IOSR Journal of Agriculture and Veterinary Science (IOSR-JAVS)
e-ISSN: 2319-2380, p-ISSN: 2319-2372. Volume 7, Issue 7 Ver. I (July. 2014), PP 59-65
www.iosrjournals.org

Effect of *Lactobacillus Acidophilus*, *Lactobacillus Acidophilus Plus Pichia Anomola* and *Lactobacillus Acidophilus Plus Pichia Anomola Plus Bacteriophage* on Immune Responsiveness, Intestinal Enumeration of *Clostridium Perfringens, Salmonella Typhimurium,* Colisepticaemia, and Gut Integrity of Broiler Chickens

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Abstract: *The immune status assessment clarified that both phagocytic percentage and indices significantly increased (P≤0.05) in the 3 studied probiotic groups as compared with their untreated control group at all studied intervals (1-5 weeks of age). Obtained findings showed significant clear tendency for elimination of S. Typhimurium intestinal enumeration at all studied intervals (P≤0.05), provided that complete intestinal clearance could be achieved in Lactobacillus acidophilus plus Pichia anomola (Lactolife-Av+) and Lactobacillus acidophilus plus Pichia anomola plus Bacteriophage (Lactolife-Av+B) treated groups at 14 days post infection. A significant reduction (P≤0.05) in intestinal and cecal enumeration of C. perfringens were obtained on using the studied probiotics provided that best results have been obtained on using Lactolife-AV+B followed by Lactobacillus acidophilus (Lactolife–Av) then Lactolife-Av+. Experimental infection with pathogenic E. coli serogroup O78 resulted in reduction of chicken mortality with a lesion scoring reaching 1.27, 2.53 and 0.63 in Lactolife–Av, Lactolife-Av+ and Lactolife-AV+B treated groups as compared with 4.36 in untreated control group respectively. There was increase in jejunal villus length in all treated groups provided that the highest villus length and villus height crypt depth ratio were recorded in Lactolife-Av+ treated group when compared with the other 2 treated groups. The crypt depth of the jejunum was slightly increased in Lactolife-Av group and Lactolife-Av+B group as compared to the control group while decreased in Lactolife-Av+ group. The ileum revealed increased villus height in all treated groups as compared with the control but highest ileal villus height was recorded in Lactolife-Av+ group. While the crypt depth was decreased in all treated groups as compared with their controls and the highest villus height crypt depth ratio was achieved in Lactolife-Av+ group.*

Key words: *Probiotics, Broiler chicken gut integrity, Immune response, Chicken bacterial Enteropathogen.*

I. Introduction

Chicken gastrointestinal tract (GIT) provides a means by which the body derives nutrition, furnishes protective mechanisms to safeguard the host and serves as an environment for other living organisms. Accordingly; the economic losses associated with enteric bacterial diseases can be summarized in; high mortality, loss of weight, poor feed conversion, down grading of carcasses, lowering fertility and hatchability as well as severe drop in egg production. Colibacillosis, salmonellosis and clostridial infections incriminated as the major bacterial poultry enteric problems that adversely affect gut integrity leading to severe economic losses in poultry industry.

Infection with *E. coli* varies from an acute septicemia and even sudden death in younger age (2-4 weeks) to subacute fibrinopurulence in the older birds (5-8 weeks) (**Sojka, 1965)**. **Yogaratnam (1995)** reported that 43% of broiler carcasses were condemned due to E. coli septicemia. Salmonellosis acquired vertically from parents or horizontally in the hatchery can cause significant growth depression or even mortality in young chicks (**Gast, 1997**). A major portion of human salmonella food poisoning outbreaks were associated with poultry meat (30.6% of incidents and 42.7% of cases) (**Todd, 1989 and Gast 1997**). Clostridium perfringens (C. perfringens) is an obligate anaerobic bacterium in the intestinal tract of chickens (**Johansson and Sarles, 1948; and Shapiro and Sarles, 1949**). These organisms are relatively innocuous unless there cofactors occur such as dietary ingredients or changes, severe stress, coccidiosis, or immunosuppressive affections (**Barnes, 1997**). C. perfringens is responsible for severe food borne enteritis in man and its enterotoxin has been shown to be responsible for food poisoning (**Brynestad, 2002**).

The direction towards the use of competitive exclusion products "probiotics" (previously known as ecological health control products) as natural control method was emerged. They are cultures of living microorganisms which are able to proliferate in the host-bird's intestinal tract, resulting in a balanced microbiota. **Fuller (1989)** mentioned that the auspicious effect of probiotics over the organism is due to the better adhesion of the lactic acid bacteria to the intestinal epithelium in comparison with the pathogenic bacteria, and stooping the implementation of those bacteria over the mucus membranes of the intestine.

This study was conducted to compare the possible effect of drinking water administration of three probiotic compounds containing basically Lactobacillus acidophilus on immune responsiveness, intestinal enumeration of *C. perfringens* type C and *S. Typhimurium*, colisepticaemia, and gut integrity of broiler chickens.

II. Material and Methods

The probiotics:

Three probiotics containing dried Lactobacillus acidophilus avian strain produced by kanzymedipharm, Canada under the trade names of Lactolife-Av, Lactolife-Av+ and Lactolife-Av+B were used in this experiment:

1-Lactolife-Av: Lyophilized cake containing ≥ $1x10^8$ CFU/g Lactobacillus acidophilus, Batch No. LL1170 .

2-Lactolife-Av+: Lyophilized cake containing ≥$1x10^8$ CFU/g Lactobacillus acidophilus + Pichia anomola containing ≥$1x10^7$ CFU/g, Batch No. LA1172 .

3-Lactolife-Av+B: Lyophilized cake containing ≥$1x10^8$ CFU/g Lactobacillus acidophilus + Pichia anomola containing ≥$1x10^7$ CFU/g + Bacteriophage Containing ≥$1x10^6$ PFU/g, Batch No. LB1177 .

Experimental design:

One day-old male Arbor Acres plus broiler chickens (n = 240) were used in this study. These birds have been allotted into 16 equal groups (1-16) consisting of 15 birds. All groups were assigned into 3 equal replicates.

Birds of groups 1-4 received Lactolife-Av, birds of groups 5-8 received Lactolife-Av+, birds of groups 9-12 received Lactolife-Av+B, in a dosage of 10^4 CFU/bird in drinking water at 1st and 10th day of age. While chickens of groups 13-16 fed on plain water without treatment.

At 2nd day of age, chickens of groups 2, 6, 10 and 14 orally inoculated with $4x10^5$ CFU/bird of S. Typhimurium after **Awaad et al. (2003 b)**. At 21st day of age; chickens of groups 1, 5, 9 and 13 inoculated subcutaneously with $4.5x10^6$ CFU/bird of E. coli serogroup O78 after **Awaad et al. (2008)**.

While those of groups 3, 7, 11 and 15 subcutaneously inoculated with $0.5x10^8$ CFU/bird of C. perfringens in phosphate buffered saline (PBS) after **Awaad et al. (2011)**. All experimented birds vaccinated against different diseases according to the vaccination programs usually adopted in Egyptian broiler chicken farms.

Diets:

Chickens fed ad libitum a commercial starter diet (23% crude protein and 3000 k cal ME/kg diet) during the first 2 weeks of age, commercial grower diet (21% crude protein and 3100 kcal ME/kg diet) from 2-4 weeks of age, and then commercial finisher diet (19% crude protein and 3200 kcal ME/kg diet).

Measured parameters:

I. Bioassay:

For intestinal enumeration of *S. Typhimurium*; 3 random selected birds of groups 2, 6, 10 and 14 at zero, 3, 7, 14 and 17 days post infection (PI) were sacrificed and 0.2 g of intestinal contents from each were collected and serially diluted to 1:100, 1:1000, and 1:10000 in sterile PBS and 0.1 ml of each dilution was plated on brilliant green agar (BGA) plates, incubated aerobically at 37°C for 24 hours and colonies counted and calculated per gram. Typical salmonella colonies were confirmed by biochemical tests on triple sugar iron agar.

For intestinal colonization of *C. perfringens* 3 birds were randomly sacrificed at zero, 3, and 7 days PI from groups 3, 7, 11 and 15 (one bird/replicate) and subjected to post mortem examination and 0.2 g of intestinal contents from each bird were serially diluted in sterile PBS to 1:100, 1:1000, and 1:10000 and 0.1 ml of each dilution and poured on the surface of sheep blood agar plates and tryptose sulfite-cycloserine (TSC) agar (supplemented by D-cycloserine) with egg yolk emulsion. This was overlaid with the same medium but without egg yolk. After anaerobic incubation at 37°C for 24 hours; typical *C. perfringens* colonies (black colonies) on TSC agar or large dome-shaped colonies with a double zone of hemolysis on blood agar plates were counted

and reported as colony-forming units (CFU) per gram. The colonies picked and confirmed by criteria of **Harmon (1984)** and **Carrido et al. (2004)**.

For *E. coli* infection; birds of groups 1, 5, 9 and 13 were kept under observation for 14 days PI with recording mortality and post mortem changes. Lesion scores were recorded in sacrificed survived birds at end of observation period (35 days of age).

II. Immune status assessment:

For determination of the effect on cell mediated immunity; measurement of phagocytic activity of peripheral blood monocytes using Candida albicans has been adopted on 6 randomly selected birds at weekly intervals (1-5 weeks of age) from groups 4, 8, 12 and 16 (2 birds/replicate) as described by **Anthony et al. (1985)** and **Chu and Dietert (1989)**.

III. Histomorphometric assay for gut integrity:

At the end of the experiment, five birds from groups 4,8,12 and 16 were sacrificed and small intestine was collected and immediately immersed in 10% buffered formalin. After fixation, 1 cm-thick samples were taken from the jejunum and ileum [the intestinal segmentation was according to **Samanya and Yamauchi (2002)** as jejunum from the bile duct to Meckel's diverticulum and ileum from the Meckel's diverticulum to ileo-cecalcolonic junction]. Routine histological laboratory methods including dehydration, clearing and paraffin embedding used and paraffin blocks made. The slides were stained by haematoxylin and eosin. Histological indices were measured using digital photography and light microscopy the photos were taken and morphometric analyses were performed by means of an image analysis program (Image J software). The villus height was measured from the apical to the basal region, which corresponded to the superior portion of the crypts. Crypts were measured from the basis until the region of transition between the crypt and the villus. Three measurements per slide were made for each parameter and averaged into one value.

Statistical analyses:

One-way analysis of variance has been adopted using SAS software general liner models procedure (**SAS Institute, 1999**). The main factor was probiotic supplementation. Mean values were assessed for significance using Duncan's multiple range test with significance set at $P<0.05$.

III. Results and Discussion

Results of experimental infection of *S. Typhimurium* in treated and untreated broiler chickens with the investigated probiotics to determine its intestinal enumeration showed significant clear tendency for its elimination at all studied intervals provided that complete intestinal clearance could be achieved in Lactolife-Av+ and Lactolife-Av+B treated groups at 14 days PI (Table 1). Food borne Salmonellae are estimated to cause 1.3 million illnesses, 15,000 hospitalizations and 500 deaths and cause severe economic losses of $ 0.5-2.3 billion per year in the United States and throughout the world (**Mead et al., 1999**). Most reports have mentioned *S. enterica serovar Tyhimurium* and *S. enteric serovar Enteritidis* as the most common causes of human salmonellosis worldwide (**ECSRZ, 2007**). **Public health Services laboratory (1991**) reported that different analysis present that 67% of the feed containing animal proteins and 69% from the mixtures at the feed factories were infected with Salmonellae. **Cardoso and Carvalho (2006**) mentioned that salmonellosis account for a severe problem of public health both in developing and developed countries and several controlling mechanisms of such pathogens have been applied to poultry production. Coinciding with the above reported data, **Barrow et al. (1987**) reported that intestinal colonization is normally the first step in the infection process for orally introduced paratyphoid Salmonellae, frequently leading to the persistent shedding of Salmonellae in the feces. The inhibition effect produced by probiotics on the population of salmonella through the competitive exclusion (CE) mechanism is widely documented in the literature (**Ghadban et al., 1998; Jeffrey, 1998; Reid and Friendship, 2002; Awaad et al., 2003 a; Hariharan et al., 2004; Dahiya et al., 2006; Callaway et al., 2008**).

A significant reduction in intestinal as well as cecal colonization of *C. perfringens* on using the studied probiotics at 3 and 7 days PI provided that best results have been obtained on using Lactolife-AV+B followed by Lactolife–Av then Lactolife-Av+ (Table 2). *C. perfringens* is responsible for the rare but severe food borne necrotic enteritis in man (enteritis necroticans or pigbel disease) which is fatal specially in young and elderly and its enterotoxin has been shown to be the virulence factor responsible for causing the symptoms of *C. perfringens* type A food poisoning which is the more common in the industrialized world (**Ghadban et al., 1998, Brynestad, 2002** and **Awaad et al., 20011**). **Fukata et al. (1991)** reported that the probiotic *Lactobacillus acidophilus* and *Streptococcus faecium* reduced the severity of necrotic enteritis.

Experimental infection with pathogenic *E. coli* serogroup O78 resulted in reduction of chicken mortality in probiotic treated birds. The lesion scoring reached 1.27, 2.53 and 0.63 in Lactolife–Av, Lactolife-

Av+ and Lactolife-AV+B treated groups as compared with 4.36 in untreated group respectively (Table 3). These findings might be attributed to the high performance of birds induced by the probiotic used which possibly indirectly improved the immune status of the treated chickens. **Jeffrey (1998)** reported that the use of competitive exclusion products can protect newly hatched, highly susceptible chicks being placed into commercial production systems and could be of great benefit in reducing colonization and disease caused by *E. coli, Campylobacter jejuni, Clostridium botulinum* and *Clostridium perfringens.*

Although bacterial enteropathogens can be prevented by prophylactic antibiotics, a serious drawback of such chemotherapy is the development of resistance to most antibiotics (**Chansiripornchai et al., 1995**). Genes located on plasmids often encode resistance to antibiotics which easily spread through bacterial populations leading to spread of resistance, rendering drugs ineffective and making their subsequent use for therapy difficult. Additionally; prophylactic antibiotics result in occurrence of antibiotic residues in poultry products (**Dupont and Steele, 1987**). The direction towards the use of environmentally friendly alternatives as natural control method has been emerged to reduce the risk factors associated with enteropathogens. As the food safety is probably the biggest issue facing poultry production systems today; consumer confidence has a direct correlation to the safety and wholesomeness of the product they will purchase. Accordingly; the global potential for animal health products are on rise for higher need of top quality poultry and for preventing contamination of poultry products with food borne pathogens which remains a considerable challenge for producers and integrations (**Awaad et al., 2011**).

Results of immunoassay are illustrated in Tables 4-5. Although both Phagocytic percentages and indices significantly increased in the 3 studied probiotics as compared with their untreated control group at majority of studied intervals (1-5 weeks of age); Lactolife-Av+B gave the superior results followed by Lactolife-Av+ then Lactolife-Av. **Goldin and Gorbach (1984)** reported that probiotics stimulate natural resistance of the organism through increasing the number of antibodies and increasing the effectiveness of macrophages. **Borchers et al. (2009**) reported that the boost produced by the colonization of probiotics are essential for the development of functional immune system including the presence of T and B lymphocytes in the lamina propria and the expansion and maturation of IgA and also induction of tolerance by the present antigens.

The effect of different water supplementations on small intestinal histomorphological parameters is presented in Table (6) and Plate 1. In jejunum the villus length was increased in all treated groups vs. blank control group. However; the highest villus length and villus height crypt depth ratio were achieved in Lactolife-Av+ treated group when compared with the other 2 treated groups. The crypt depth of the jejunum was slightly increased in Lactolife-Av group and Lactolife-Av+B group as compared with the control while decreased in Lactolife-Av+ group. On other hand the histomorphometric analysis of ileum revealed increased villus height in all treated groups compared with the control but highest ileal villus height was recorded in Lactolife-Av+ group. While the crypt depth was decreased in all treated groups compared with their controls and the highest villus height crypt depth ratio was achieved in Lactolife-Av + group. No significant difference has been noticed in the total number of goblet cells in intestinal mucosal lining of all treated groups indicating that there was no increase in mucin content on mucosal surface.

In conclusion the current study has shown the interest of using probiotics in broiler chickens to struggle *S. Typhimurium, E.coli* and *C. perfringens* infection. Taking in consideration the facts that they do not require withdrawal period, they can make a valuable contribution to flock health and safety of poultry products as food. This may provide a significant tool for the poultry industry in controlling these major enteric infections and in reduction of food borne pathogens.

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Table 1. Results of intestinal colonization of *S. Tyyphimurium* in treated and untreated broiler chickens with different probiotics (Mean bacterial count / gm intestinal contents x 10^3).

Group No.	Time post *S. Typhimurium* inoculation					
	0 hr.	3 days	7 days	10 days	14 days	17 days
1-Lactolife-Av	0.00 ± 0.00	1333333.30 ± 300462.61 [b]	800.00 ± 332.92 [b]	0.50 ± 0.00 [b]	0.02 ± 0.02	0.00 ± 0.00
2-Lactolife-Av+	0.00 ± 0.00	116666.70 ± 16666.67 [b]	5.83 ± 0.83 [b]	0.05 ± 0.00 [b]	0.00 ± 0.00	0.00 ± 0.00
3-Lactolife-Av+B	0.00 ± 0.00	9666.70 ± 333.33 [b]	8.00 ± 3.33 [b]	0.05 ± 0.00 [b]	0.00 ± 0.00	0.00 ± 0.00
4- Blank Control	0.00 ± 0.00	20000000.0 ± 2886751.35 [a]	175000.00 ± 14433.76 [a]	3.50 ± 1.50 [a]	1.70 ± 1.65	0.02 ± 0.02
Probability	.	0.0001	0.0001	0.0318	0.4203	0.4411

* Means with different superscripts, within age, are significantly different ($P \leq 0.05$).

Table 2. Means of *C. perfringens* colonization in experimentally infected broiler chickens (10^3 CFU / g).

Group No.	No. of birds/group	Organ					
		Intestine			Cecum		
		0 hr.	3 days	7 days	0 hr.	3 days	7 days
1-Lactolife-Av	15	0.00 ± 0.00 [b*]	1.36667 ± 66.67 [c]	0.15000 ± 11.55 [c]	0.00 ± 0.00 [c]	1.96667 ± 33.33 [c]	0.71667 ± 109.29 [c]
2-Lactolife-Av+	15	0.00 ± 0.00 [b]	4.33333 ± 1201.85 [b]	0.66667 ± 88.19 [b]	0.01000 ± 0.00 [b]	6.33333 ± 1201.85 [b]	2.06667 ± 33.33 [b]
3-Lactolife-Av+B	15	0.00 ± 0.00 [b*]	0.16333 ± 6.67 [c]	0.10000 ± 0.00 [c]	0.00 ± 0.00 [c]	1.96667 ± 33.33 [c]	0.30000 ± 57.74 [c]
4-Blank Control	15	0.01000 ± 0.00 [a]	12.33333 ± 1201.85 [a]	2.66667 ± 145.30 [a]	0.02000 ± 0.00 [a]	16.00000 ± 577.35 [a]	3.83333 ± 333.33 [a]

*Means within time and within region of GIT with different, superscripts are significantly different (P≤0.05).

Table 3. Results of *E. coli* O78 infection in treated and untreated broiler chickens with different probiotics.

Group No.	No.of birds	Dead birds	Surv. birds	Lesion scores of survived birds						Mean lesion score
				Peri-carditis	Pre-hepatitis	Air saculitis	Peri-tonitis	Caseus Exudate	Total	
1-Lactolife-Av	15	1(6.7%)	14	3	2	13	1	0	19	1.36
2-Lactolife-Av+	15	1(6.7%)	14	8	4	18	6	2	38	2.71
3-Lactolife-Av+B	15	0(0%)	15	1	0	9	0	0	10	0.67
4-Blank Control	15	2(13.3%)	13	9	10	23	13	6	61	4.69

Table 4. Results of Phagocytic % of broiler chicken groups treated and untreated with probiotics.

Group No.	Week1	Week2	Week3	Week4	Week5
1-Lactolife-Av	56.17 ± 0.79 [b*]	53.67 ± 0.33 [c]	55.17± 0.60 [c]	56.00± 0.58 [c]	55.83± 0.87 [bc]
2-Lactolife-Av+	56.50 ± 0.62 [b]	57.50 ± 0.85 [b]	57.33± 0.80 [b]	59.00± 0.58 [b]	57.83± 0.70 [b]
3-Lactolife-Av+B	60.67 ± 0.99 [a]	61.67 ± 0.80 [a]	65.33± 0.49 [a]	64.17± 0.79 [a]	66.33± 0.71 [a]
4- Blank Control	51.17 ± 0.79 [c]	52.50 ± 1.43 [c]	53.67± 0.80 [c]	54.67± 0.71 [c]	54.17± 1.30 [c]
Probability	0.0001	0.0001	0.0001	0.0001	0.0001

* Means with different superscripts, within age, are significantly different (P ≤ 0.05).

Table 5. Results of Phagocytic index of broiler chicken groups treated and untreated with probiotics.

Group No.	Week1	Week2	Week3	Week4	Week5
1-Lactolife-Av	0.42 ± 0.004 [b*]	0.43 ± 0.004 [c]	0.44 ± 0.008 [b]	0.44 ± 0.009 [b]	0.43 ± 0.010 [b]
2-Lactolife-Av+	0.43 ± 0.007 [b]	0.45 ± 0.006 [b]	0.44 ± 0.009 [ab]	0.45 ± 0.011 [b]	0.45 ± 0.006 [b]
3-Lactolife-Av+B	0.48 ± 0.007 [a]	0.47 ± 0.005 [a]	0.46 ± 0.004 [a]	0.50 ± 0.009 [a]	0.61 ± 0.010 [a]
4-Blank Control	0.35 ± 0.006 [c]	0.36 ± 0.008 [d]	0.35 ± 0.010 [c]	0.36 ± 0.008 [c]	0.35 ± 0.009 [c]
Probability	0.0001	0.0001	0.0001	0.0001	0.0001

* Means with different superscripts, within age, are significantly different (P ≤ 0.05).

* Number of macrophages engulfs 3 Candida spores / Total numbers of phagocytic macrophages

Table 6. The effect of different water supplementations on small intestinal hitomorphological parameters.

Group No.	Histomorphological parameters					
	Jejunum			Ileum		
	Villus height (μm)	Crypt depth (μm)	Villus length/crypt depth ratio	Villus height (μm)	Crypt depth (μm)	Villus length/crypt depth ratio
1(Lactolife-Av)	560±30	169±22	3.31	402±50	114±16	3.53
2(Lactolife-Av+)	630±20	130±6	4.85	502±26	115±12	4.36
3(Lactolife-Av+B)	576±30	175±35	3.29	400±9	100±6	4.00
4-Blank Control	549±47.18	160±9	3.43	322±28.4	122±11.9	264

Plate (1) Figures 1-8 illustrated the small intestinal histomorphological parameters

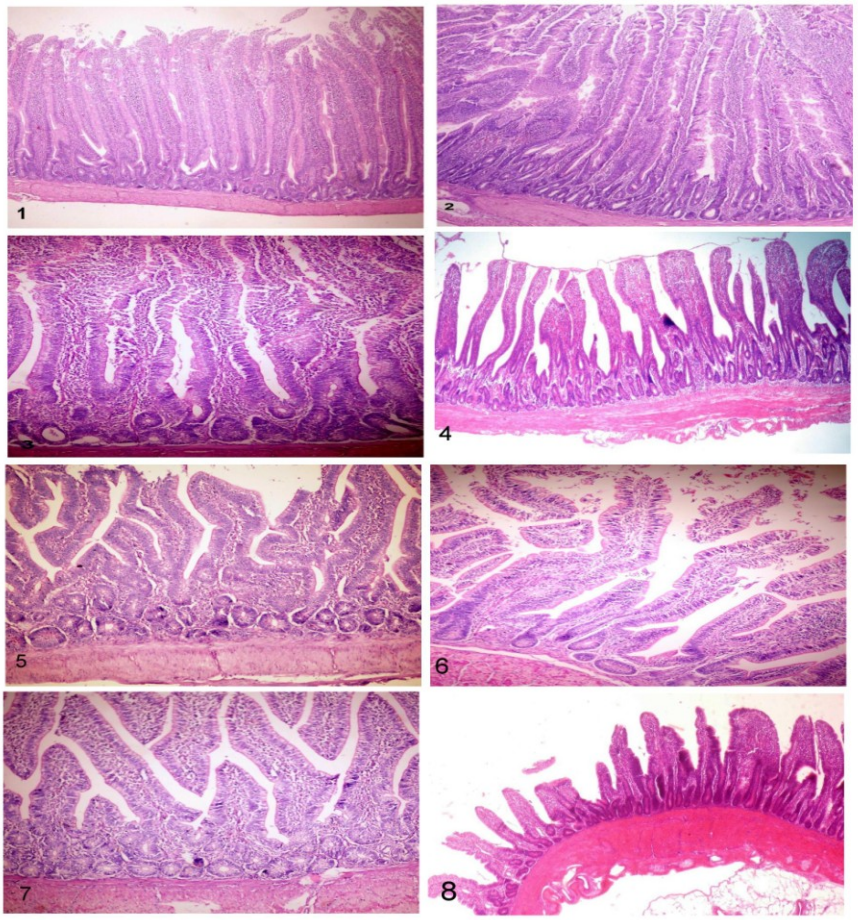

Fig.1. Photomicrography of jejunal villous and crypt of broiler chickens treated with Lactolife-Av group (40x).
Fig.2. Photomicrography of jejunal villous and crypt of broiler chickens treated with Lactolife- Av+ group (40x).
Fig.3. Photomicrography of jejunal villous and crypt of broiler chickens treated with Lactolife- Av+B group (100x).
Fig.4. Photomicrography of jejunal villous and crypt of broiler chickens control group (40x).
Fig.5. Photomicrography of ileal villous and crypt of broiler chickens broiler chickens treated with Lactolife-Av group (100x).
Fig.6. Photomicrography of ileal villous and crypt of broiler chickens broiler chickens treated with Lactolife-Av+ group (100x).
Fig.7. Photomicrography of ileal villous and crypt of broiler chickens broiler chickens treated with Lactolife-Av+B group (100x).
Fig.8. Photomicrography of ileal villous and crypt of broiler chickens control group (40x).