).

Moreover, various studies by different authors have reported significant reductions in crypt depth and increased villus height/crypt depth ratios in the jejunum of ducks (47), laying hens (48), and quails (49), following supplementation with different essential oils. However, our study did not observe similar improvements in broiler chickens. This discrepancy could be attributed to inherent physiological and metabolic variances between species and variations in different essential oils' chemical composition and properties.

The biochemical parameters assessed in this study showed no significant alterations upon adding M-Mv-EO. Although the difference in cholesterol levels did not reach statistical significance, it is particularly interesting, as it suggests a potential positive effect of M-Mv-EO on cholesterol metabolism modulation. One potential mechanism for lipid alteration could be the cholestatic effect of essential oils in the liver, possibly through enhanced removal or catabolism of lipoproteins or inhibition of hepatic 3-hydroxy-3-methylglutaryl coenzyme A (HMG-CoA) reductase activity, a key regulatory enzyme in cholesterol synthesis (50). Consistent with our findings, previous studies have reported reductions in cholesterol levels in broiler chickens following the addition of essential oils (51,52). Further research could explore the underlying mechanisms involved in regulating cholesterol metabolism by M-Mv-EO, as well as its potential impact on poultry health.

The bone marrow micronuclei assay results in this study align with previous research carried out by our group, demonstrating the lack of genotoxic and cytotoxic effects of Mv-EO both *in vitro* and *in vivo* (22). In a previous study, Mv-EO was administered up to 7 g/kg feed to rats over 90 days, and the results showed no evidence of cytogenotoxicity (23). These findings demonstrate the safety of M-Mv-EO and suggest that it is a safe feed additive for broiler chickens.

The influence of essential oils on the gut microbiota is complex. It involves interactions between bioactive compounds in the oil and diverse microbial populations in the gastrointestinal tract, which play important roles in the digestion and absorption of nutrients (36). In this study, the results of quantifying total aerobic bacteria, Enterobacteria, and *Lactobacillus* spp. suggest that dietary supplementation with microencapsulated M-Mv-EO modulates the establishment of beneficial intestinal microflora in broiler chickens. These findings are consistent with a recent study by Montironi *et al.* (24), who observed that oral supplementation with nanoencapsulated *M. verticillata* essential oil in weaned piglets promoted the development of beneficial intestinal microorganisms and potentially improved parameters associated with early weaning stress in piglets. This suggests a potential modulatory role of Mv-EO in the gut microflora across different animal species. Several research studies showed the effects of stimulating beneficial bacteria and inhibiting pathogenic microbes, inducing positive effects on productive parameters by including essential oil (53-55). In this sense, previous studies on the antibacterial properties in vitro of the *M. verticillata* essential oil have shown that it was active against *Bacillus subtilis*, *Staphylococcus aureus*, *Streptococcus faecalis*, *Bacillus cereus* var. *mycoides*, *Proteus mirabilis*, *Escherichia coli* and *Salmonella typhi* (16,37). Our results on the *lactobacillus* population are partially consistent with Cross *et al.* (56), who reported that carvacrol of thyme essential oil can stimulate the growth and proliferation of *Lactobacillus*.

Similarly, Agostini *et al.* (57) and Mohammadi *et al.* (58) found that *Lactobacilli* counts were increased for broiler chickens treated with clove essential oil. On the other hand, Geravand *et al.* (35) reported that adding 200 and 400 mg of licorice (*Glycyrrhiza glabra*) essential oil in broilers cannot stimulate the growth and proliferation of *Lactobacillus.* Although the precise mechanisms that cause the improvement in productive parameters are not yet fully understood, the modulation of intestinal microflora plays an important role. The improvement in productive parameters associated with the higher bacterial counts observed in the M-Mv-EO group than in the antibiotic group highlights the potential of this oil as a phytogenic additive. Further advanced techniques in future studies are crucial for a more complete understanding of broilers' microbial changes associated with dietary M-Mv-EO supplementation.

The results of this study underscore the potential of M-Mv-EO as a safe alternative to antibiotics in poultry feed. It can contribute to reduced antibiotic dependence and promote food safety and sustainable production practices in the poultry industry. Future research should explore the economic evaluation of *M. verticillata* essential oil in the diet of farm animals to optimize its application in animal production practices.

Conclusion

This study demonstrated that including *M. verticillata* essential oil as a phytogenic feed additive in broiler chicken diets improved growth performance, weight gain, feed intake, and feed conversion ratio. Additionally, M-Mv-EO did not induce genotoxic or cytotoxic effects. Moreover, it did not induce genotoxic or cytotoxic effects. It also enhanced the quantification of the total aerobic bacteria, Enterobacteria, and *Lactobacillus* spp. These findings support the recommendation of M-Mv-EO at a diet dosage of up to 100 mg/kg as a viable alternative to antibiotics for promoting the growth performance of broiler chickens.

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Conflict of interest

The authors declare no conflicts of interest.

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تأثير استخدام زيت مينثوستاتشيس فيتيسيلاتا العطري المغلف بالغشاء الدقيق كبديل للمضادات الحيوية على أداء الدجاج اللاحم

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الخلاصة

هدفت هذه الدراسة إلى تقييم تأثيرات استبدال المضادات الحيوية بالزيت الأساسي لمينثوستاكيس فيرتيشيلياتا المغلفة بالميكرو (M-Mv-EO) كإضافة تغذوية نباتية (PFA) على أداء النمو، والتغيرات النسيجية للكبد والأمعاء، ومعايير الكيمياء الحيوية في المصل، والنشاط الجيني السمي، والميكروبات القولونية في دجاج التسمين. تم التغليف الدقيق للـ Mv-EO باستخدام مادة الجدار المجففة بالرش (WM) المكونة من صمغ العربي، والمالتوديكسترين، ومركز بروتين مصل اللبن (١: ١: ١، وزن / وزن / وزن). تم تعيين ثمانية وأربعين دجاجة تسمين من نوع كوب في عمر يوم واحد عشوائياً إلى أربع معاملات غذائية (ثلاث مكررات لكل معاملة) وتم تغذيتها بنظام غذائي أساسي (CON)، أو نظام غذائي مكمل بالمضادات الحيوية (ملح زنك الباسيتراسين، ٧,٥ ملجم/كجم) (BZS)، أو نظام غذائي مكمل بـ M-Mv-EO بتركيز ١٠٠ ملجم/كجم، أو نظام غذائي مكمل بـ WM فقط (CON-WM). أظهرت النتائج أن الكبسولات الدقيقة التي تم الحصول عليها عن طريق التجفيف بالرش ظلت مستقرة دون تعديل تركيبة Mv-EO. أدى التكميل الغذائي بـ M-Mv-EO إلى زيادة في الوزن وزيادة في تناول العلف وتحسين نسبة تحويل العلف (FCR؛ $P<0.05$) مقارنة بمجموعات CON وCON-WM وBZS. علاوة على ذلك، لم يتسبب التكميل بـ M-Mv-EO في تغييرات نسيجية في الأمعاء الدقيقة أو الكبد. خفض التكميل بـ M-Mv-EO مستويات الكوليسترول الكلية وأظهر تأثيرات غير سامة للجينات في اختبار النوى الدقيقة لنخاع العظام. علاوة على ذلك، عزز التكميل بـ M-Mv-EO نمو Lactobacillus spp. وEnterobacteria وتكاثر البكتيريا الهوائية الكلية مقارنة بمجموعة BZS ($P<0.05$). تشير هذه النتائج إلى أن M-Mv-EO كإضافة تغذوية نباتية، بتركيز ١٠٠ ملجم/كجم، يمكن أن تحل محل المضادات الحيوية في أنظمة دجاج التسمين الغذائية مع أداء نمو ومعايير صحية مماثلة.