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Effects of a Dietary Herbal Mixture on Growth Performance, Metabolic Status, and Intestinal Health Parameters in Broiler Chickens

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Abstract:

Background:

In this study, the effects of supplementation with a dietary herbal mixture on growth performance, metabolic parameters, intestinal health, and nutrient transporter expression through Immunohistochemistery in broiler chickens is analyzed. This study was conducted in the specific environmental conditions of Wasit Province, Iraq. Ross 308 broiler (Twenty-day-old) chicks were randomly assigned to three dietary treatment groups during the study period (10 days) as followings: control group (basal diet without additives); T1 group (basal diet with 0.5 g/kg herbal mixture), and T2 group (basal diet with 1.0 g/kg herbal mixture). The herbal mixture was made of thyme (*Thymus vulgaris*), oregano (*Origanum vulgare*), and ginger (*Zingiber officinale*). These herbs mixture ratio was 40:40:20, respectively. The findings of the current study revealed significant ($p < 0.05$) differences among treatment groups. T2 group showed the highest enhancements (6.1%) of body weight (1.75 ± 0.03 kg) than in control chickens (1.65 ± 0.05 kg). It also demonstrated 8.0% improvement in feed conversion ratio (1.49 ± 0.02) than in controls (1.62 ± 0.02). Moreover, it revealed a significant up regulation of nutrient transporters and tight junction proteins. Overall, these results indicate that the supplementation of the herbal combination at a concentration of 1.0 g/kg of feed does promote growth performance, metabolic status, and intestinal health by improving absorption capacity and gut barrier function in broiler chickens in Iraq.

Keywords: Broilers, Growth Performance, Gut Health, Nutrient Transporters, Herbal Mixture, Iraq

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Introduction

In the past several decades, the poultry sector has become one of the most efficiently produced livestock protein systems around the world. This intensive production system, however, has unprecedented challenges in ensuring animal health and welfare, and in keeping an optimal production levels. In particular, the issue of antibiotic growth promoters (AGPs) (Windisch *et al.*, 2008; Dhama *et al.,* 2021). There is an increasing demand for AGP-free livestock and the challenge of antimicrobial resistance, which has pushed for the use of AGPs to be substituted by other natural means.

In Wasit Province of Iraq, the poultry industry is an important part of the agrarian economy, securing food and generating job opportunities. During the hot season, stormy and dusty summer months, the extreme and rapid daily temperature changes become an additional challenge to the poultry production systems (Al-Abbad *et al.,* 2019; Al-Hilali and Al-Mayah, 2020). These environmental stressors become more problematic in broilers during their 20 to 30 days of age where they underwent a rapid growth period. At this specific age of broilers, the muscle deposition is coupled with elevated metabolic demands and is challenged immunologically due to vaccine induced immunity or exposure to pathogens, or both.

The hot months of the year in Wasit Province of Iraq present unique environmental stressors to poultry production systems. The withdrawal of AGPs from poultry has increased the need of alternatives that can mitigate the gut inflammatory response, improve nutrient absorption, and promote overall gut health are a good option for overcoming the challenges that inflammatory response poses to production and profit in poultry (Zeng *et al.,* 2020; Abdelli *et al.,* 2021). The modulation of gut microbiota, improvement of enzyme activity, and nutrient absorption is a function of the variety of bioactive and phytogenics.

Thyme (Thymus vulgaris), oregano (Origanum vulgare), and ginger (Zingiber officinale) represent three specific phytogenic value plants which possess distinctive phytobiological activities. Thyme has high concentration of thymol and carvacrol, which are well known for their strong anti-microbial activity towards pathogens like *Escherichia coli* and *Salmonella* species (Hashemipour *et al*., 2013). Oregano has a large amount of carvacrol which is known for its strong anti-microbial activity and antioxidant properties (Giannenas *et al.,* 2014). Ginger has glucosides and shogaols that have anti-inflammatory, antioxidant, and digestive stimulating attributes (Zhang *et al.*, 2009).

There are a number of new analytical methods that explore the mechanistic phytogenic effects on gut health on a molecular level. The methods provide spatial resolution of the molecular profiling of the proteins that are responsible for the transport of nutrients and gut permeability. These are of great potential value in the study of the gut physiology impacted by phytogenic compounds (Broom and Kogut, 2018; Yang *et al.,* 2020). The molecular explanation of the enhancing effects of the glucose and peptide transporters SGLT1 and PepT1, and the tight junction proteins ZO-1 and occludin on gut health helps researchers to propagate the herb blends that improve gut health (Zhang

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et al., 2009).

As research on phytogenic feed additives continues to grow, there still needs for more information on their influence regarding environmental conditions, such as those found in the Wasit Province in Iraq. Notably, there is limited number of comprehensive studies using advanced molecular approaches, including methods like immunohistochemistry, to explain the mechanisms of action of herbal mixtures on broiler chickens. Thus, this study is proposing to fill this research gap to investigate the influence of a dietary herbal mixture of thyme, oregano, and ginger on growth performance, metabolic parameters, and intestinal health using immunohistochemistry in broiler chickens raised in a unique environmental situation of Wasit Province, Iraq (Al-Abbad *et al.*, 2019).

Materials and Methods

Ethical Approval and Study Location

In spring 2024 (March-April), the experiment took place at a commercial broiler farm located in Al-Hai district of Wasit Province, Iraq. Approval of the research protocol was granted by Animal Care and Use Committee at the College of Veterinary Medicine, University of Diyala (ethical approval number VM222). All procedures were performed according to international standards concerning with the ethical treatment of animals in research.

Experimental Birds and Management

From a local hatchery, 300 of Ross 308 broiler chicks (one-day-old) were obtained. The birds were placed in an environmentally controlled shed with a concrete floor and wood shavings as litter material. During the first week of the experiment, the temperature was kept at 32°C and then was gradually lowered by 2.5°C each week until it reached 22°C. The humidity was maintained at 60-70. During the entire experimental period, birds were provided 23 hours of light. Feed and water were freely accessible (*ad libitum*) to all birds through automatic feeders and nipple drinkers.

Vaccination Program

All birds underwent the same vaccination program as the followings:

- Day 1: Infectious Bronchitis (Massachusetts strain) via spray.
- Day 7: Newcastle Disease (LaSota strain) and Infectious Bronchitis via drinking water.
- Day 14: Newcastle Disease (LaSota) and Gumboro disease vaccines via drinking water.

Experimental Design and Dietary Treatments

On day 20, birds were assigned to three experimental groups (5 replicates per group, 20 birds per replicate):

1. Control group: Basal diet without additives.
2. T1 group: Basal diet + 0.5 g/kg herbal mixture.
3. T2 group: Basal diet + 1.0 g/kg herbal mixture.

The herbal mixture was made up of 40% thyme (Thymus vulgaris), 40% oregano (Origanum vulgare), and 20% ginger (Zingiber officinale) powders. The herbs were sourced from a certified

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supplier. The basal diet was prepared to meet nutrient requirements according to Ross 308 guidelines.

Growth Performance Measurements

After the beginning of the experiment, the birds were individually weighed on days 20 and 30 using a scale that measured to the nearest 0.1 gram. The amount of feed that each bird ate was calculated for each experimental period. Also, the following parameters were determined: Average weight (grams), gained weight (grams), feed conversion ratio (FCR), and total weight attained (grams). The FCR (Feed conversion ratio) was calculated by counting the total feed weight and dividing it by the total live weight (Karadas *et al.*, 2007).

Blood Sample Collection and Analysis

Two birds were taken from each experimental group and blood was collected by brachial vein puncture on day 30 of the experiment. Blood was placed in sterilized containers and later on was stored at a temperature of -20 C°. Then, serum was prepared and analyzed for the biochemical evaluation such as total protein (albumin, globulin), AST, and ALT (Marc *et al.*, 2010).

Amino Acid Analysis

Serum amino acid was spearheaded by high-performance liquid chromatography and o-phthaldialdehyde pre-column derivatization. Analysis included only an essential amino acids such as lysine, methionine, threonine (Farikha *et al.*, 2012).

Intestinal Tissue Sampling and Histomorphometry

On day 30, jejunal tissue samples were taken (approximately 2 cm length) from two birds per replicate. This tissue was fixed in 10% neutral buffered formalin for 73 hours, processed through a series of graded alcohols, embedded in paraffin, and sectioned to 5 µm thickness. The sections were stained with hematoxylin and eosin for morphological examination. An image analysis software NIRS was used to measure villi height, crypts depth, and muscularis thickness (Wang *et al.*, 2021).

Immunohistochemical Analysis

For the jejunal tissue sections, the immunohistochemical staining was performed using the avidin-biotin-peroxidase complex approach. The tissue sections were deparaffinized and rehydrated through xylene and a series of graded alcohols. The antigen was retrieved using a citrate buffer (pH 6.0) at 95°C for 20 minutes. Blocking of the endogenous peroxidase activity was performed using 3% hydrogen peroxide for 10 minutes. In addition, 5% normal goat serum was used for 30 minutes to block the non-specific binding.

Sections were incubated with primary antibodies overnight at 4°C:

- Anti-SGLT1 (1:200 dilution) for sodium-glucose transporter
- Anti-PepT1 (1:200 dilution) for peptide transporter
- Anti-ZO-1 (1:100 dilution) for tight junction protein
- Anti-occludin (1:100 dilution) for tight junction protein

Color development was obtained using DAB, and subsequent counterstaining was performed using hematoxylin. Sections were washed, and then biotinylated secondary antibody and streptavidin-HRP

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complex were added sequentially for 30 minutes, each. Negative controls were prepared with no primary antibody.

Image Analysis and Quantification

Immunohistochemistry was quantified using ImageJ software. Five 400× images were taken per section from randomly chosen fields, and the integrated optical density (IOD) of the positive staining was assessed and normalized to the area measured. Results were reported as IOD/μm².

Statistical Analysis

Statistical analysis was performed using SPSS, version 26.0. Normality was assessed using the Shapiro-Wilk test, and for multiple comparisons, One-way ANOVA with Tukey's HSD test was applied. Results are displayed as mean ± standard deviation, and significance was considered at $p < 0.05$.

Results

Growth Performance Parameters

As indicated in Table 1, improvements in growth performance parameters were noted in trete groups T1 and T2 due to the complementary herbal mixture. The starting body weights were statistically similar for all the treatment groups ($p > 0.05$). Still, by the 30-day mark, there were differences in the final body weights recorded, with the T2 group obtaining the heaviest weight of 1.75 ± 0.03 kg, which is a 6.1% improvement compared to the control group that recorded 1.65 ± 0.05 kg. T1 group showed intermediate results with a final body weight of 1.72 ± 0.04 kg, which illustrates a clear dose-response relationship.

T2 group exhibited the greatest weight gain (0.95 ± 0.02 kg) which significantly outperformed the T1 group (0.92 ± 0.02 kg) and the control group (0.85 ± 0.03 kg). Although the amount of feed consumed did not differ significantly between the groups ($p = 0.125$), the feed conversion ratio (FCR) showed significant differences. T2 group had the most optimal FCR of 1.49 ± 0.02 (8.0% improvement over the control group) which had an FCR of 1.62 ± 0.02. T1 group was again in the middle of the FCR values, which was 1.52 ± 0.01, thus reinforcing the idea that the improvements due to the herbal mixture in the FCR were dose dependent.

Table (1): Growth performance of broilers fed with different dietary treatments (20-30 days)

PARAMETER	CONTROL	T1 (0.5 G/KG)	T2 (1.0 G/KG)	P-VALUE
INITIAL BW (KG)	0.80 ± 0.02	0.80 ± 0.03	0.80 ± 0.02	0.985
FINAL BW (KG)	1.65 ± 0.05c	1.72 ± 0.04b	1.75 ± 0.03a	<0.001
BWG (KG)	0.85 ± 0.03c	0.92 ± 0.02b	0.95 ± 0.02a	<0.001
FI (KG)	1.38 ± 0.04	1.40 ± 0.03	1.42 ± 0.05	0.125
FCR	1.62 ± 0.02a	1.52 ± 0.01b	1.49 ± 0.02c	<0.001

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Serum Biochemical Parameters

There are statistically significant improvements regarding protein metabolism and liver function in the herbal-supplemented groups (T1 and T2, Table 2). Total proteins increased in a dose-dependent manner and reached the highest concentration in the T2 group (4.6 ± 0.3 g/dL), a 21.1% increase compared to the control group (3.8 ± 0.2 g/dL). Albumin levels also increased significantly and in the T2 group, final values reached 2.1 ± 0.1 g/dL as compared to 1.6 ± 0.1 g/dL in the control group which reflects the improved capacity of protein synthesis.

Chickens that received herbal supplementation had lower values of AST and ALT. These enzymes are indicators of stress and injury. The T2 group had the lowest levels of AST (38.7 ± 1.8 U/L) and ALT (23.1 ± 1.1 U/L) compared to the control group, this reduction was 14.6% and 19.2%, respectively. These combinations of results indicate that the herbal mixture protects the liver and further supports the idea that the mixture is including antioxidant and anti-inflammatory characteristics.

Table (2): this table outlines the serum biochemical parameters of broilers taken on day 30.

PARAMETER	CONTROL	T1 (0.5 G/KG)	T2 (1.0 G/KG)	P-VALUE
TOTAL PROTEIN (G/DL)	3.8 ± 0.2c	4.2 ± 0.2b	4.6 ± 0.3a	<0.001
ALBUMIN (G/DL)	1.6 ± 0.1c	1.8 ± 0.1b	2.1 ± 0.1a	<0.001
GLOBULIN (G/DL)	2.2 ± 0.1b	2.4 ± 0.1ab	2.5 ± 0.2a	0.023
AST (U/L)	45.3 ± 2.1a	42.1 ± 1.9ab	38.7 ± 1.8b	0.031
ALT (U/L)	28.6 ± 1.3a	25.3 ± 1.2b	23.1 ± 1.1b	0.028

Total protein concentration for the control group was 3.8 ± 0.2g/dL, and it was 4.2 ± 0.2 for T1 (0.5 g/kg), and 4.6 ± 0.3 for T2 (1.0 g/kg), which was significant (P<0.001).

The serum biochemical parameters was significant (P<0.001) for albumin since the control group was 1.6 ± 0.1, T1 was 1.8 ± 0.1, and T2 was 2.1 ± 0.1. For globulin, it was significant (P = 0.023) since the values for control was 2.2 ± 0.1, for T1 was 2.4 ± 0.1, and was 2.5 ± 0.2 for T2. For AST the control group was 45.3 ± 2.1, T1 was 42.1 ± 1.9, and T2 was 38.7 ± 1.8, which was significant (P=0.031). For ALT the control group was 28.6 ± 1.3, T1 was 25.3 ± 1.2, and T2 was 23.1 ± 1.1, which was significant (P=0.028).

Serum Amino Acid Profile

Lysine concentration was 185.3 ± 8.2 µmol/L in the control group and was 218.7 ± 9.1 µmol/L in the T2 group. Of note, there was an 18% increase of lysine in the T2 group. Methionine had a 29.6% increase for the T2 group (58.6 ± 2.8 µmol/L) compared to control (45.2 ± 2.1 µmol/L). Threonine concentrations was higher in T2 group (182.4 ± 8.3 µmol/L) compared to the control (156.7 ± 6.8 µmol/L); which showed an increase of 16.4%.

Among non-essential amino acids, glutamine increased by 18.7% in the T2 group as compared to the control. In the T2 group, arginine concentrations increased by 26.4% compared to

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the control group.

Table (3): Serum amino acid profile of broilers on day 30 (µmol/L)

AMINO ACID	CONTROL	T1 (0.5 G/KG)	T2 (1.0 G/KG)	P-VALUE
ESSENTIAL AA				
LYSINE	**185.3 ± 8.2c**	**201.5 ± 7.6b**	**218.7 ± 9.1a**	**<0.001**
METHIONINE	**45.2 ± 2.1c**	**52.3 ± 2.3b**	**58.6 ± 2.8a**	**<0.001**
THREONINE	**156.7 ± 6.8c**	**168.9 ± 7.2b**	**182.4 ± 8.3a**	**<0.001**
NON-ESSENTIAL AA				
GLUTAMINE	**285.4 ± 12.3c**	**312.6 ± 13.1b**	**338.9 ± 14.7a**	**<0.001**
ARGININE	**95.3 ± 4.2c**	**108.7 ± 4.8b**	**120.5 ± 5.3a**	**<0.001**

Intestinal Histomorphometry

The improvements in gut architecture as evidenced by the histomorphometric analysis of the intestines in Table 4 are considerable. The T2 group (1120 ± 35 µm) had villi height increase of 17.9% in comparison to control (950 ± 25 µm). Crypt depth in the control (180 ± 10 µm) was reduced by 8.3% compared to the T2 group (165 ± 7 µm). The Villus Hight: Crept Depth (VH: CD) ratio of controls (5.27 ± 0.3) improved by 28.6% in the T2 group (6.78 ± 0.5).

The T2 group showed a reduction of 15.3% in the muscularis thickness compared to control group. This reduction in muscularis thickness indicate of a positive response to the reduction of inflammation of the intestines for the herbal supplemented groups. Herbal supplementation positively improves inflammation in the intestines as seen in the muscularis thickness reduction and also improves the absorbance of nutrients as seen in the increase of the intestinal villi.

Table (4): 30 Day Intestinal Histomorphometry

PARAMETER	CONTROL	T1 (0.5 G/KG)	T2 (1.0 G/KG)	P-VALUE
VILLUS HEIGHT (MM)	950 ± 25c	1050 ± 30b	1120 ± 35a	<0.001
CRYPT DEPTH (MM)	180 ± 10a	170 ± 8ab	165 ± 7b	0.032
VH:CD RATIO	5.27 ± 0.3c	6.18 ± 0.4b	6.78 ± 0.5a	<0.001
MUSCULARIS THICKNESS (MM)	85 ± 4a	78 ± 3b	72 ± 3c	0.021

Immunohistochemical Analysis

Table 5 summarizes of the results of immunohistochemical analysis.

SGLT1 expression in the T2 group increased by 45.2% (1.23 ± 0.06 IOD/µm²) compared to control (0.85 ± 0.04 IOD/µm²). PepT1 expression also increased by 38.7% in the T2 group (1.08 ± 0.05 IOD/µm²) compared to control (0.78 ± 0.03 IOD/µm²).

Regarding tight junction proteins, ZO-1 expression showed a 52.3% increase in the T2 group (0.99 ± 0.05 IOD/µm²) in comparison to the control (0.65 ± 0.03 IOD/µm²), while occluding showed a 48.6% increase in the T2 group (1.06 ± 0.05 IOD/µm²) relative to control (0.71 ± 0.03 IOD/µm²). The increased expression of these proteins indicates strengthened transport of nutrients and better gut barrier in the herbal-supplemented groups.

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Immunohistochemical analysis of jejunal tissues (IOD/μm²) is shown in Table 5.

Table (5): Immunohistochemical analysis of jejunal tissues (IOD/μm²)

PARAMETER	CONTROL	T1 (0.5 G/KG)	T2 (1.0 G/KG)	P-VALUE
SGLT1	0.85 ± 0.04c	1.12 ± 0.05b	1.23 ± 0.06a	<0.001
PEPT1	0.78 ± 0.03c	0.98 ± 0.04b	1.08 ± 0.05a	<0.001
ZO-1	0.65 ± 0.03c	0.89 ± 0.04b	0.99 ± 0.05a	<0.001
OCCLUDIN	0.71 ± 0.03c	0.94 ± 0.04b	1.06 ± 0.05a	<0.001

Hematological Parameters

Improvement of the immune status of the herbal-supplemented groups is reflected in the hematological parameters shown in Table 6. Although total white blood cell count did not reveal significant differences (p = 0.215), the differential counts did show some significant changes. The percentage of heterophils decreased by 16.2% in the T2 group (35.6 ± 1.8%) as compared to the controls (42.5 ± 2.1%), and the percentage of lymphocytes increased by 15.5% in the T2 group (55.8 ± 2.8%) compared to control group (48.3 ± 2.4%). Heterophils to lymphocyte ratio (H/L ratio), an indicator of stress, dropped by 27.3% in group T2 (0.64 ± 0.03), compared to the controls (0.88 ± 0.04), suggesting a decrease in stress levels and an increase in immune competence in the groups supplemented with herbs.

Table (6): this table reveals hematological parameters of broilers

PARAMETER	CONTROL	T1 (0.5 G/KG)	T2 (1.0 G/KG)	P-VALUE
WBC (×10³/ML)	25.3 ± 1.2	26.1 ± 1.3	26.8 ± 1.4	0.215
HETEROPHILS (%)	42.5 ± 2.1a	38.2 ± 1.9b	35.6 ± 1.8c	<0.001
LYMPHOCYTES (%)	48.3 ± 2.4c	52.6 ± 2.6b	55.8 ± 2.8a	<0.001
H/L RATIO	0.88 ± 0.04a	0.73 ± 0.03b	0.64 ± 0.03c	<0.001

Discussion

Based on the results of this study, dietary supplementation with an herbal mixture of thyme, oregano, and ginger boosts growth performance, metabolic status, and intestinal health in broiler chickens. There was a dose-response relationship in all parameters, with the greatest effects seen in the 1.0 g/kg mixture (T2 group).

The boosts of growth performance, the FCR increase by 8.0% in the T2 group in particular, come from multiple synergistic effects. Bioactive compounds of thyme, oregano, and ginger (thymol, carvacrol and gingerols, respectively) stimulate the secretion of digestive enzymes and improve nutrient digestibility (Windisch *et al.*, 2008). With an antimicrobial effect, these compounds likely assisted in minimizing nutrient competition and toxic metabolite production, and maintain a balanced gut microbiota (Hashemipour *et al.*, 2013).

The results of immunohistochemistry provide interesting clues to understand the improvements in the molecular mechanisms. The major increase of the SGLT1 and PepT1 transporters range explains the improvement in the capacity of absorption of the gut, while greater

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levels of ZO-1 and occludin explain the enhanced gut barrier function. These molecular changes explain the gut histomorphometry improvements, the increase in villi height, and the improvements of the VH: CD ratio (Broom and Kogut, 2018).

The improvement in serum amino acids in the groups treated with herbs indicates improvemed metabolism and utilization of the proteins. Moreover, the increased essential amino acids like lysine and methionine indicate that the protein digestibility and absorption were enhanced. Better availability of these amino acids explains the improvement in growth performance observed in treated groups (Yang *et al.*, 2020).

The reductions of the liver transaminases (AST and ALT) in the herbs treated groups suggest potential hepatoprotective effects, perhaps due to the antioxidant capacity of the herbal compounds. Improved values of the hematological parameters, especially the lower H/L ratio, explain the treated birds have enhanced immune competence with lower levels of stress (Dhama *et al.*, 2021).

The implications of this study are significant for chicken farming in Wasit Province, Iraq. Supplemental herbal mixture at 1.0 g/kg feed level with region's harsh environmental constraints, positively impacted productivity and health status. The present study also give benefits for the herbal extract in 'low' class broiler performance h(thyme, oregano, and ginger). The targeted feed formulation at 1.0 g/kg level was tremendously contributed to the health improvement. The immunohistochemical analysis confirms the health benefits gained with the strengthened nutrient transporter and tight junction proteins expressions. The improvement in all parameters suggests great potential of the use of herbal mixture as a substitute for antibiotic growth promoters in poultry production systems in Wasit Province, Iraq. Future research should investigate different environmental conditions and the long-term effects of supplementation on various parameters.

Conclusions:

The present study concluded the herbal extracts mixture give good body performance and healthy intestinal environment support the immune statues for broilers.

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