EFFECT OF DIETARY SUPPLEMENTATION OF MIACLOST ON PERFORMANCE AND GUT MORPHOLOGY IN BROILER CHICKENS CHALLENGED WITH *ESCHERICHIA COLI*

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ABSTRACT

This experiment was aimed to investigate the efficacy of MiaClost (*Bacillus subtillis* PB6 and *Enterococuss faecium*) as an alternative to antibiotic (zinc bacitracin) on performance and gut health of broiler chickens under *Escherichai coli (E. coli)* challenge model. A feeding study was conducted using 240-day-old Ross 308 chicks that were randomly assigned to 5 treatments each with 4 replicates and reared in two different rooms. Four treatments including (positive control, antibiotic, MiaClost 1 and MiaClost 2) were challenged with *E. coli* and reared in room1, however the fifth treatment was used as Negative control (nonchallenged) and reared in the second room. Birds were challenged with *E. coli* at day 8 and 9 of age. On days 24 and day 35 of birds age, the live body weight was lower ($P < 0.01$) and feed conversion ratio was higher ($P < 0.01$) in positive control birds than that of other experimental groups. Both levels of MiaClost significantly increased live body weight over other treatments. Birds in negative control, antibiotic, MiaClost 1 and MiaClost 2 increased villus height ($P > 0.03$) and increased villus height/ crypt depth ratio ($P > 0.04$) in compare to positive control. Furthermore, the serum concentration of alanine transaminase (ALT) was significantly lower ($P < 0.001$) in negative control and MiaClost 2 supplemented birds compared to positive control, antibiotic and MiaClost 1. The highest concentration of glucose was observed in the serum of positive control. In this study the MiaClost was as effective as antibiotic in preventing the expression of the negative impacts of *E. coli* on the performance and gut health of broiler chickens. This study indicates that MiaClost has promise as a tool for controlling *E. coli* in broiler production.

Key words: Broiler chicken, gut morphology, *E. Coli,* challenge

تاثير اضافة المياكلوست على الاداء الانتاجي و الصفات النسيجية لامعاء فروج اللحم المعرضة للايكولاي

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المستخلص

اجريت هذه التجربة لدراسة تاثير المعزز الحيوي (مياكلوست) كبديل للمضاد الحيوي على الاداء الانتاجي و الصفات النسيجية لامعاء فروج اللحم المعرضة لبكتريا الايكولاي. مجموع مائتان و أربعون من دجاج فروج اللحم بعمر يوم واحد استخدمت في هذه التجربة ووزعت بشكل عشوائي على خمس معاملات مع ٤ مكررات لكل معاملة في غرفتين. معاملة السيطرة الموجبة و المضاد الحيوي و مستويان من المعزز الحيوي (مياكلوست) استخدمت على الدجاج المعرض للايكولاي في نفس الغرفة أما المعاملة الخامسة المتمثلة بمعاملة السيطرة السالبة ربيت في غرفة ثانية. تعرضت الدجاج للايكولاي في اليوم الثامن و التاسع من العمر. اثبتت النتائج ان وزن الجسم كان معنويا الأقل وكفائة التحويل الغذائي كانت الأعلى (الاسوأ) في دجاج معاملة السيطرة الموجبة مقارنة مع بقية المعاملات في اليوم ٢٤ و ٣٥ من العمر . وزن الجسم كان الأعلى في الدجاج التي غذيت على المعزز الحيوي مقارنة مع باقي المعاملات. طول الزغابات و كذلك نسبة الزغابات الى عمق الخبايا كانت معنويا الأقل في معاملة السيطرة الموجبة مقارنة مع بقية معاملات التجربة. بالإضافة الى ان مستوى إنزيم الالانين ترانسفيرين كان الأقل في معاملة السيطرة السالبة و المستوى الأعلى من المعزز الحيوي مقارنة مع المعاملات الأخرى. ايضا مستوى الكلوكوز كان الأعلى في مصل دم طيور معاملة السيطرة الموجبة. المعزز الحيوي المستخدم كان فعالا مثل المضاد الحيوي في منع التأثيرات السلبية للايكولاي على الاداء الانتاجي و صحة الجهاز الهضمي للدجاج في هذه التجربة. أظهرت هذه التجربة على إمكانية استخدام المعزز الحيوي (مياكلوست) كمضاد للايكولاي في انتاج الدواجن

كلمات مفتاحية: المضاد الحيوي ، المعزز الحيوي، السيطرة السالبة، الزغابات.

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INTRODUCTION

Enteric diseases are one of the most important illnesses in the poultry industry because of high economic losses due to decreased weight gain, increased mortality rates, feed conversion ratio and medication costs (15). Avian colibacillosis caused by *Escherichai coli (E. coli),* consequences in significant economic losses every year in the global poultry production as a result of its high mortality and morbidity rates(4, 12). *E. coli* characterized by varied array of lesions, including perihepatitis, air sacculitis, and pericarditis lesions(20). Recent studies have also shown that E. Coli cause gastroenteritis and diarrhea in young animal and could impair intestine including inflammatory response (3, 17, 18). To control enteric infection, Antibiotics have been used as an effective tool to improve animal performance, by selectively modifying the gut microflora, decreasing bacterial fermentation, reducing thickness of the intestinal wall and suppressing bacterial catabolism and also control enteric disease outbreak(10). Although different antibiotics have been used to control and prevent the colibacillosis, the emergency of antibiotic resistant bacteria have decreased the antibiotics effectiveness and may pose a human health hazard(1, 2). In addition, drug resistance of *E. Coli* has increased resistance genes such as plasmid -mediated Amp-C beta-lactamases (Amp-C) and/or extended-spectrum beta-lactamases (ESBL) (13). Thus, new methods for controlling *E. coli* must be investigated to improve gut health or reduce the severity of *E. coli*. Probiotics as a natural alternative to infeed-antibiotics have received much attention duet to their antimicrobial activities in gastrointestinal of poultry. The modes of action of probiotics include maintaining gut microflora by competitive exclusion, stimulating the immune system, altering metabolism through increased digestive enzyme activity, ammonia production decreasing bacterial enzyme activity (5, 21, 26, 29). The mechanisms of competitive exclusion of pathogens include mucosal binding sites and nutrients competition , or SCFAs production and low pH, which are bactericidal or bacteriostatic for pathogenic bacteria (19). Several studies demonstrated that different kinds of probiotics may have effect on specific pathogens. *Bacillus subtilis* PB6 improved body weight, FCR and increased concentration of *lactobacillus* spp in broiler chickens infected with a pathogenic strain of *E. coli* (28)and also gut health and gut integrity by increasing the villus height and villus height to crypt depth ratio and controlled induced necrotic enteritis in broiler chickens (7). Cao (4) reported that *Enterococuss faecium*, as lactic acid bacterium, improved growth performance, intestinal morphology and usefully manipulate the cecal microbiome in broiler chickens challenged with *E. Coli* K88. Thus, the present study was designed to investigate the effect of probiotics (*Enterococuss faecium* and *Bacillus subtilis* PB6) on the performance, gut morphology, lymphoid organs weight, serum biochemical and nutrients digestibility of broiler chickens challenged with *E. coli* isolated from local commercial farms.

MATERIALS AND METHODS

The experiment was approved by the Animal production department scientific committee of college of agriculture, University of Duhok.

Animal husbandry

A total of 240 d-old Ross 308 chicks were placed in 20 floor pens in the University of Duhok, college of agriculture, Animal House Complex. All the birds were vaccinated against Newcastle disease and infectious bronchitis. These birds were randomly assigned to 5 treatments with four replicate pens per treatment and 12 birds in each pen. Pens (wire mesh partitioned at 100 × 100 cm) were assigned into two partitions to prevent Negative control birds from infection of *E. Coli* in the same environmentally controlled facility. The room temperature was set at 33-34°C initially and gradually decreased by 3°C per week until 22-24°C was reached by the third week. Birds were subjected to artificial fluorescent illumination light of 23 hours between d 0-7, then 18 hours from d 8 to 30, and 23 hours from d 30 to 35. Each pen was equipped with a separate tube feeder and nipple drinkers with water and feed provided *ad libitum*. During the trial period, starter diets were fed during d 0-10, grower diets between d 10-24, and finisher diets between d 24-35. The primary determinants of performance, i.e., cumulative pen weight, feed intake (FI) and feed conversion ratio (FCR) were measured at day 10, 24 and 35.

Dietary treatment

Three diets were formulated according to Ross 308 nutrient specifications (Table 1). Treatments were negative control (no challenge and no additive), Positive control (challenged and no additive), antibiotic (challenged and control diet supplemented with antibiotics 0.33 g/kg zinc bacitracin in starter, grower and finisher diets), MiaClost 1 (challenged and control diet supplemented with 0.2 g/kg MiaClost in starter, grower and finisher diets), and MiaClost 2 (challenged and control diet supplemented with 0.4 g/kg MiaClost in starter, grower and finisher diets). MiaClost (Probiotic) contains $50000x10^{7}$ CFU/Kg *Bacillus subtillis* PB6 and $1500x\ 10^{9}$ CFU/Kg *Enterococuss faecium*.

***E. Coli* challenge**

The *E. Coli* used in this study was isolated in our laboratory from local commercial farms. *E. Coli* was incubated overnight at 37˚C in 100 mL of sterile MacConkey broth followed by subsequent overnight incubations of 0.1 mL of the previous broth in Eosin methylene blue agar (EMB) for colony counting. A colony from EMB inculcated to 1000 mL of MacConkey broth to obtain the challenge inoculum. On days 8 and 9, challenged birds were inoculated with 1.5 ml *E. Coli* suspension (3.8×10^{8} CFU/mL).

Sample collection

On d 14 and 24, two birds and one bird, respectively, were randomly selected from each pen, weighed, and euthanized by cervical dislocation. Digesta samples from the ileum and caeca were collected. Around 1 g of content was used to measure the pH. Approximately 1 g of cecal digest was collected in a 2mL Eppendorf tube, stored at -20°C for bacteria quantification. Approximately 1 cm of the illume from one bird in each pen was collected for morphometric analysis. The tissue was opened and flushed clean with phosphate buffered saline (PBS, pH 7.4) and fixed in 10% buffered formalin for 24 hours. Formalin was subsequently replaced by 70% ethanol for long-term storage.

Measurements and analysis

Ileal and Cecal Ph

The ileal and cecal pH values were measured at d 14 and 24. Approximately 1 g of contents was diluted in 9 mL of distilled water. The suspension was mixed with a stirrer and the pH was determined by the EcoScan 5/6 pH meter (Eutech Instrument Pty Ltd., Singapore).

Histology

Fixed samples were dehydrated, cleared and embedded in paraffin wax for subsequent histological analysis. Consecutive longitudinal sections (7 μm) were placed individually onto Superfrost® slides (Thermo Scientific, Rockville, MD, USA) and stained with hematoxylin and eosin. Villus height and crypt depth were measured by the Dino-eye program and the images captured with a color video camera (Dino-eye 20). The height of 10 villi. depth of 10 crypts and thickness of 10 muscles were measured from each replicate. The means were obtained from villus height and crypt depth, the villus height/crypt depth ratio (VH:VD) was determined.

Serum biochemical

At day 24 of age, blood samples were collected from the jugular vein and serum was separated for determination of glucose, creatinine, total protein, albumin, cholesterol, triglyceride, alanine transaminase (ALT) and aspartate aminotransferase (AST). For individual serum sample determination, an automatic analyzer (TOKYO BOEKI MEDICAL SYSTEM), and using commercial kits (prestige 24i LQ CHOL and Glucose (COD-PAP)).

Statistical analysis

The SAS statistical package (PROC GLM) was used to determine significance of main effects (SAS, 2013). Duncan's multiple range test was used to detect the differences between individual treatment means.

Table1. Ingredient and composition of the basal starter, grower and finisher diets as percentage.

Ingredients	Starter	Grower	Finisher
Corn	47	49.9	51.5
Wheat	5	5	5
Wheat bran	5	3	5
Soybean meal	37	34	30
Vegetable oil	1.5	3.4	4.5
Limestone	1.8	1.7	1
Dicalcium phosphate	0.7	0.5	0.5
Salt	0.05	0.01	0.05
Vitamin premix	2.5	2.5	2.5
Nutrient composition %			
ME (kcal/kg)	2878	3035	3116
Crude protein	22.86	21.33	19.58
Crude fiber	3.02	2.76	2.92
Fat	3.76	5.64	6.80
Linoleic acid	1.92	2.88	3.48
Lysin	1.58	1.47	1.37
Methionine	0.66	0.64	0.63
Tryptophan	0.37	0.36	0.34
Methionine + cystine	1.05	1.01	0.96
Threonine	0.97	0.92	0.86
Arginine	1.59	1.5	1.37
Calcium	1.08	0.99	0.73
Phosphor	0.54	0.5	0.49
Sodium	0.19	0.18	0.19
Chloride	0.26	0.24	0.26

RESULTS AND DISCUSSION

Broiler performance: Performance results are present in table 2. At day 10, broiler chickens fed MiaClost 1 and 2 had higher live body weight ($P < 0.003$) compared to negative control, positive control and antibiotic. Although inclusion of antibiotic, MiaClost 1 and 2 increased feed intakes over negative and positive control ($P < 0.0001$), no significant different detected among experimental treatments for FCR. At days 24 and 35, the effect of the challenge was clearly visible. The live boy weight and FCR of positive control birds were significantly poorer than negative control, antibiotic, MiaClost 1 and 2. Feed conversion ratio and live body weight of the birds supplemented with MiaClost 1 and 2 were not different from negative control. In the birds given antibiotic, the feed intake was significantly increased over all dietary treatments at days. However, birds fed antibiotics had significantly higher live body weigh compared with negative control at day 35.

Organs percentage

Organs percentage from live body weight were measured at day 14 and 24 in birds fed treatment diets (Table 3). There were no significant differences of liver, bursa, spleen, and pancreas percentage between birds fed different experimental diets at days 14 and 24.

Ileum and caeca pH

The effects of different levels of MiaClost on ileal and cecal pH are summarized in table 4. At days 14 and 24, no significant differences were observed in ileum and caeca digesta pH of birds fed different levels of MiaClost.

Gut morphology

The morphology of jejunal samples was studied after *E. Coli* challenge and the data are presented in Tables 5. At day 24, the effect of the challenge was clearly visible. The negative control, antibiotic, MiaClost1 and MiaClost 2 birds had higher villus height ($P < 0.03$) and villi/crypt ratio ($P < 0.04$) than positive control birds. The muscle thickness of positive control was numerically higher overall treatments.

Serum biochemical parameters

The effect of treatments on the serum biochemical parameters in broiler at day 24 are presented in table 6. The result revealed that serum glucose of positive control birds increased ($P < 0.03$) over negative control, antibiotic, MiaClost 1 and MiaClost 2. The results of serum biochemical parameters at day 24 showed that total protein concentration decreased significantly in MiaClost 1 and MiaClost 2. Serum Albumin in negative control, MiaClost 1 and MiaClost 2 significantly reduced compared to positive control and antibiotic. The result showed that ALT concentrations in negative control and MiaClost 2 were lower (P < 0.001) than positive control, antibiotic and MiaClost 1. However, no significant changes (P > 0.05) were observed in cholesterol, triglyceride and AST concentrations between treated groups

Table 2. Effect of treatments on bird's performance at day 10, 24 and 35

Period	Negative control	Positive control	Antibiotic	MiaClost1	MiaClost2	P-value	Pooled SEM
Live body weight (g/bird)							
0-10d	252[b]	258[b]	264[b]	289[a]	290[a]	0.003	4.56
0-24d	1073[a]	925[b]	1123[a]	1039[a]	1082[a]	0.009	5.05
0-35d	1910[b]	1755[c]	2159[a]	1980[ab]	2024[ab]	0.001	0.01
Feed intake (g/bird)							
0-10d	242[b]	251[b]	275[a]	291[a]	289[a]	0.0001	20.32
0-24d	1401[b]	1352[b]	1505[a]	1407[b]	1404[b]	0.01	15.46
0-35d	2792[b]	2768[b]	3119[a]	2834[b]	2854[b]	0.030	0.02
Feed conversion ratio (FCR)							
0-10d	1.14	1.16	1.22	1.18	1.16	0.24	40.31
0-24d	1.36[b]	1.56[a]	1.39[b]	1.41[b]	1.35[b]	0.01	41.56
0-35d	1.46[b]	1.58[a]	1.45[b]	1.44[b]	1.41[b]	0.01	0.02

[a, b, c] means in rows with different superscripts are significantly different (P< 0.05).

Table 3. Effect of treatments on organs percentage from live body weight of birds in different experimental treatments

Treatments	Negative control	Positive control	Antibiotic	MiaClost1	MiaClost2	P-value	Pooled SEM
Day 14							
Liver %	3.69	3.47	4.14	3.28	3.83	0.61	0.17
Gizzard %	5.71	5.64	5.5	5.26	5.97	0.30	0.10
Bursa %	0.21	0.21	0.18	0.22	0.19	0.48	0.007
Spleen %	0.07	0.09	0.09	0.09	0.07	0.51	0.004
Pancreas %	0.52	0.52	0.57	0.50	0.50	0.70	0.017
Day 24							
Liver %	2.54	2.78	3.57	3.14	2.81	0.15	0.14
Gizzard %	3.42	3.78	3.89	3.39	3.46	0.37	0.098
Bursa %	0.20	0.19	0.21	0.2	0.21	0.98	0.009
Spleen %	0.07	0.09	0.12	0.09	0.11	0.15	0.007
Pancreas %	0.36	0.33	0.29	0.33	0.35	0.22	0.01

Table 4. Effect of treatments on illeal and caecal digesta pH in birds fed dietary treatments

Treatments	Negative control	Positive control	Antibiotic	MiaClost1	MiaClost2	P-value	Pooled SEM
Day 14							
Illum	6.07	6.93	6.49	6.44	6.39	0.09	0.1
Caeca	6.59	6.28	6.3	6.78	6.75	0.28	0.09
Day 24							
Illum	6.26	6.15	6.35	6.48	5.89	0.34	0.09
Caeca	7.54	7.85	7.49	7.93	7.93	0.26	0.13

Table 5. Effect of treatments on jejunal muscle thickness, villus height and crypt depth at day 24

Treatments	Negative control	Positive control	Antibiotic	MiaClost1	MiaClost2	P-value	Pooled SEM
Villus height *um*	1244[a]	1052[b]	1316[a]	1320[a]	1302[a]	0.03	34
Crypt depth *um*	224	268	241	222	235	0.53	8.94
Villi/crypt ratio	5.55[a]	4.04[b]	5.53[a]	6.05[a]	5.55[a]	0.04	0.23
Muscle thickness *um*	209	237	2016	209	212	0.15	7.17

[a, b] means in rows with different superscripts are significantly different (P< 0.05).

Table 6. Effect of treatments on serum biochemical parameters at day 24

Treatments	Negative control	Positive control	Antibiotic	MiaClost1	MiaClost2	P-value	Pooled SEM
Glucose (mg/dl)	239[b]	335[a]	290[b]	247[b]	254[b]	0.03	12.07
Creatinine (g/dl)	0.16	0.19	0.18	0.14	0.16	0.88	0.017
Total protein(g/dl)	3.04[a]	3.29[a]	3.37[a]	2.32[b]	2.41[b]	0.01	0.129
Albumin(g/dl)	1.61[a]	1.85[a]	1.86[a]	1.28[b]	1.35[b]	0.004	0.086
Cholesterol(mg/dl)	116	169	150	104	111	0.18	10.12
Triglyceride(mg/dl)	72	79	89	62	71	0.86	7.25
AST (U/L)	229	283	247	200	197	0.16	12.56
ALT(U/L)	2[b]	6[a]	5.5[a]	4.9[a]	2.5[b]	0.001	0.43

[a, b] means in rows with different superscripts are significantly different ($P< 0.05$).
ALT= alanine transaminase, and AST = aspartate aminotransferase (AST).

It is well known that gut microflora of chickens has wide metabolic potential. It affects the host health and nutrition. Amplified counts of some pathogen bacteria, such as *E. Coli,* may negatively affect broiler chickens body weight, feed intake, feed conversion ratio, nutrients absorption and gut health which it is an indicator for digestion and intestinal integrity. Supplementation of probiotics can be promising strategies for preventing and treating *E. Coli* infection. Probiotics are becoming popular worldwide because of its growth promoting, improve intestinal morphology, and beneficially manipulate gut microflora in broiler chickens (4). *E. coli* is a gram-negative bacterium and its pathogenic element is lipopolysaccharides, which can cause inflammation. Limitation of muscle protein and mobilizes energy synthesis to support the immune by inflammation resulting in poor growth (27). Evidence has been presented that probiotic improve growth performance. The results of current study demonstrate that MiaClost (*Bacillus subtillis* PB6 and *Enterococuss faecium*) was effective in curbing performance decline, live body weight and FCR, as antibiotic in birds challenged with *E. coli*. This is in agreement with findings of other researchers (4, 6) who reported that the addition of *E. Faecium* in broiler diets had positive effect on growth performance of broiler chickens after *E. coil* challenge. Teo and Tan (28) reported that adding *Bacillus subtillis* PB6 to the broiler chickens’ diet improved FCR and body weight under E. coli challenge condition. The improved body weight and FCR are probably due to the beneficial effect of MiaClost on gut health. *Bacillus subtillis* PB6 and *Enterococuss faecium* are natural strains found in the intestine of healthy chicken gut which have positive improvement on performance, intestinal morphology, enhancing the humoral immune response and preventing intestinal tracts disorder (6, 7). The current results showed that MiaClost increased villi height / crypt ratios, showing a long, matured and functionally active villus, in company with a thin crypt with constant renewal of cells. *Bacillus subtillis* PB6 and *Enterococuss faecium* increases the concentration of Short chain of fatty acids (SCFAs) and bacteriocin (14). It has been reported that SCFA stimulate gastrointestinal cell proliferation through the increase of plasma glucagon-like peptide-2 (GLP-2) and ileal proglucagon, glucose transporter (GLUT2) expression and protein expression. Intestinal morphology reflects the integrity and health of the digestive tract. Pathogens or toxin can damage the gut. *E. coli* challenge disturb intestinal morphology (4, 6, 30). Similarly, current study showed that the birds in challenged control (positive control) decreased villus height and villi/crypt ratio. Furthermore, several studies have been conducted on the effect of probiotics on gut morphology. Dietary supplementation of *E. faecium* in broiler chickens diet efficiently improved the intestinal mucosal architecture by increasing villus height and villi / crypt ratio (24). *E. faecium* can inhabit the adhesion of *E.coli* through altering steric hindrance (9). It has been confirmed that the addition of *E. faecium* (4, 6) and *Bacillus subtills* (16)in broiler chickens diet improved intestinal histomorphology with an increased villus height and villus/ crypt ratio under *E. coli* challenge. Consistently, our results showed that the MiaClost was effective as antibiotics in maintaining the gut morphology integrity by increasing villus height and villus/crypt ratio after *E coli* challenge and MiaClost villus height and villus/crypt ratio were not different than unchallenged control (negative control). The present study showed that *E. coli* challenge significantly affected the birds' serum biochemical parameters such as glucose and ALT. Similar significant increase in serum ALT activities have been reported by other workers in *E. coli* infection in broiler (11, 22) Elevation of serum ALT is mostly due to hepatic injuries (25). However greater liver enzymes (ALT) reduction were detected associated with a greater improvement in liver enzymes by supplementing MiaClost group when compared with positive control and antibiotics groups. Similarly, Rishi (23), found that probiotic supplementation resulted in decreased bacterial translocation in the liver of mice challenged with *Salmonella* typhimurium and decreased levels of serum aminotransferases, suggesting the protection role against Salmonella infection. Also, the present study showed that positive control had higher serum glucose compared to negative control. The increased serum glucose may be due to *E coli* infection. During infection stress, epinephrine (adrenaline), glucagon, growth hormone and cortisol play a role in blood sugar levels, which trigger release of glucose to ensuring that enough sugar or energy is readily available. Glucan and epinephrine levels increase and Insulin level decreases then more glucose is released from the liver. At the same time, cortisol and growth hormone rise, which causes body tissues to be less sensitive to insulin (8). This study was successful in demonstrating the *E. coli* challenge model. It showed that MiaClost was as effective as antibiotic in controlling performance including live body weight and feed conversion ratio. And improving gut integrity by increasing VH and VH:CD ratio in *E. coli* challenged broiler chickens. These results indicate that MiaClost can act as a replacement for antibiotic for controlling *E coli* infection.

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