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USING *LACTOBACILLUS bacteria* AS *IN-OVO* INJECTION OR ORAL ADMINISTRATION TO IMPROVE THE PERFORMANCE OF BROILER CHICKS

Amal M. Hassan[1*], I. El-Wardany[2] and M.I. Shourrap[2]

1- Animal and Poultry Physiology Department, Desert Research Center, Cairo Egypt, * Email: amalmhassan@yahoo.com), 2- Poultry Production Department, Faculty of Agriculture, Ain Shams University, Cairo, Egypt.

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SUMMARY

One hundred and sixty broiler fertile eggs with an average weight of 68.61 g were used to determine the best way to deliver probiotic and prebiotic to the chicken embryos. The eggs were obtained from a commercial Hubbard parent flock at 48 weeks of age. At 17th day of incubation, the eggs were divided into four main groups, each of 40 eggs. The first group served as control (C), while the second and third were subjected to in ovo injection with Lactobacillus bacteria (LB) at concentration of 9.8 X 10^{9} cfu (0.1 ml/egg) either into air cell (Br group) or the amniotic fluid (Bm group). The hatched chicks of the 4th group were orally inoculated (O) with the same dose of bacteria immediately after post hatching.

The obtained results can be summarized as follows:

- Administration of LB either orally or in – ovo injection had a positive effect on feed consumption and live body weight at 5th week of age.

- The chicks of Bm group showed the highest relative weight of carcass components, while those of O group showed the lowest abdominal fat. In addition, administration of LB resulted in significantly (P<0.05) higher relative weight of liver.

- Plasma total protein, globulin and A/G ratio did not significantly (P<0.05) influenced by treatments.

- Chicks of O group showed a significant (P<0.05) decrease in albumin level.

- Total bacterial count and total lactic acid bacteria were increased significantly (P<0.05) in the O group compared to the C group.

Histological examination of tissues showed an improvement in ilium villi height of treated groups than in the control group.

Keywords: In ovo injection, Lactobacillus, blood, ilium histomorphology, lymphoid organs and broiler chicks

INTRODUCTION

There are a few ways to prescripetion of probiotics, the most common way is adding it to feed or drinking water, while spraying and oral gavage are other ways. Conventionally, in-feed or in-water supplementation has been used at the first hours/days post hatching. However, this approach relies on the amount of feed and/or water intake, the quality of water and other experimental factors (Biggs *et al*., 2007). Moreover, during early post-hatching period, infection of chicks by detrimental bacteria is also possible.

In ovo approach for injection of probiotics directly to the incubating egg has been developed. It allows for a precise delivery of the bioactive substance to all embryos at early stage of development, which unifies the effects of probiotics across the flock and assures proper development of gut microflora in all chicks. In ovo methodology may be applied in order to supply the embryo with additional nutrients prior to hatching and those nutrients will continue to be utilized by the chick post-hatch during the fasting period (SLawinska *et al.*, 2014).

Based on chemical and physical features and dose of the injected substance, different site of injection (i.e. the embryo, the amnion, the allantois, the air cell or the yolk sac) and embryo age (0, 12, 17 or 18 days of incubation) have been used (Pilarski *et al.*, 2005). Uni, *et al*., (1995) indicated that day 12 of incubation is the optimal time for probiotic injection into the air cell of the incubating egg, where at this time, embryo is totally immersed in amniotic fluid and is completely developed and highly vascularized, allowing for transfer of the bioactive solution from air cell to embryonic gastrointestinal tract.

This method (*in ovo* technique) has been successfully used for prebiotics and probiotics (Bednarczyk *et al.*, 2010) or synbiotics (Slawinska *et al.*, 2014 and Pruszynska-Oszmalek *et al.*, 2015). In ovo delivery of prebiotics was found to improve growth rate, feed intake, nutrient digestibility (Bednarczyk *et al.*, 2010) and meat quality Moreover, significant increase was found in total activity of pancreatic enzymes (Pruszynska-Oszmalek *et al.*, 2015) and immune system development and function (Slawinska *et al.*, 2014 b).

Injection of probiotic bacteria intra egg may be an alternative to microbiota acquisition by chicks before

hatching and may reduce or avoid the gastrointestinal colonization by pathogens. Three species of bacteria, *Bacillus subtilis*, *Enterococcus faecium*, and *Pediococcus acidilactici*, are naturally occurring microbiota in the intestine of birds and common in commercial probiotic products (Manes *et al.*, 2017). Recently, Bacillus, Lactobacillus and Saccharomyces are the major strains applied in broilers. The mechanisms of action of in ovo injected bioactive substances are complex (Slawinska *et al.*, 2014), but researchers still predict their positive effects on organism growth and body weight.

Therefore, the objective of the current study was to determine the effect of prebiotics administered in ovo at the 17th day of incubation on growth performance, carcass traits, and histomorphological traits of the small intestine, intestinal microflora and some plasma biochemical parameters of broiler chicks at the 5th week of age. The main aim was to find the best way to introduce *Lactobacillus bacteria* to broiler chicks.

MATERIALS AND METHODS

The present study was carried out at the poultry research unit belonging to Faculty of Agriculture, Ain Shams University, during the period from December (2015) to January (2016). Two hundred Hubbard fertile eggs with an average weight of 68.61±0.01 g were used in the present study. At day 14th of the embryonic development, eggs were candled and the infertile eggs or early embryonic dead were culled. One hundred and sixty eggs were randomly divided into four treatment groups (40 eggs for each). The first group did not received any supplementation and served as control (C), while the 2nd and 3rd groups were subjected to *in ovo* injection with *lactobacillus bacteria* at a concentration of 9.8 x 10^9 cfu (0.1ml /egg) into either air cell (Br) or the amniotic fluid (Bm), respectively. Egg injection procedure was carried out at day 17th of the embryonic development. Hatched chicks of fourth group was orally inoculated (O) with the same dose of bacteria immediately post hatching.

The fertile unhatched eggs were broken out, the percentages of late embryonic mortality, and hatchability percent were calculated based on the number of fertile eggs. At hatch, a total of 120 chicks representing the four treatment groups were randomly taken and transferred into four groups of 30 chicks, in three replicates (10 chicks each). All chicks were brooded in wire battery brooder. The chicks were fed *ad libitum* on commercial starter ration until 15 day of age (23% crude protein, 3050 Kcal/Kg metabolizable energy,). From 16 to 35 day of age, the chicks were fed on grower ration (21% crude protein, 3150 Kcal/Kg metabolizable energy). Chicks were vaccinated against the common viral diseases in the local area. No antibiotics were added to drinking water or feed during the whole experiment period, but multivitamins were added weekly to drinking water during the whole experimental periods.

Chicks were individually weighed at hatch and then at 2 and 5 weeks of age till the 5th week of age, then average body weight gain (BWG) was calculated (g/bird/period). Feed consumption (FC) was determined as g/bird/period. Feed conversion ratio (FCR) was calculated according to the following equation:

$$FCR = FC(g) / BWG(g)$$

A total of 24 blood samples (six samples per group) were collected at the 5th week of age during their exsanguinations into heparinized Wassermann tubes. Plasma samples were harvested directly after bleeding and centrifugation of blood samples were done at 4000 rpm for 10 min using laboratory Centrifuge (Hettich Zenttrifugen, Germany). The plasma samples were stoppered tightly and stored in a deep freezer at -20ºC until the biochemical analysis were done. Plasma total proteins (g/dl) were determined according to the method described by Henry (1974). Plasma albumin (g/dl) was measured as described by Doumas *et al.* (1971). Globulin (g/dl) was calculated by subtracting plasma albumin from total protein, then A/G ratio was calculated.

The material for the morphological and histological analysis of the duodenum was collected at 35-day-old chickens of each. Before slaughter, a total of 20 chickens (a representative selection) from each group were weighed, and their mean body weight was calculated. Subsequently, 10 chickens per group, with the body weight similar to the mean for the group were selected. After slaughter, the small intestine was removed out and the duodenum was dissected, measured and weighed. Samples for histomorphometric analyses (approx. 2 cm) were taken from the midway of the duodenum.

Data were subjected to a one-way analysis of variance using the General Linear Models procedure of SAS, (2004), according to the following model:

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

Where:

Y_{ij} = the j observation of the i^{th} bacterial treatment;

μ = an effect of the overall mean;

T_i = a fixed effect of i^{th} bacterial treatment;

ε_{ij} = a random experimental error assumed NID $(0, \sigma e^2)$

The differences among means were determined using Duncan's new multiple range test (Duncan, 1955). Statistical significance was accepted at a probability level of 0.05 ($P \leq 0.05$).

RESULTS AND DISCUSSION

Growth performance:

The effect of prebiotics administered *in ovo* at the 17th day of incubation on growth performance is illustrated in table (1). The live body weight (LBW) was not significantly affected by treatment except at 2 weeks of age, where the chicks of (Br) group achieved the highest weight. At the end of

experiment, the chicks of both *in ovo* and oral inoculation of *LACT* showed the highest LBW and BWG.

Table 1. Effects of experimental treatments on live body weight and gain (g) of broiler chickens

Age	Treatment			
	C	Br	Bm	O
	Live body weight (g)			
1d	49.93±0.621	48.79±0.125	48.81±0.245	49.22±0.547
2 wks	476.75^{b}±4.151	548.67^{a}±43.956	489.60^{b}±8.465	527.00^{ab}±5.568
5 wks	1985.50±70.638	2075.67±62.691	2066.40±26.628	2075.33±7.881
	Body weight gain (g)			
0-2 wks	426.75^{bc}±4.662	500.00^{a}±44.276	440.60^{bc}±8.704	478.00^{ab}±6.028
3-5 wks	1509.00±67.513	1526.33±28.038	1577.00±23.843	1548.33±4.177
0-5 wks	1935.50±70.918	2026.67±62.691	2017.40±26.593	2026.33±8.453

[a,b] Means within the same row with no common superscript differ significantly. C, (negative control); O, (oral with *Lactobacillus bacteria*); Br, (*in ovo* injection with *Lactobacillus bacteria* in air sac); Bm, (*in ovo* injection with *Lactobacillus bacteria* in amniotic fluid). *P≤ 0.05, NS= non -significant.

These results are in full agreement with that reported by Dankowiakowska *et al.* (2013); Cox (2013) and Slawinska *et al.* (2014), who found that *in ovo* inoculation of *LACT* bacteria strains resulted in improving the productive performance of broiler chicks. The improvement in both LBW and BWG by *in ovo* injection of *LACT* might be due to reduction of pathogenic bacteria number in the intestine by competitive exclusion, production of antimicrobial substances such as lactic and acetic acids that inhibit a wide range of gram-positive and gram-negative bacteria (Williams and Zedeka, 2010) and/or modulation of immune responses of chicks during post hatching growth. This means that treating chicks by *LACT* which improved feed utilization might be through improving some metabolic processes.

On the other hand, same investigators found no effect on post hatch growth performance of broilers through *in ovo* inoculation of *LACT* bacteria, (Edens *et al.*, 1997 and Stern *et al.*, 2001).

Feed consumption and feed conversion ratio:

The treatments did not affect feed consumption (Table 2). The Br group showed a significant improvement (P<0.01) in feed conversion ratio at the period 0– 2 weeks of age compared with control. At the end of the experiment (five weeks of age), all groups showed nearly the same feed conversion ratio. Cox (2013) and Oliveira de *et al.* (2014) found the same trend; *in ovo,* treatment with different doses of primalac (a probiotic compound) had no significant effect on feed consumption or feed conversion of broiler chicks. On the other hand, Bednarczyk *et al.* (2010) found improvement in nutrient digestibility and Pruszynska-Oszmalek *et al.* (2015) observed significant increase in total activity of pancreatic enzymes (amylase, lipase and trypsin).

Table 2. Effect of experimental treatments on feed consumption and feed conversion ratio of broiler chicks at 35 day of age

Age	*Treatment*			
wks	C	Br	Bm	O
	Feed consumption (g)			
0-2	524.50±4.173	526.67±41.273	526.80±11.876	558.33±11.465
3-5	2399.75±73.673	2464.00±77.078	2497.60±46.427	2494.33±78.252
0-5	2924.00±74.720	2990.33±107.667	3024.00±56.384	3053.00±76.167
	Feed conversion ratio			
0-2	1.23^{ab}±0.020	1.06^{c}±0.062	1.20^{b}±0.019	1.17^{b}±0.016
3-5	1.59±0.022	1.61±0.037	1.59±0.037	1.61±0.054
0-5	1.51±0.019	1.48±0.023	1.50±0.027	1.51±0.044

[a,b,c] Means within the same row with no common superscript differ significantly. C, (negative control); O, (oral with *Lactobacillus bacteria*); Br, (*in ovo* injection with *Lactobacillus bacteria* in air sac); Bm, (*in ovo* injection with *Lactobacillus bacteria* in amniotic fluid). *P≤ 0.05, ** P≤ 0.01, NS= non-significant.

Carcass traits:

Carcass traits as affected by treatments are shown in Table (3). At marketing age (5 weeks), the chicks of Bm group showed the highest (P<0.05) carcass relative weight with significantly different than O group only. Chicks of Br and Bm groups had the highest (P<0.05) abdominal fat, while those of Bm and O groups showed the highest (P<0.05) liver relative weight. Relative weight of heart, gizzard and gizzard fat were not significantly (P<0.05) affected by treatments. Chicks of the oral inoculated (O) group has the significant abdominal fat (%) compared to Br and Bm. Our results are in full agreement with that reported by El-Husseiny *et al.*

(2001); Yosrizal and Chen (2003) and Tollba and Mahmoud (2009) who showed that *Lactobacillus acidophilus bacteria* in the broiler diets decreased abdominal fat percentage. Moreover, Willis *et al.* (2007) observed a decrease in carcass yield percentage as a result of oral inoculated of LB. However, Awad *et al.* (2009) reported that the carcass percentage was not affected by probiotic-supplementation.

Table 3. Effect of experimental treatments on relative weight of carcass and relative org and weight of broiler chicks

Traits (%)	*Treatment*			
	C	**Br**	**Bm**	**O**
Carcass	74.13^{ab}±0.541	73.967^{ab}±1.536	76.40^{a}±1.384	72.65^{b}±0.211
Heart	0.46±0.022	0.50±0.023	0.45±0.015	0.440.023
Liver	1.96^{b}±0.106	2.54^{a}±0.161	1.91^{b}±0.096	2.48^{a}±0.139
Gizzard	0.85±0.067	1.10±0.110	1.07±0.163	1.02±0.061
Abdominal Fat	1.11^{ab}±0.173	1.22^{a}±0.158	1.29^{a}±0.118	0.72^{b}±0.090
Gizzard Fat	0.46±0.011	0.48±0.087	0.49±0.055	0.46±0.077

[a,b,c] Means within the same row with no common superscript differ significantly.
C, (control); O, (oral with *Lactobacillus bacteria*); Br, (*in ovo* with *Lactobacillus bacteria* in air sac); BM, (*in ovo* with *Lactobacillus bacteria* in amniotic fluid). *P≤ 0.05, NS= non-significant.

Plasma total protein, albumin and globulin:

It is clear from our results in table (4) that total protein (TP) and A/G ratio were not significantly (P<0.05) influenced by treatments. Chicks of O group showed the lowest (P<0.05) albumin level. These results are in agreement with Mountzouris *et al.* (2010) and Torshizi *et al.* (2010).

Table 4 Effect of experimental treatments on plasma protein fractions (g/dl) of broiler chicks

Items	*Treatment*			
	C	**Br**	**Bm**	**O**
Total Protein	4.44±0.131	4.47±0.147	4.17±0.79	4.29±0.121
Alb (A)	2.38^{ab}±0.082	2.41^{a}±0.083	2.28^{ab}±0.040	2.21^{b}±0.021
Glo (G)	2.07±0.057	2.06±0.077	1.89±0.052	2.09±0.103
A/G Ratio	1.15±0.032	1.17±0.034	1.21±0.031	1.06±0.051

[a,b,c] Means within the same row with no common superscript differ significantly.
C, (control); O, (oral with *Lactobacillus bacteria*); Br, (*in ovo* with *Lactobacillus bacteria* in air sac); Bm, (*in ovo* with *Lactobacillus bacteria* in amniotic fluid). *P≤ 0.05, ** P≤ 0.01, NS= non-significant.

The Ilium histology:

The data on ilium histology is shown in table (5). The transverse section through ilium revealed that musculosa depth was not affected by treatment. Oral inoculation with *Lactobacillus bacteria* (O group) in significant (P<0.05) ilium villi height, villi diameter (Fig. 1) and the ratio of villus height to crypt depth (Table 5). Injection *in-ovo* of *Lactobacillus* into air sac (Br group) resulted in the highest (P<0.05) crypts depth (Table 5). Also, chicks of both groups C and Br showed a good development in crypts in the sub-mucosa layer (Fig. 1). On the other hand, the chicks of Bm group showed the lowest villi height and crypts depth (Table 5).

Oral inoculation hatched chicks with Lactobacillus bacteria (O group) and in ovo- amnion injection (Bm group) caused an increase in villi diameter with many well developed crypts in the sub-mucosa layer as shone in Histological sections. The villi and the size of crypts appeared shorter and blunt for both groups compared with the control and in ovo administration of Lactobacillus into air sac group of chicks (Fig. 2- C and Br). This holds true as the crypt depth of Br-treatment and oral-treatment groups was significantly higher than the other groups (Table 5). The crypts of Lieberkuhn secreted fluids containing different vital substances essential for enhancing the internal micro-environment of the intestine segments. These fluids are rapidly absorbed from the villi lumens elaboration and production of antibodies and lymphocytes along with an increase in goblet cells which secrets substances responsible for reducing PH of the intestinal segment, Hodes (1974); Roberfroid (2000) and Pelicano *et al*. (2005).

Table 5. Effect of *Lactobacillus acidophilus* administration on ilium histomorphological parameters of broiler chicks at 35 day of age

Items	*Treatment*			
	C	Br	Bm	O
Musculosa depth (µm)	115.67±6.614	120.60±2.540	116.96±2.785	114.63±7.010
Villus height (µm)	637.49^{b}±22.820	610.08^{b}±24.238	598.73^{b}±21.913	755.86^{a}±20.452
Crypt depth (µm)	88.67^{bc}±2.422	115.25^{a}±4.139	76.21^{c}±3.688	96.029^{b}±7.759
Villus height/crypt depth	7.11^{b}±0.425	5.49^{b}±0.311	8.09^{ab}±0.474	9.82^{a}±1.037

^{a,b,c} Means within the same row with no common superscript differ significantly. C, (control); O, (oral with *Lactobacillus bacteria*); Br, (*in ovo* with *Lactobacillus bacteria* in air sac); Bm, (*in ovo* with *Lactobacillus bacteria* in amniotic fluid); *P≤ 0.05, NS= non-significant.

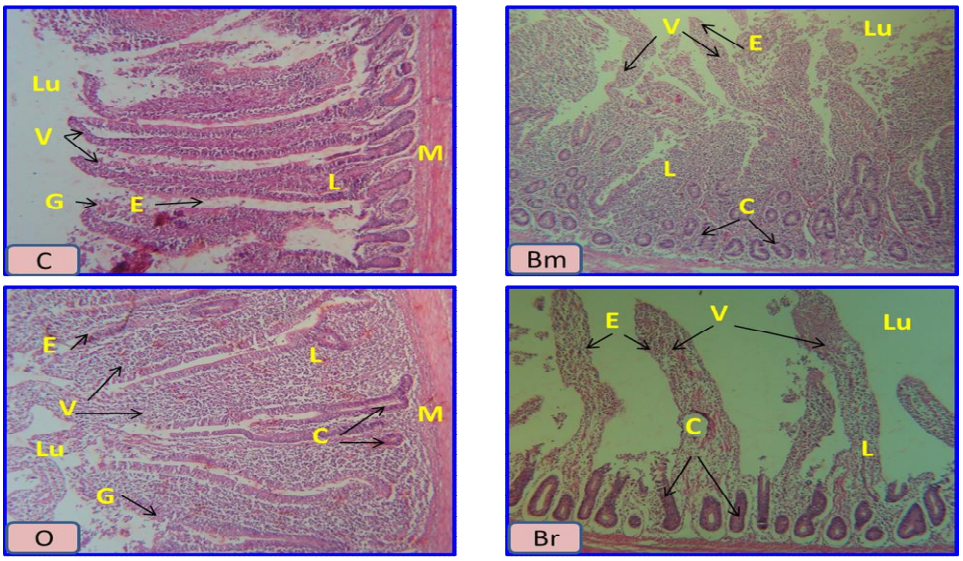

Figure 1. Transverse section through ilium from birds of different treatments at 35 day of age: C, (control); O, (oral with *Lactobacillus bacteria*); Br, (*in ovo* with *Lactobacillus bacteria* in air sac); Bm, (*in ovo* with *Lactobacillus bacteria* in amniotic fluid). (Lu) lumen; (E) epithelial lining; (G) Goblet cells; (V) Villi; (C) Crypts of Lieberkuhn; (M) Muscular is mucosa;(L)Lamina propria. (H & E ×10).

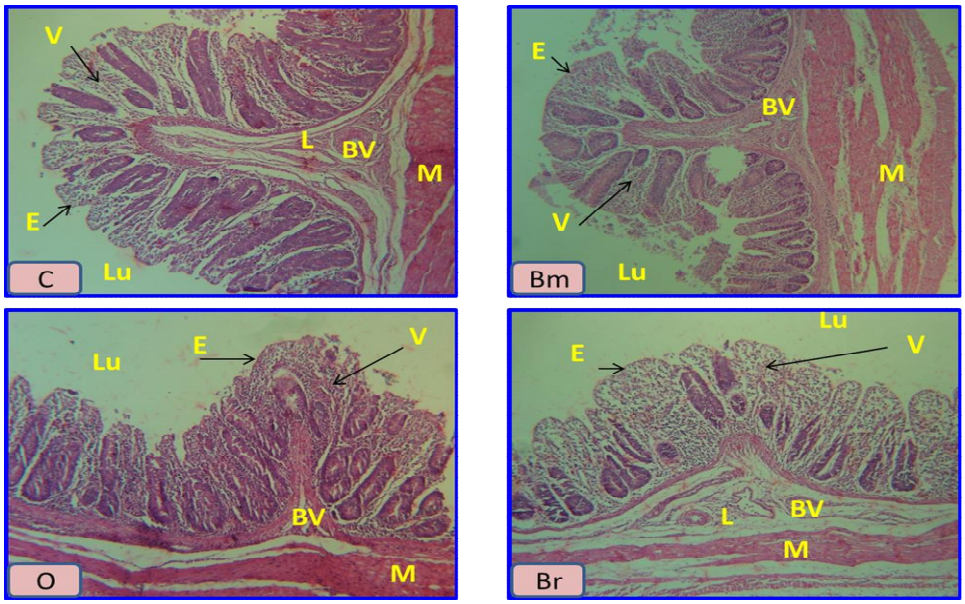

Figure 2. Transverse section through cecum from birds of different treatments at 35 day of age. C, (control); O, (oral with Lactobacillus bacteria); Br, (in ovo with Lactobacillus bacteria in air sac); Bm, (in ovo with Lactobacillus bacteria in amniotic fluid).(Lu) lumen; (E) epithelial lining; (Bv) Blood vessels; (V) Villi; (M) Muscularis mucosa; (L) Lamina propria; (H & E ×10).

The mucosa layers:

The caecum sections were taken from the middle cecum and composed of similar layers as the other small intestine segments, with only slight variation due mainly to the part where the caecal specimen was dissected (proximal, middle, or distal caecum). Since the muscular is mucosa layer (m) and is well developed with an outer longitudinal and inner circular layers being thick in the BM and Br sections than C and O ones. There are many lymph nodules containing numerous aggregates of lymphocytes with different size. The crypts of Lieberkuhn are short tubular ducts and laying in the base of the villi, where they occupy most of the tunica propria layer between the bases of the villi and the muscular is mucosa layers. The villi being well developed both in length and breadth but they are very short and blunt with a very well epithelial lining containing many goblet cells. These results are in accordance with the findings by Edens *et al.* (1997); Chichlowisk, *et al.* (2007), de Oliveira, *et al.* (2014) and Yamawaki, *et al.* (2013). They used *in ovo* injection of different probiotics and found similar and promising results.

Table 6. Effect of *Lactobacillus acidophilus* administration on cecum histomorphological parameters of broiler chicks at 35 day of age

Items	***Treatment***			
	C	**Br**	**Bm**	**O**
Musculosa depth(μm)	$119.00^{b}\pm6.00$	$179.29^{a}\pm4.911$	$143.50^{ab}\pm9.633$	$150.83^{ab}\pm8.619$
Mucosa height(μm)	$173.2^{c}\pm11.972$	$190.76^{b}\pm3.199$	$156.80^{c}\pm5.374$	$211.45^{a}\pm4.517$

[a,b,c] Means within the same row with no common superscript differ significantly; C, (control); O, (oral with *Lactobacillus bacteria*); Br, (*in ovo* with *Lactobacillus bacteria* in air sac); Bm, (*in ovo* with *Lactobacillus bacteria* in amniotic fluid); *P≤ 0.05, NS= non-significant

CONCLUSION

This study demonstrates that *in ovo* injection of probiotic into the air chamber of egg significantly influences the histomorphological parameters on d 17 of rearing without negatively affecting productivity in chickens at the end of rearing.

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استخدام بكتريا الكتو باسلس كحقن داخلي للبيضة أو التجريع الفموي لتحسين أداء كتاكيت بداري التسمين

امال محمد حسن[١]،إبراهيم الورداني[٢]، محمد إبراهيم شراب [٣]

١ـقسم فسيولوجيا الحيوان و الدواجن- مركز بحوث الصحراء- القاهرة- مصر، ٢ـقسم انتاج الدواجن، كلية الزراعة، جامعة عين شمس، القاهرة مصر

أستخدم عدد مائة وستين بيضة تفريخ دجاج تسمين مخصبه سليمة بمتوسط وزن ٦٨.٦١ غرام من قطيع Hubbard تجاري في عمر ٤٨ أسبوع لتحديد أفضل طرق إعطاء بكتريا اللاكتوباسيلاس. في اليوم السابع عشر من التفريخ، تم تقسيم البيض إلى أربع مجموعات رئيسية، كل منها به ٤٠ بيضة. المجموعة الأولى لم تعطى اى معاملة وأستخدمت كمجموعة مقارنه (C)، في حين تعرضت الثانية والثالثة لحقن البيضة مع بكتيريا *Lactobacillus bacteria* (LB) عند تركيز 9.8×10^9 cfu (0.1 ml/egg) إما في الغرفه الهوائية (مجموعة Br) أو السوائل الجنينيه (مجموعةBm). الكتاكيت الفاقسه من المجموعة الرابعة تم إعطائها نفس الجرعة من البكتيريا على الفور بعد الفقس عن طريق الفم (O).

النتائج المتحصل عليها يمكن تلخيصا فيما يلى:

- إعطاء البكتريا عن طريق الفم أو بالحقن فى البيضة له تأثير إيجابي على استهلاك العلف والوزن الحي للكتكوت في الأسبوع الخامس من العمر.
- اظهرت المجموعة Bm المحقونة في الغشاء الأمنيونى (السوائل الجنينة) أعلى وزن نسبي لمكونات للذبيحة ($P < 0.05$) بينما اظهرت مجموعة المعطاة للبكتريا فى الفم بعد الفقس اقل نسبة لدهن البطن. بالإضافة إلى ذلك، أدى إعطاء LB إلى ارتفاع ($P < 0.05$) الوزن النسبي للكبد.
- لم يتأثر البروتين الكلي للبلازما، الجلوبيولين ونسبة A / G بصورة معنويه بالمعاملات.
- أظهرت إجمالي عدد البكتيريا وإجمالي بكتيريا حمض اللاكتيك زيادة كبيرة في مجموعة O مقارنة مع مجموع C.
- أظهر الفحص الهستولوجى للأنسجه تحسنا في ارتفاع الخملات فى الأمعاء الدقيقه فى المجاميع المعاملة مقارنة بالكنترول.