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Original Article

Effects of Supplementation of *Brassica Juncea* Seed Extract in Drinking Water on Intestinal Histomorphometry, Bacteriology, and Serum Biochemistry Parameters of Broiler Chicken

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Abstract

Brassica juncea (*B. juncea*) is an erect, and often an unbranched plant that belongs to the family Brassicaceae. The plant's seeds have been used in many countries as a folk remedy to treat considerable common and chronic diseases. The current study aimed to investigate the possible effects of *B. juncea* seed extract supplementation in the drinking water as an alternative antibiotic growth promoter on poultry production. In a completely randomized design, 308 unsexed Ross broilers were allocated into 4 treatments with4 replicates, and each replicate was run on10 birds. Aqueous *B. juncea* seeds extract (MSE) was administered to drinking water at levels of 0, 3, 5, and 7 ml/liter to T1, T2, T3, and T4, respectively, from day 1to day 35. No significant effects were reported regarding jejunum villi height and villi thickness ($P \geq 0.05$). However, the ratio of villus height to crypt depth was increased ($P<0.05$), and the crypt depth was reduced ($P<0.05$) in birds that had been fed *B. juncea* seeds extract, compared to control treatment (T1) at 35 day. The *B. juncea* seeds extract (MSE) at the level of 7 ml (T4) yielded the highest serum total protein, phosphorus, and calcium. The T2, T3, and T4 had the lowest values of cholesterol (160, 180mg /L) and the highest value ($P<0.05$) of alkaline phosphatase. On day 35, the birds receiving different levels of *B. juncea* seed extract had lower total aerobic bacteria counts in the ileum, compared to birds fed with control treatment. The administration of *B. juncea* seeds extract at 3, 5, and 7 ml levels can be added to drinking water to improve gut morphology, blood biochemical traits, and intestinal bacterial load.

Keywords: *Brassica juncea* Seed, Broilers, Intestinal health

1. Introduction

In the poultry production industry, antibiotic growth promoters (AGP) have been used to reduce morbidity, mortality, and intestinal problems (1). In recent years, the growing concern about the spread of bacterial resistance to antibiotics has severely restricted the use of antibiotics in animal feeds (2, 3). Restrictions in the use of antibiotic growth promoters had a negative effect on the growth performance and intestinal health (4). For these reasons, producers increasingly manipulate the balance of beneficial bacteria (*Lactobacillus*, *Enterococcus*, and *Bifidobacteria*) and pathogenic bacteria (preferences *Clostridium*, *Escherichia coli*, *Salmonella*, *Spp*), which should be 90 and 10% of the total intestinal bacteria, respectively, to overcome the effect of banned AGP (5). The application of locally available natural resources may have some beneficial effects to alleviate this problem. Therefore, various

investigations have recently been conducted to examine the use of bioactive agents that have a positive effect on gut health (6, 7). There is evidence to suggest that some of these components have appetite-stimulating (menthol) and antibacterial effects such as chaminoil, or may provide an antioxidant function (8). Mustard seeds (Brassica) are a very good source of organo-Sulphur compound, known as glucosinolates and phosphorus, manganese, dietary fiber, magnesium, selenium, iron, calcium, protein, niacin, zinc, and omega-3 fatty acids (9). Sinigrin, sinalbin, and glucobrassicin are the most prevalent glucosinate found in mustard (10). The activation of sinigrin metabolism is believed to lead to the synthesis of isothiocyanates, which contributes to antibacterial activity in animal production (11). The results of some studies showed that sinigrin acts as an anti-cancer, anti-inflammatory, anti-bacterial, anti-fungal, antioxidant, and wound-healing agent. Moreover, mustard seeds have been shown to stimulate growth and antioxidants, and play a role in improving metabolic activity, intestinal structure, and intestinal bacterial load (10). Although natural growth stimulants (NGPs) are commonly included in broiler chickens' feed as alternative products, not much is known about their possible effect on the health and productivity of these chickens. Therefore, new compounds will appear as an alternative to the antibiotic in poultry feed, and other nutraceuticals may be tested as well.

2. Material and Methods

2.1. Animals and Rearing

The experimental procedure was approved by the Animal Care Unit of Al-Qasim Green University in Iraq, from April to June 2020. In this completely randomized design study, 160 unsexed Ross 308 broiler chicks (with the mean ± SD body weight of 45±5 g) were selected from a local hatchery and randomly assigned into four equal-sized groups. Each group consisted of four replicates, with ten birds in each replicate. The chicks were housed in floor pens of 1.2×1 m in length and subjected to conventional management methods and environmental conditions for 35 days. The chickens were kept under the condition of 23 h of lighting and 1 h of darkness, and the temperature of33°C in the first week which was gradually reduced by 3°C per week until it reached 21°C in the third week. Each pen was supplied with aspirate feeding and one bell water drinker and bedded with wood shavings (7 cm deep). A free supply of food and water was made available to the chickens, and a constant lighting schedule remained in place during the whole trial.

2.2. Experimental Diets

Broiler chickens were supplied with a starter diet (21.7 g/ Kg CP, 11.87 MJ ME /Kg from day 1 to 21 and with a finisher diet (18.5 g/ Kg CP, 12.16 MJ ME/ Kg) from day 22 to day 35, respectively (Table 1, Calculated as-fed basis).The components of diets were created using the UFFDA feed formulation program (isocaloric and isonitrogenous diets were managed to meet the nutrient requirements of Ross 308 broiler chickens). UFFDA feed formulation program was developed by J. Hargrave at the University of Georgia in Athens, Georgia, United States (Aviagen, 2007). The experimental groups included T1: Basal diet with water,T2: Basal diet with water containing3 ml of Aqueous extract of *Brassica juncea* seeds MS per 1 lit. of drinking water, T3: Aqueous extract of *B. juncea* seeds MS5ml per 1lit of drinking water, and T4: Aqueous extract of *Brassica juncea* seeds MS7 ml per1 lit of drinking water.

Table 1. Items containing ingredients and nutritional levels in baseline diets% (as fed basis) [1]

Ingredients%	Starter(1-21 d)	Finisher(22-35d)
Corn	58.50	63.10
Soybean meal	35.99	31.39
Vegetable fat	1.25	2.21
Dicalcium phosphate	1.75	1.25
$CaCo_3$	1.57	1.39
Lysine-Hcl	0.02	0.03
Dl-Methionine	0.37	0.23
Salt	0.25	0.20
Mineral-vitamin premix[¥]	0.30	0.21
Nutrients composition		
Energy (metabolic) (kcal/kg)	2,960	3,085
Protein (Crude)	22.00	20.2
Calcium%	1.00	0.94
Available phosphorus%	0.71	0.63
Methionine + cysteine%	0.92	0.77
Lysin %	1.13	1.05

[¥]Per kilogram of food, mineral-vitamin premix supplied the following: 9,000 IU vitamin A; 2,100 IU vitamin D3; Vitamin E, Magnisum (30mg);Nicotinic acid vitamin (30 mg); Vitamin B12 (0.12 mg); Calcium pantothenate (10 mg); Vitamin K3(5 mg); Thiamine or B1(1.1 mg); Riboflavin(4.5 mg); Vitamin B6 (2.0 mg); Folic acid (0.5 mg); Biotin (0.5 mg); Fe (50 mg);Copper (10 mg); Manganese (70 mg)
[1]Estimated from NRC (1994) composition table.

2.3. Blood Parameters and Bacteriological Test

On day 35, female birds (n=8) were chosen randomly after 6 h of feed starvation and were weighed afterward. Blood samples were taken from the brachial vein, placed in identified tubes, and centrifuged at 2700 rpm for 15 min at 25 C^0 to separate the serum, which was then stored in Eppendorf tubes at -15 C^0. Total protein, total cholesterol, calcium (Ca), phosphorus (P), and alkaline phosphatase (ALP) were all measured using the Reflotrone plus analyzer (Roche, USA).These chosen broiler chickens were killed following the blood tests using a halal procedure to assess morphological and bacteriological characteristics of the gastrointestinal tract. The bacterial population in the small intestinal contents was used to determine some selected micro-organisms. The contents of the ileum (10 cm middle part) were separately collected, cooled, and used for microbial methods. The populations of *Escherichia coli*, *Salmonella* spp., and *Staphylococcus aureus* were diagnosed and then reported as cfu g-1. Sterilized Phosphate-buffered saline (PBS) (99ml) was added (1:100) to 1 g of fresh material to prepare the required dilutions. The diagnosed bacteria were cultivated in pure cultures and subcultured on nutrient agar, MacConkey agar, blood agar, eosin methylene blue (EMB), and triple sugar iron agar. All the isolates were stored in brain heart infusion broth containing15% glycerol at 4°C in the refrigerator to maintain the stock culture. Cultures were identified using conventional biochemical tests. Samples were diluted using sterile saline or phosphate buffer saline until the serial dilution was reached to the expected count through the conventional plate count procedure. Moreover, the last plates in the series should have between 30 and300 colonies. More than the 300 colonies on a plate are likely to generate colonies that are too close together to be recognized as different colony-forming units (they may not be representative

of the sample), and less than 30 colonies on a plate is not statistically acceptable (they may not be representative of the sample). The idea is that each viable bacterial cell is different from the others, and they will be aggregated and multiplied many times to form single separate colonies (CFU). Therefore, the count of colonies should be equal to the number of bacteria that develop under the employed incubation conditions. The number of bacteria per milliliter or gram of sample will be obtained by the number of colonies divided by the dilution factor multiplied by the volume of specimen supplied to liquefied agar (Figure 1).

CFUs = Numbers of bacteria/mlX invert dilution factor.

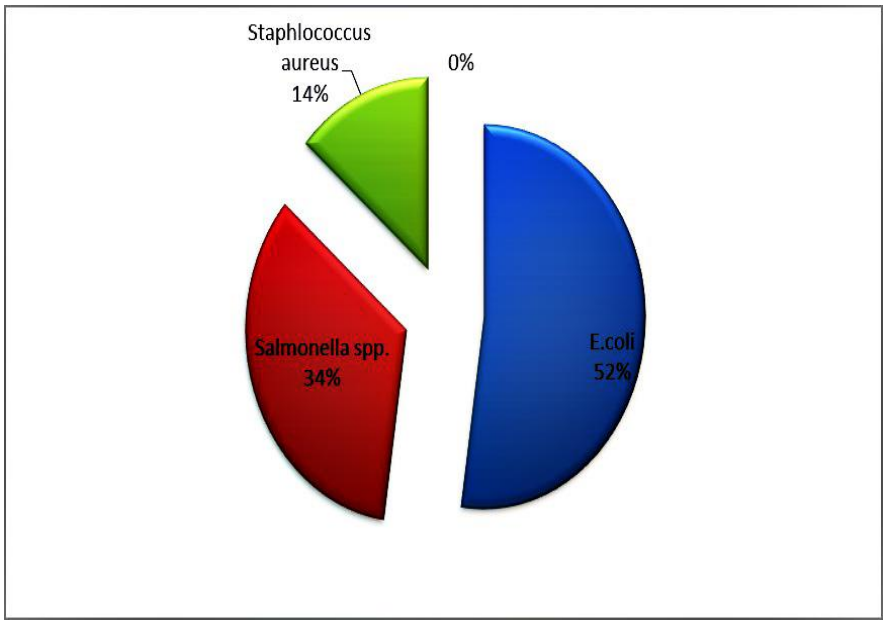

Figure 1. Percentage of the bacterial isolates

2.4. Histological Test

The middle third of the jejunum was excised approximately 2 cm. The pieces were immediately washed in distilled water, identified, and fixed using 10% buffered formalin for one week and dehydrated by immersion in a series of alcohols with increasing concentration (from 70% to absolute100%), infiltrated with xylene, and embedded in paraffin. The rotary-type microtome was used to cut the paraffin sections (7µm). Afterward, the samples were prepared and stained using hematoxylin and eosin at the histological lab in Al-Qasim Green University, Al Qasim, Iraq, to estimate villus height(VH), the junction of villus crypt, villus width (VW) (at half height), crypt depth (CD; defined as the depth of the invagination between adjacent villi), and VH to CD ratio (VH/CD). All specimens were examined using multiple magnifications (100 and 400X). VH and CD were measured using Sigma Scan Pro 5software (Olympus America Inc., Melville, NY.).

2.5. Plant Extract

Mustard seeds were purchased at a local market in Hilla, central south of Iraq. The extract was prepared using the procedure described by Arogundade, Enaibe (12). A blender was used to turn the mustard seeds (Brassica nigra) into powder. Subsequently, 300 gof crushed seeds were immersed in 1000 ml of deionized water, mixed, and stored in a refrigerator at 4°C for 36 h. The mixture was filtered using Whatman No. 1 filter paper. The filtrated fluid was then dried for 96 h at 40°C in a water bath to get a product that was then

diluted to make the stock. The extract was administered orally to the participants.

2.6. Statistical Analysis

The study had a completely randomized design with a 1× 4 factorial arrangement. The data were analyzed using SPSS software 2001 (20.20) and analysis of variance (ANOVA).The study values were compared using LSD tests at P<0.05.The mathematical model was as the following:

$Y_{ij} = \mu + T_i + e_{ij}$

Where Y_{ij} = Responded variables (intestinal histology, biochemical, and bacteriology);

μ = Overall mean

T_i = Treatment effect

eij = Random error. A p-value ≤ 0.05 was statistically significant.

3. Results

3.1. Blood Biochemical Parameters

The effect of MSE and water on blood biochemistry is presented in table 2. The groups that received 5 and 7 ml MSE (T3 and T3) had higher total protein values, compared to the control group (T1). Elevated levels of ALP, Ca, and P were observed in all MSE treated groups (Table 3), compared with the controls (P<0.05). The increased level of the MSE was associated with a decrease in serum total cholesterol.

Table 2. The effect of incorporating MSE into drinking water on the blood parameters of broilers at 35 days

Treatments	Hb (mg/dl)	Total Protein(g/dl)	Alkaline phostase(µ/L)	Calciummg/dL	Phosphor mg/dL	Cholestrol mg/dL
T1	3.4[b]	5	16.7 [c]	9 [ab]	6.1 [b]	191.2
T2	3.9[b]	4	20.7 [ab]	7[b]	5.5 [b]	200
T3	3.4[b]	5	21.7 [ab]	9[a]	5.4[b]	186
T4	3.5[b]	6	25.7 [ab]	10[a]	7.7 [a]	165
Pooled SEM	0.29	0.42	1.2	0.39	0.31	9.1
P-value	0.06	0.08	0.04	0.04	0.04	0.07

[1]Values are the mean of four replicateonevery10 chicks; SEM: standard error of the mean. [2] T1: control group; T2: 3% MSE in drinking water; T3: 5% MSE in drinking water; T4: 7% MSE in drinking water

SEM: Error of the mean [a-b]. At P<0.05, the means of each parameter in a column with the same superscripts do not differ significantly.

Table 3. Effects of MSE on histomorphometry of intestine in Broilers[1]

Intestinal histological	T1	T2	T3	T4	Pooled SEM
Villi hight(µm)	1277.7	1244	1118	1090	38.7
Villi thickness(µm)	150.4	146	178	155	10
Crept depth(µm)	343[a]	216[b]	208[b]	242 [b]	12.4
VH/CD	3.9[b]	6[a]	5[a]	4[b]	0.24

[1]Values represent the means off replicates on every ten chicks in each pen, SEM: Standard error of the mean.

T1: Control group; T2: Drinking water + 3% MSE; T3: Drinking water+5% MSE; T4: Drinking water +7% MSE

SEM: Standard error of mean

a-b: Means in the same row with no superscript or with common superscripts are not significantly different (P<0.05)

VH = Villus height, VW = Villus wide, CD = Crypt depth, VH/CD = Villus height to cryptdepth, Pooled SEM = Pooled standard error of means

3.2. Histomorphometry Parameters

The effects of MSE on the morphological development of the small intestine in the jejunum of broiler chickens are presented in Table 3 (Figure 2). The incorporation of MSE treatments in the drinking water significantly improved VH: CD, compared to that in the control group (P<0.05). Mustard seed extract levels did not affect VH and V thickness significantly, compared to the birds in the group of control. The CD was reduced with all treatments of MSE, compared to the control group (217, 208, 242 vs. 343) (P<0.05), while VH/CD was increased significantly (P<0.05) by the addition of MSE at levels of 3 and 5 ml per lit.

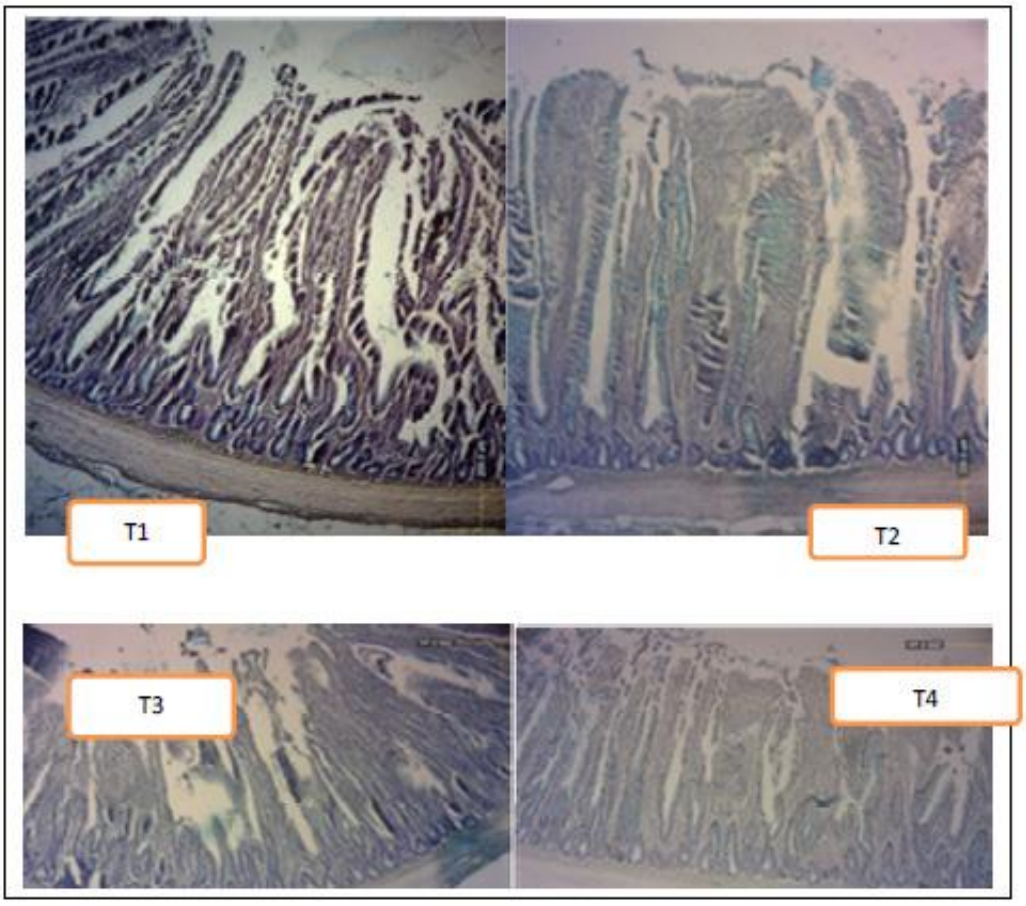

Figure 2. Measurement of Jejunum villus height, villi width and crypt depth with 40X magnification

Table 4. Effect of inclusion of mustard seed extract MSE into drinking water on intestinal microbial count of Ross 308 Broilers[1]

Items (log 10/c.f.u)	T1	T2	T3	T4	Total SEM
Total Bacteriacount ×10^5	6.1^{d} ± 0.3	4.6^{c} ± 0.27	1.5^{b} ± 0.5	0.5^{a} ± 0.01	0.40

[1]Values represent the means of replicate one very ten chicks, SEM: Standard error of the mean.
T1: Control group; T2: Drinking water + 3% MSE; T3: Drinking water+5 % MSE; T4: Drinking water+7% MSE
SEM: Standard error of mean
a-b Means with no superscript or with popular superscripts in the same row werenot significantly different (P<0.05)

3.3. Bacteriological

3.3.1. Isolation of Bacteria

On day 35, out of the total samples (n=44), there was 23(52.27%), 15(34.09%), and 6 (13.64%) isolates of E.coli, Salmonella spp., and staphylococcus aureus in the ileum contents in the control group, respectively (Figure 1). The incorporation of MSE treatments into broiler diets at levels of 3 ml, 5ml, and 7 ml reduced the bacterial total count in the chickens' gut ($P<0.05$), in comparison with the control group T1 (Table 4).

4. Discussion

4.1. Blood Biochemical indices

Blood components are an indicator of the normal functions in animals and a diagnostic method form any organs dysfunction (13). The impact of phytogenic in the reduction of oxidative stress may be explained by the low cholesterol concentration observed in broiler chickens in this study. The lowered cholesterol concentrations in the blood have been caused by alpha-linolenic acid in the MSE. The alpha-linolenic acid reduces the harmful effects of stress (14, 15). The ALP level in the plasma a is a good pointer of cellular metabolism and overall health condition (16). The elevated ALT concentration observed in the broiler chickens after the administration of MSE might have been caused by bio-agent composition (flavonoids and phenol). In line with the results of the present study, (15), Adegbeye, Oloruntola (17), (18) observed enhanced blood ALP after the administration of MSE at the level of 200 mg/kg BW. They also reported that increasing the level of MSE up to 300MG\KG resulted in the elevation of the serum concentration of ALP. The higher serum TP concentration recorded in birds administrated with high levels of MSE may be attributed to polyphenolic compounds that act as scavengers for free radicals and enhance liver metabolic function (19). The high concentration of Ca and P following the administration of MSE can be attributed to the high mineral composition in the mustard seed meal (20). Moreover, and it was found that the mustard seed meal is richer in calcium, phosphorus, sodium, and iron, and lower in potassium, manganese, zinc, and copper. According to the USDA National Nutrient Database for Standard Reference, ground mustard seeds constitute 26.6, 51.2, 92.5, 40.5, and 82.8% of one's daily calcium, iron, magnesium, zinc, and phosphorus, respectively (21). These findings were consistent with those obtained by (11), Abbasi, Seidavi (22) who found that a high dosage of MSE might alter serum calcium, phosphorus, and AKP levels. These results were inconsistent with those obtained in the study conducted by Adeyeye, Ayodele (23) who reported that the administration of MS as feed additives (up to a level of 15 g/kg) did not affect the blood biochemical parameters, including T.P, cholesterol, and albumin.

4.2. Histomorphometry

Gross morphological characteristics of the intestinal epithelium, such as height and surface area were used to assess intestinal integrity (24). The role of the herbal plant as a feed additive on the intestinal morphology of the poultry was explained by Giannenas, Bonos (25). The components of MSE, such as Sinigrin, are not usually antibacterial; however, when degraded enzymatically to produce allyl isothiocyanate, it displays strong antimicrobial action against food spoilage and pathogenic organisms. Moreover, the flavonoids and tannins may increase the number and width of the villi due to the complete maturation of the intestine with these plant components (26, 27). The addition of 15% of mustard oil in vitro can reduce the methane formation that has a deleterious effect on intestinal structure (11). Mustard seed promotes intestinal growth and makes the villi stronger due to the presence of Vitamin A and E along with calcium, protein, and omega fatty acids (18, 28). The effect of mustard oil has been attributed to alpha-linolenic acid (15). This finding was inconsistent with that obtained in the study performed by Azubuike, Okwuosa (18) who found that this material had an adverse effect on the person's heart. Moreover, the findings of this study did

not confirm those obtained by VanDono (29) who concluded that the intestinal length could have a profound effect on the whole body energetic. The increase in VW could represent an attempt to increase the intestinal surface area to maximize absorption area. This result confirmed the findings of the study conducted by Abdulameer, Modirsanei (30). Zhang, Sun (31) pointed out that the enhancement in the absorption function of the intestine depends on an increase in WV rather than an increase in the total number of villi. The partial enhancement of the VH/CD ratio in the jejunum is correlated with the overall gut health. An enhanced VH/CD ratio and reduced CD might be associated with an increase in the population of beneficial bacteria in the gut lumen (31, 32).

4.3. Bacteriology

The obtained results in the present study indicated the reduction of microbial count in the gut as a result of the administration of MSE at the levels of 3, 5, and 7%. Similarly, Bidarnamani, Shargh (33), (34) stated that MSE is a good source for the reduction of the pathogenic microbial population in the gastrointestinal tract. Maina, Misinzo (35) claimed that the bioagent compounds in the mustard seed and antimicrobial action of such isothiocyanates as allyl isothiocyanate are mediated through the induction of the intracellular cell cycle. The allyl isothiocyanate disrupts the cell membrane of bacteria through the generation of holes which allows chemicals to seep into the cytoplasm, inactivates functioning enzymes, and alters cellular metabolic processes. It also creates homeostatic pressure and enhanced b-galactosidase activity (35). Alteration in intracellular structure, as observed by Listeria monocytogenes, is another mechanism of isothiocyanate action (36). The thioredoxin and acetate kinase of Escherichia coli is inhibited by allyl isothiocyanate (10). Methane production in vitro was reduced by 15%after the inclusion of mustard oil, without altering fermentation processes or by-products, and the protozoa population was lowered as well (17). In the same line, Abukhabta, Khalil Ghawi (37) also reported that mustard seeds were more effective on microbes. Mustard seeds contain the highest levels of glucosinolates, and it is believed that myrosinase enzyme present in mustard seeds can contribute to its antimicrobial effect against Escherichia coli O157:H7 through the hydrolysis of glucosinolates (37). This result is inconsistent with that obtained by Adegbeye, Oloruntola (17), (38, 39) who reported that the administration of MSE at the level of15g/kg did not affect the total bacterial count in the cecum.

In conclusion, the health and morphological structure of the intestine as revealed in this study confirmed the hypothesis that mustard seed extract up to 7 ml per liter of drinking water can increase gut integrity in the broiler chicks.

Authors' Contribution

Study concept and design: Y. S. A.
Acquisition of data: H. H. A.
Analysis and interpretation of data: Z. B. A.
Drafting of the manuscript: Y. S. A.
Critical revision of the manuscript for important intellectual content: H. H. A.
Statistical analysis: Y. S. A.
Administrative, technical, and material support: Z. B. A.

Ethics

The experimental procedure was approved by the Animal Care Unit of Al-Qasim Green University in Iraq, from April to June 2020.

Conflict of Interest

The authors declare that they have no conflict of interest.

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