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The use of probiotics to enhance immunity of broiler chicken against some intestinal infection pathogens

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Abstract

This study was conducted on 120 one day old broiler chicks which were divided into six groups, 20 birds each. Group 1 (control), group 2 (supplemented with probiotic), group 3 (challenged with S*almonella* and receive no probiotic), group 4 (challenged with *E coli* and receive no probiotic), group 5 (challenged with *Salmonella* and supplemented with probiotic), group 6 (challenged with *E coli* and supplemented with probiotic). The experiment extended for 30 days starting from one-day-old chicks. Body weights, clinical symptoms, haematological analysis and postmortem lesions were demonstrated on 8th, 15th and 30th day of the experiment. Also, histopathological studies of the intestinal mucosa, liver, spleen, thymus and bursa of Fabricius, as well as immunostaining of surface antigens (CD3A in the thymus and CD79A in the spleen and bursae of Fabricius), were also investigated. The current study revealed that supplementation of probiotic alone obviously improved weight gains as compared to the control group.

Furthermore, probiotic supplementation decreased the colony forming a unit (CFU) of *Salmonella enteritidis* and *E. coli (strain O2: H45)* in the intestinal mucosa. Histopathologically, the intestinal mucosa showed an improvement which indicated by hyperplasia of the lining epithelium and abundance of goblet cells, but this local effect did not extend to other organs in the body that demonstrated mild to severe histopathological changes in challenged groups. The haematological analysis also verified that treatment with probiotics had no significant effect on most blood values (RBCs, WBCs and Hb). However, the differential leucocytic counts were significantly influenced by dietary treatment with probiotics which caused a highly significant decrease in lymphocyte percentage. In conclusion, probiotics obviously improved the growth performance and local immune response in the intestine, however no clear evidence of improvement of the general immune status of the experimental birds.

Keywords: Probiotics, chicken, immunity, pathology.

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Introduction

The increase in productivity of the poultry industry has been accompanied by various impacts, including the emergence of a large variety of pathogens and bacterial resistance. These impacts are in part due to the indiscriminate use of chemotherapeutic agents as a result of management practices in rearing cycles (Kabir, 2009). The increased use of antibiotics for therapeutic, prophylactic and growth promotion purposes led to the presence of antibiotic residues in poultry, meat and eggs which have the deleterious effect on human consumers that can cause the resistance of human flora and pathogenic microbes to those antibiotics (Abd-el-rahman et al., 2012).

In Europe and South Korea, growth promoting antibiotics have been banned since 2006 and 2012 respectively, and such bans are further expected to affect the rest of the world. The development topic for animal science research involved in developing sustainable animal production system, that in the absence of alternatives to antibiotics chicken raised under current intensive production systems face a higher risk of infection by enteric pathogens (Lillehoj and Lee, 2012). Probiotics are being considered to fill this gap, and already some farmers are using them instead of antibiotics (Trafalsk and Grzybowski, 2004).

Probiotics are "live microorganisms when administered in adequate amounts conferring a health benefit to the host". The most important advantage of probiotics is that they neither have any residues in animal products nor exerts any antibiotic resistance by consumption and probiotics have a good impact on the poultry performance (Koenen et al., 2004 and Mountzouris et al., 2007).

Lactobacilli species are commonly selected as probiotics since they express many crucial properties such as high tolerance to acid and bile, capability to adhere to intestinal surfaces, withstanding low PH, gastric juice (antimicrobial activity), resisting antibiotics, producing exopolysaccharides and removing cholesterol (Ruiz et al., 2013 and Tulumoglu et al., 2013). *Lactobacillus acidophilus* or mixture supplementation of lactobacilli cultures to chickens significantly increase the levels of amylase enzyme through its colonising of the intestine and thus increasing the digestibility of nutrient (Dierck, 1989).

In broiler nutrition, probiotic species belonging to Lactobacillus, Streptococcus, Bacillus, Bifidobacterium, Enterococcus, Aspergillus, Candida and Saccharomyces have a beneficial effect on broiler performance, modulation of intestinal flora, intestinal histological changes, immunomodulation, in addition, have an impact on specific haemato-biochemical parameters and improving microbiological meat quality of broilers (Matsuzak and Chin, 2000; Islam et al., 2004; Matsuzaki et al., 2007; Apata, 2008 and Ashayerizadeh et al., 2009). Probiotics also regulate the microbial environment in the gut, thereby improve feed conversion ratio. In vitro and in vivo studies have demonstrated that lactic acid producing bacteria can inhibit the growth of poultry pathogens like S*almonella* and *E coli* by lowering the PH of the gut (Chaucheyras et al., 1995; Lee et al., 2003 and Frizzo et al., 2010).

Probiotics have been reported to cause enhancement of colonization resistance against pathogens as S*almonella* enterica which colonize and penetrate the mucosal barrier. Probiotics also strengthen tight

junctions between enterocytes and enhance the mucosal immune response to pathogens (Lei and Allan, 2001).

Probiotics reduce colonization and shedding of *Salmonella* and Campylobacter and are a useful measure to protect newly hatched chicks and other birds against *Salmonella* and other entomopathogens (Line et al., 1998; Fritts et al., 2000 and Schneitz, 2005).

Brisbin et al. (2008) investigated the spatial and temporal expression of immune system genes in chicken cecal tonsil in response to structural constituents of *L. acidophilus* which induced T-helper-1 cytokines in cecal tonsil cells. Also, several investigations demonstrated the potential effect of probiotic on immune modulation (Matsuzaki and Chin, 2000; Mathivanan and Kalaiarasi, 2007 and Apata, 2008).

The current study aims to evaluate the beneficial effect of probiotic on growth performance, haematological picture and favorable impact on immunity of broiler chicken against *Salmonella enteritidis* and *E coli* infection and their consequent histopathological changes in different organs.

Materials and Methods

Cloacal swabs:

Sampling was done in the period between September-2017 to February - 2018 in ten broiler chicken farms located in Assuit city, Egypt, whose birds ageing 27 to 35 days. From each farm, 20 individual cloacal swabs were randomly collected from birds suffered from respiratory and intestinal signs. Samples were transported in 1.5 ml test tubes containing 750 µL of brain heart infusion (BHI) broth then refrigerated in an ice box and sent to the laboratory of animal health research institute in Assuit governorate.

Isolation and Identification of the suspected bacteria:

For isolation of *E coli*, BHI broth that used for transporting samples were incubated at 37ºC for 18 hours, then a loopful of the incubated BHI broth was streaked in the plate containing Eosin Methylene Blue (EMB) agar. Colonies with the characteristic morphology (dark coloured colonies with a brilliant green sheen) were selected and identified with biochemical reactions (Quinn et al., 2002).

For the isolation of *Salmonella* sp., as done previously with *E coli* a loopful of incubated BHI broth was transferred to Rappaport-Vassiliadis broth then incubated at 37ºC for 24 hours. Samples were streaked on Brilliant Green agar added Novobiocin (40 µg/mL) and Salmonella-Shigella agar and left for 24 hours at 37ºC. After incubation, colonies from each sample with characteristics morphology to *Salmonella* sp. were subjected to biochemical identification. Isolates with biochemical profile compatible with *Salmonella* spp. were identified serologically (Al-Aalim, 2017).

Experimental birds:

One-hundred- and twenty of one-day-old chicks were divided into six groups according to the ration received, 20 birds each group:

Group 1: control (only standard ration).
Group 2: (standard ration and probiotics).
Group 3: (standard ration and challenged with S*almonella*).
Group 4: (standard ration and challenged with *E coli*).
Group 5: (standard ration challenged with *Salmonella* and received probiotics).
Group 6: (standard ration challenged with *E coli* and received probiotics).

The birds were reared under hygienic management practices throughout the entire period of study. Commercially available standard poultry feed (Feed mix, Egypt) was used for all groups throughout the experiment. The broiler chicks were fed with typical broiler starter, broiler grower and broiler finisher rations. As per instruction, probiotics were added to drinking water at a ratio of 0.5g/1litre water and was given daily to birds belonging to groups 2 and 5 and 6. The experiment extended for 30 days from one-day-old until 30 days old chicks.

Body weight:

All chicks were individually weighed at 8^{th}, 15^{th} and 30^{th} days of the experiment and the average bird weight gains were determined.

Clinical signs and lesions:

Clinical signs and PM lesions were investigated throughout and at the end of the experiment.

Haematological examination:

Blood samples were collected via the wing vein from all groups at 8^{th}, 15^{th} and 30^{th} days of the experiment. About 2 ml of blood were drained from each bird into a tube containing 1mg ethylene tetraacetic acid (EDTA). Total red blood cells (RBC) count, haemoglobin (Hb) concentration, total white blood cells (WBC) count and differential leucocytic count were determined within 1-2 hours of collection. Haematological parameters were determined using (Medonic Auto Hematology Analyzer CA 620/ Vet/20).

Histopathological examination:

The chickens were sacrificed, and tissue specimens from bursa, thymus, spleen, lung, liver and intestine of all experimental groups were collected and fixed in 10% neutral buffered formalin. The tissues were prepared for routine histopathological examination (Bancroft and Stevens, 1982) and examined using the light microscope (Olympus CX31, Japan) and photographed using a digital camera (Olympus, Camedia C-5060, Japan).

Immunohistochemistry investigations:

Paraffin sections from the thymus, bursa of Fabricius and spleen were used for immunohistochemical detection of CD3 (T- lymphocytes) in thymus and CD79 (B-lymphocytes) in spleen and bursa of Fabricius at the end of the experiment (30^{th} day). The tissue sections (3µm thick) were deparaffinized and hydrated then washed by distal water. Antigen retrieval was applied in a water bath using citrate buffer (pH6) for 20 minutes. The endogenous peroxidase activities were removed with 3% hydrogen peroxide (H_2O_2). Sections were then incubated in diluted polyclonal primary antibody for one hour at room temperature in a humidified chamber for CD79 (obtained from Novus Biologicus company) and CD3 polyclonal rabbit anti-human CD3 (Dako) at 1 in 300 dilutions. The primary antibodies were detected in all experimental groups. The staining was performed using Power-Stain™ 1.0 Poly HRP DAB according to the manufacturer's instructions. Then the sections were rinsed three times for 5 min each with Phosphate-buffered saline, and the sections were incubated in Poly HRP Conjugate for 15 minutes at room temperature. A mixture of DAB chromogen visualized the sections, and DAB substrate then incubated for 10 minutes. Sections were washed by distilled water then counterstained with hematoxylin and dehydrated and mounted (Anis et al., 2013).

Scoring of immunoreactivity:

The immunepositive cells were counted in 10 fields of the histological sections of tissues. Cytoplasmic CD79A positive cells were detected in the medulla of bursal follicles and white pulp of the spleen. Cytoplasmic CD3A positive cells were observed in the thymus. Positive cells were identified using digital an Axiostar plus microscope (Carl Zeiss, Thornwood, NY, USA) interfaced with an Axiostar plus digital camera and Axiovision 4.1 software (Carl Zeiss) at a magnification of 100, where B-lymphocytes and T lymphocytes were diffusely distributed, and their relative frequency per focus was calculated according to the point count method (Weibel, 1969).

Statistical analysis:

The variation in numbers of CD79A positive cells in bursa of Fabricius and spleen in addition to CD3A positive cells in the thymus (randomized block design) were compared among broilers in different experimental groups at the end of the study. Statistical analysis was performed by using the Statistical Package for the Social Sciences (SPSS), Version 16 for windows. Data are expressed as mean ± SD was evaluated by two independent samples Test "Mann-Whitney U Test".

Results

Clinical signs:

Clinical signs included depression, weak growth, weakness, diarrhoea and dehydration particularly in groups challenged with *Salmonella enteritidis* and *E coli*. Mortalities were mostly limited to the first two weeks of age and seen only in group 5 challenged with S*almonella* and supplemented with probiotics (Table 1).

Table (1): The rate of morbidity and mortality in all experimental groups

Group	Morbidity	Mortality
Group 1 (control)	1/10	0/10
Group 2 (Probiotic group)	0/10	0/10
Group 3 (*Salmonella* challenged)	10/20	0/20
Group 4 (*E coli* challenged)	6/20	0/20
Group 5 (*Salmonella* + probiotic)	10/20	10/20
Group 6 (*E coli* + probiotic)	6/20	0/20

Isolation and identification:

E coli were isolated from 45% of the examined samples. In contrast, *Salmonella* isolates were isolated from 5% of individual cloacal swab samples. According to the serotyping, 12 out of 45 of the isolated *E coli* were of strain *O2: H45*. However, 3 out of 5 positive *Salmonella* isolates were *Salmonella enteritidis*.

Body weight of the birds:

The current study revealed that the weight of the experimental birds recorded variable values between different groups (Table 2). The group receiving only probiotics in addition to the standard diet (group 2) showed the best weight gain among experimental groups. However, the group challenged with S*almonella* and received no probiotics (group 3) recorded the least weight gain compared to other groups. Besides, the group challenged with *E coli* and received probiotics (group 6) recorded better weight gain compared to that challenged with *Salmonella* and received probiotics.

Table (2): Weight of experimental birds (grams ± SD) throughout the experiment

Groups	Body weight		
	8th day	15th day	30th day
Group 1 (control)	190.0±12.9	500.5±15.2	1333.7±72.3
Group 2 (Probiotic group)	210.5±13.2	650.3±14.3	1606.7±66.2
Group 3 (*Salmonella* challenged)	180.9±12.4	490.4±12.2	1050.8±81.2
Group 4 (*E coli* challenged)	195.8±13.1	485.5±16.7	1260.1±75.4
Group 5 (*Salmonella* + probiotic)	186.2±12.7	515.5±15.2	1166.1±65.5
Group 6 (*E coli* + probiotic)	194.4±12.9	520.2±14.3	1266.4±50.6

Bacterial colony forming units (CFU):

The CFU, in the group 5 (supplemented with probiotics and challenged with *Salmonella enteritidis*), recorded no change by the beginning of the second weeks; however, it demonstrated a noticeable decrease by the 30th day of the experiment when compared to group 3. In the *E coli* challenged group and supplemented with probiotics (group 6), the CFU showed a slight decrease only by the 30th day of the experiment (Table 3).

Table (3): CFU/ml in challenged experimental groups

Groups	Colony count		
	8th day	15th day	30th day
Group 3 (*Salmonella* challenged)	$10x10^8$	$10x10^8$	$712x10^8$
Group 4 (*E coli* challenged)	$420x10^8$	$128x10^8$	$320x10^8$
Group 5 (*Salmonella* + probiotic)	$420x10^8$	$420x10^8$	$230x10^8$
Group 6 (*E coli* + probiotic)	$484x10^8$	$484x10^8$	$430x10^8$

Haematological parameters:

The result of haematological parameters in all chickens' broiler groups was presented in Table (4) and Figure (1). All measured haematological parameters for RBC, WBC, HB, and monocytes% showed no significant differences ($P > 0.05$). However, there was a highly significant decrease in lymphocytes percentage in group 2 (probiotic group) compared to the control (group 1), and a substantial decrease in lymphocytes percentage in group 3, group 5 and group 6 when compared with group 1. On the other hand, the granulocytes percentage showed a highly significant increase in groups 2 and 6 and a substantial increase in group 3.

Table (4): Hematological parameters of different experimental groups

Groups	RBC ($x10^6/mm^3$)	WBC ($x10^3/mm^3$)	Hb (g/dl)	Lymphocytes %	Monocytes %	Granulocytes %
Group 1 (control)	1.69±0.4	3.43±0.6	11.5±0.9	64.6±3.8	13.6±3.5	22.0±0.8
Group 2 (Probiotic group)	2.49±0.7	6.06±1.1	13.7±2.8	3.6±0.3	74.03±1.2	22.3±1.5
Group 3 (Salmonella challenged)	1.86±1.2	5.06±0.9	13.1±3.8	11.7±6.4	62.4±5.6	25.9±5.9
Group 4 (*E coli* challenged)	1.97±0.8	5.86±1.4	12.7±2.0	30.4±4.6	52.2±40.8	17.3±8.3
Group 5 (Salmonella + probiotic)	2.22±0.7	5.06±1.4	12.1±2.4	17±10.4	51.6±16.5	31.2±6.6
Group 6 (*E coli* + probiotic)	1.57±0.8	5.33±1.4	8.8±4.4	6.13±3.4	73.4±11.1	20.4±8.2

RBC= Red blood cells counts/ WBC= Total white blood cells counts/ Hb= hemoglobin content.

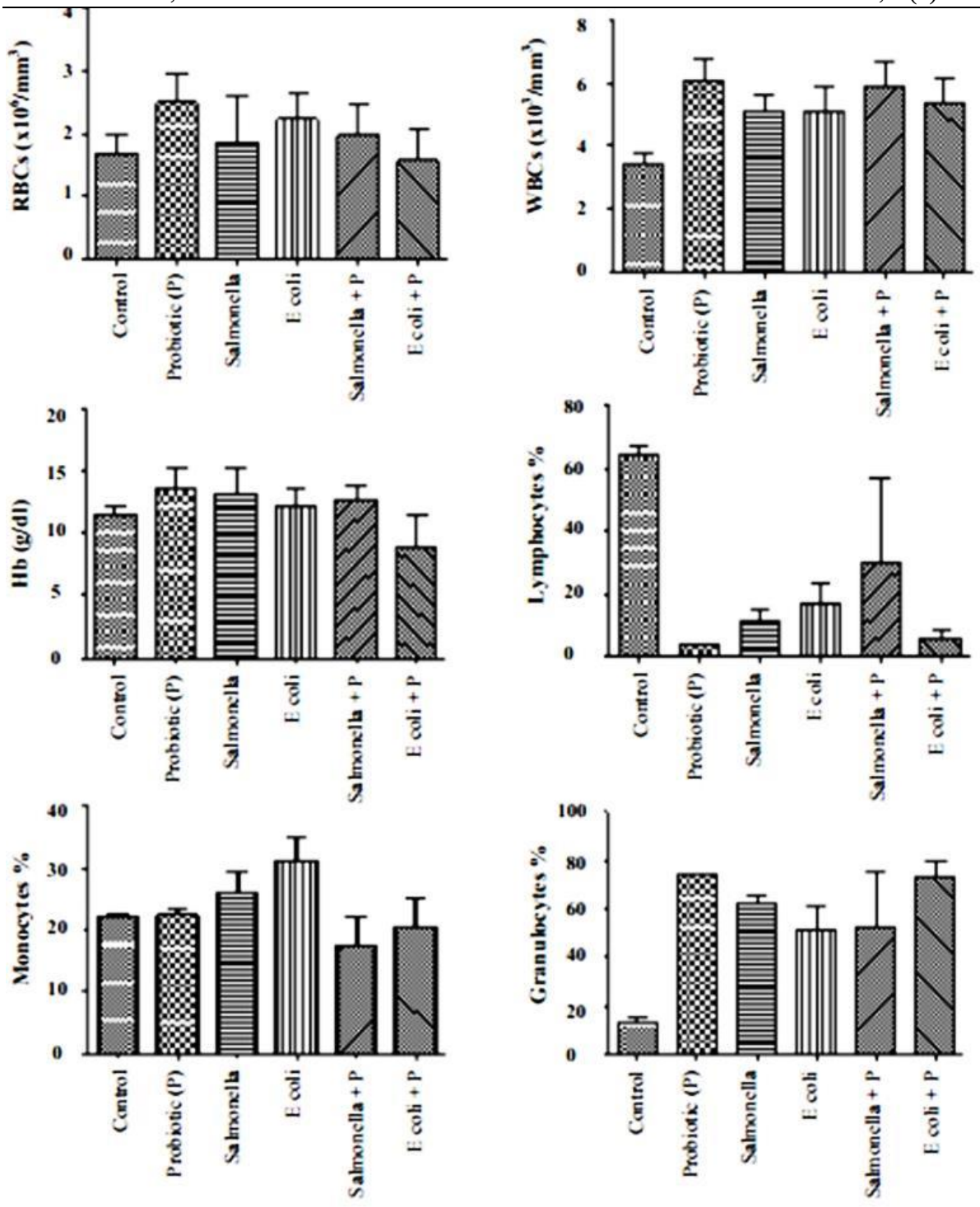

Fig. 1. Hematological parameter; RBC ($x10^6/mm^3$), WBC ($x10^3/mm^3$), Hb (g/dl), lymphocytes %, monocytes %, and granulocytes % (each value = means of the samples at 8th, 15th and 30th day) in all experimental groups including control, probiotics, salmonella challenged, E coli challenged, salmonella challenged + probiotics, and E coli challenged + probiotics.

Histopathological changes:

Lesions included an enlarged liver with necrosis, unabsorbed yolk sac and enteritis with necrotic lesions in the mucosa. Sometimes there were no lesions due to acute death caused by septicemia. Older birds could have the fever, being pale, dehydrated, and had diarrhoea. Also, the liver was swollen, brittle and often bile-stained

I . Liver:

In the control group, the liver showed normal hepatic tissue consisting of a central vein (C.V.) surrounded by radiating hepatic cords (Fig 2A). Liver of chickens supplemented with probiotics for 15 days was almost normal with uncommon, mild vacuolar degeneration of hepatocytes (Fig 2B). The *Salmonella* challenged group showed mild vacuolar degeneration of hepatocytes with the proliferation of mononuclear cells on the 15th day of the experiment (Fig 2C). These changes increased at the end of the study (30th day) and became accompanied by congestion of blood vessels. Livers of salmonella-infected birds and immunized with probiotics after 15 days and until the end of the study showed severe vacuolar degeneration in hepatocytes (Fig 2D). In *E coli* infected group, liver showed characteristic lesions of *E coli*. Liver showed perivascular haemorrhage and vacuolar degeneration of hepatocytes 15 days post-infection (Fig 2E). These changes continued until 30 days post-infection. The examined liver of *E coli* infected chickens and 15 days post-treatment with probiotics showed perivascular infiltration with inflammatory cells (Fig 2F). Birds sacrificed 30 days post-treatment showed mild vacuolar degeneration with an appearance of focal areas of Kupffer cells.

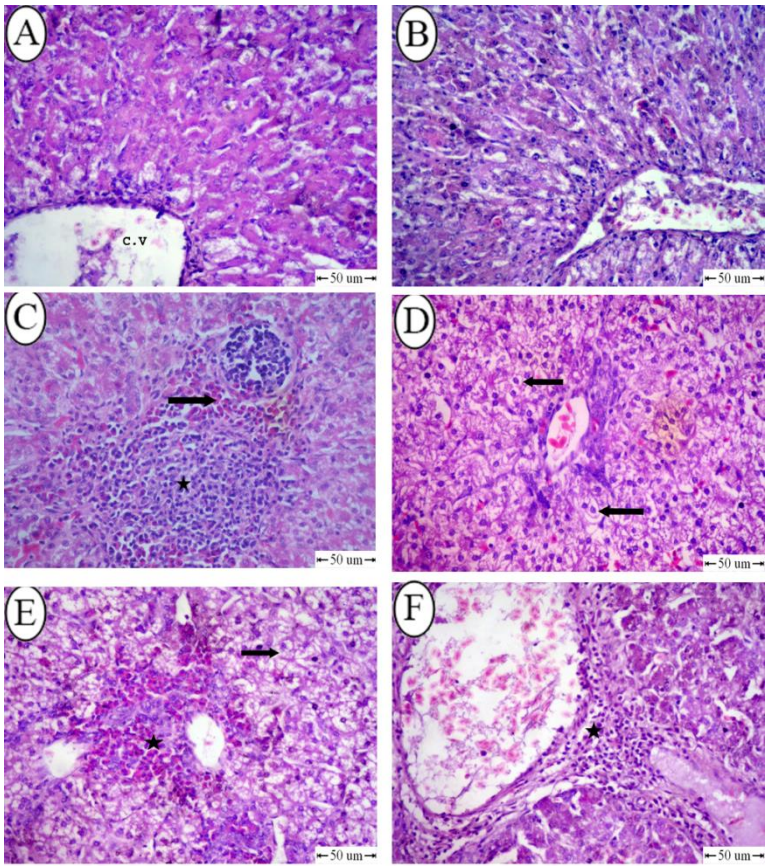

Fig. 2. Histopathological examination of the liver in (A) control group showing central vein (C.V.) surrounded by radiating hepatic cords. **(B) The probiotic-treated group** is showing mild vacuolar degeneration of hepatocytes**. (C) (Salmonella-infected group)** Fifteen days post-infection showing proliferation of mononuclear cells (star) and focal haemorrhage (arrow). **(D) (Salmonella-infected group and challenged with probiotics)** showing severe vacuolar degeneration in hepatocytes (arrow) **(E) (*E coli* infected group)** 15 days post-infection showing perivascular haemorrhage (star) and ballooning degeneration (arrow). **(F) *E coli* infected group and challenged with probiotics** showing perivascular infiltration with inflammatory cells (star), bar=50. H & E.

Ⅱ. Intestine:

Microscopically, intestine of the control group showed intestinal villi with intact epithelium (Fig 3A). After 15 days, the gut of probiotics treated group, showed hyperplasia of goblet cells which became more evident on the 30th day of the experiment (Fig 3B). In cecum of the Salmonella-infected group, desquamation of epithelium, interstitial haemorrhage in lamina propria was observed after 30 days (Fig 3C). Salmonella-infected birds and immunized 30 days post-treatment with probiotics, cecum showed intact epithelium with mild depletion in cecal tonsils (Fig 3D). Duodenum of *E coli* challenged group showed severe desquamation of epithelium after 15 days from exposure which also observed after 30 days (Fig 3E). Similar changes characterized by severe desquamation of epithelium were found in the intestine of *E coli* infected group and challenged with probiotics and examined after 15 days and 30 days (Fig 3F).

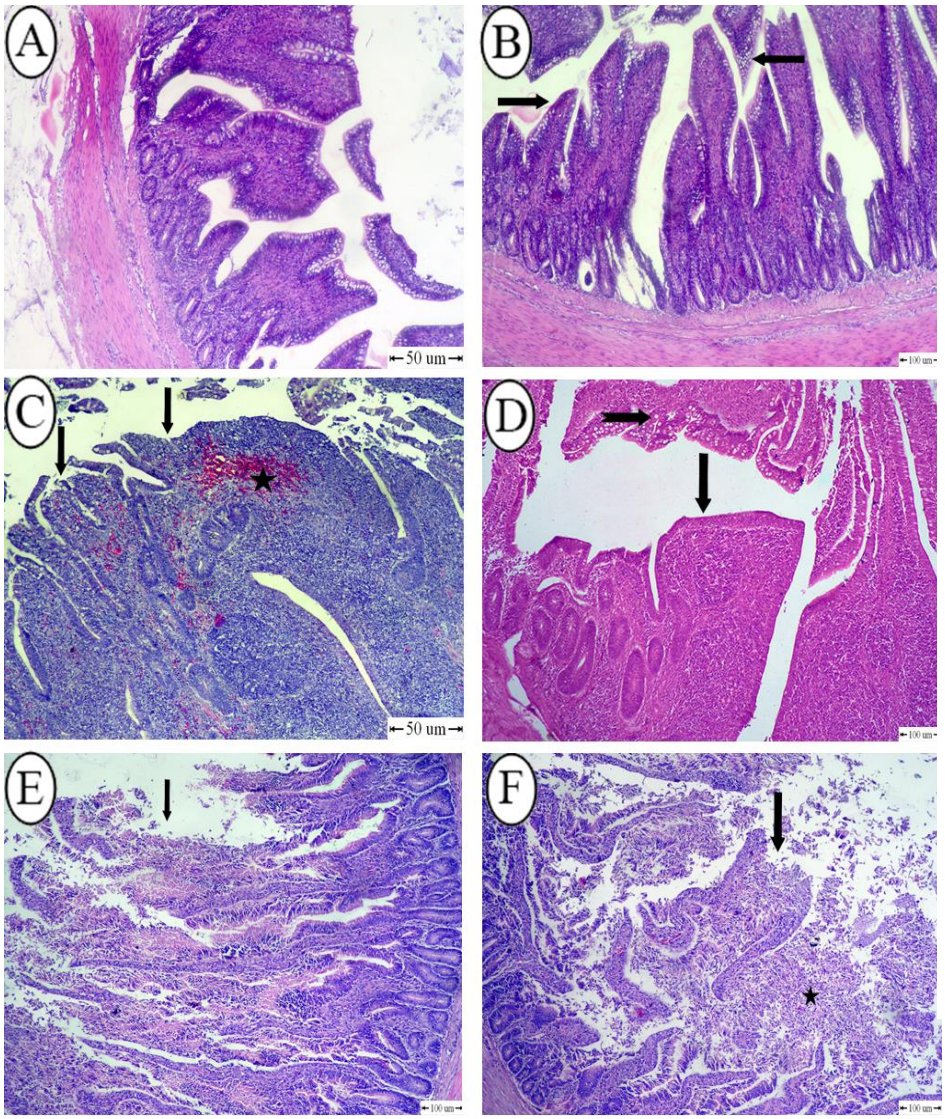

Fig. 3. Histopathological examination of the intestine in (A) Control group showing intact epithelium. **(B) Probiotics treated group** showing hyperplasia of goblet cells (arrow). **(C) (Salmonella-infected group)** Cecum 30 days post-infection showing desquamation of epithelium (arrow) and interstitial haemorrhage in lamina propria (star). **(D) (*Salmonella*-infected group and challenged with probiotics)** Cecum 30 days post-treatment showing intact mucosal epithelium (arrow) and activation with hyperplasia of goblet cells (notched arrow). **(E) (*E coli* infected group)** Intestine 30 days post-infection showing severe desquamation of epithelium (arrow). **(F) (E-coli infected group and challenged with probiotics)** Intestine 30 says post-treated showing severe desquamation of epithelium (arrow) and tissue debris filled the lumen (star), bar=100. H & E.

Ⅲ. Spleen:

Spleens of the control group showed normal architecture consists of white and red pulp (Fig 4A). At the end of the experiment, depletion in different areas of the spleen was seen in probiotics treated group (Fig 4B). Spleen showed a severe reduction in lymphoid cells of white pulp in the Salmonella-infected group (Fig 4C). In Salmonella-infected and probiotics supplemented group showed dilatation of blood sinusoids in red pulp was very obvious (Fig 4D). *E coli* infected group after 30 days from infection showed apoptosis and nuclear debris of lymphocytes in white pulps of the spleen was demonstrated in 75% of experimental chickens (Fig 4E). Apoptosis with nuclear debris of lymphocytes in white pulps associated with dilatation of blood sinusoids of red pulp in 100% of chickens on the 30th-day post infected with *E coli* and treated with probiotics (Fig 4F).

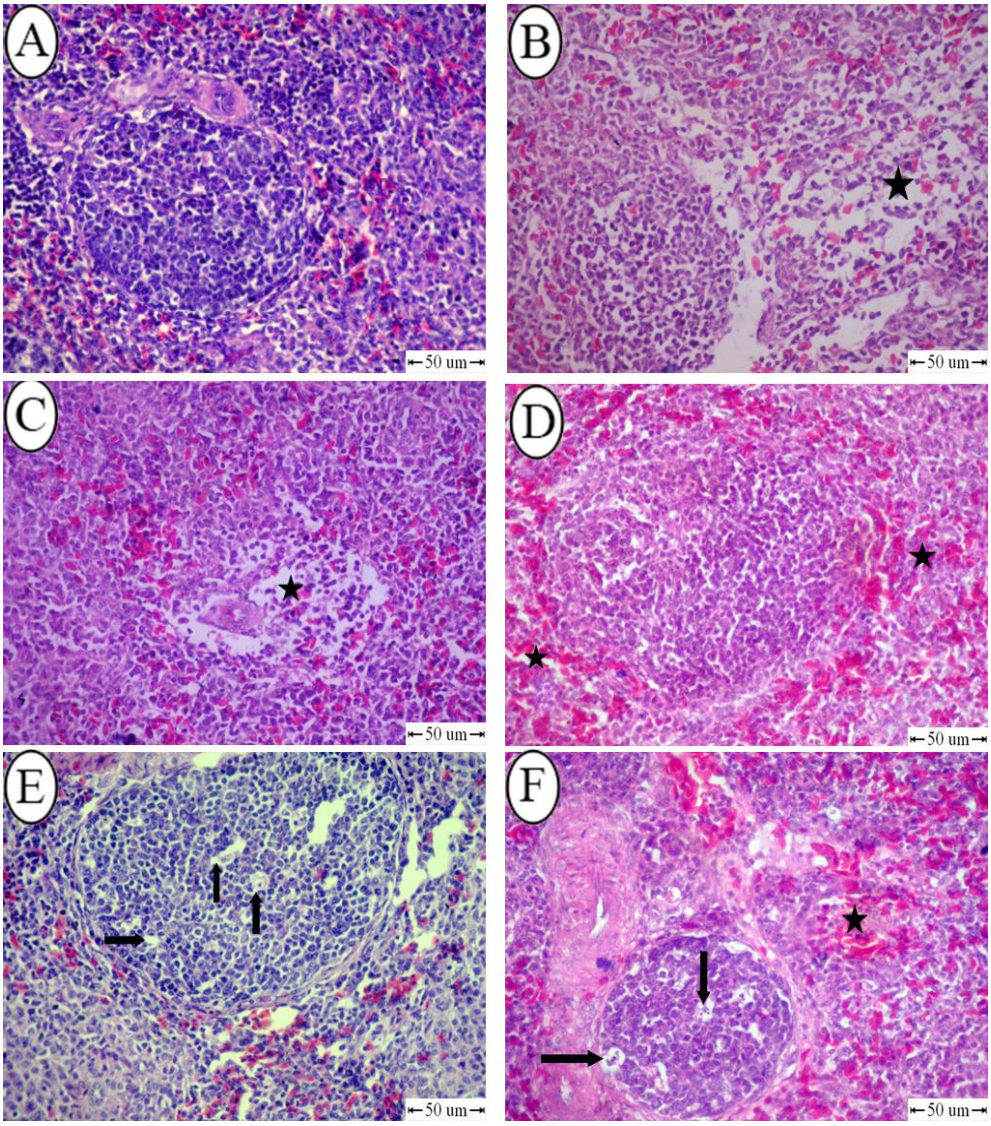

Fig. 4. Histopathological examination of the spleen in (A) Control group showing normal white and red pulp. **(B) The probiotic-treated group** was showing necrosis in red pulp (star). **(C): (Salmonella-infected group)** 30 days post-infection showing severe depletion in white pulp (star). **(D) (*Salmonella*-infected group and challenged with probiotics)** showing dilatation of blood sinusoids of red pulp (star). **(E (*E coli* infected group)**

30 days post-infection showing apoptosis with nuclear debris in white pulps (arrow). **(F) (*E coli* infected group and challenged with probiotics)** showing dilatation of blood sinusoids of red pulp (star), apoptotic lymphocytes with nuclear debris in white pulps (arrow), bar=50. H & E.

IV. Bursa of Fabricius:

Bursas of the control group showed bursal follicle consisting of cortex, medulla and follicular associated epithelium. Bursal follicles were covered with pseudostratified columnar surface epithelium and separated by inter-follicular connective tissue (Fig 5A). Probiotics treated group showed hyperplasia of epithelium, thickening of subepithelial connective tissue stroma with the formation of small cysts (Fig 5B). Slight depletion of lymphoid follicles with epithelial formation cysts was also observed in bursa of a Salmonella-infected group in all examined birds during the experiment (Fig 5C). Salmonella-infected and probiotics supplemented group showed moderate depletion of lymphoid cells in bursal follicles with the formation of epithelial cysts after 15 days which increased on reaching 30th day of the experiment and was associated with interstitial oedema (Fig 5D). Bursal follicles of *E coli* infected group showed severe depletion and lysis of lymphoid cells after 30 days from infection with the appearance of multiple epithelial cysts (Fig 5E). In *E coli* infected, and probiotics supplemented group, the changes of the bursa in birds sacrificed 15 and 30 days post-infection were minimal. They were expressed by congestion of blood vessels and subepithelial oedema in connective tissue stroma with the formation of the small number of epithelial cysts (Fig 5F).

Immunohistochemistry of (thymus, bursa and spleen):

Detection of CD3A positive cytoplasmic reaction in the thymus in different groups showed a significant decrease in group 1 in comparison to group 2. On comparing between group 3 and group 5, the quantification of CD3A positive cells displayed a significant increase in group 3. Also, the immunoreactivity increased slightly in the thymus of broiler chickens in group 4 when compared to group 6. Lymphoid follicles in bursa showed strong positive immunoreactivity of CD 79A positive cytoplasmic reaction in group 1 when compared to group 2, while, the response in other groups showed non-significant variation between them. The reactive CD 79A positive cells in the spleen of different groups showed non-significant variation between them (Table 5 and Fig 6).

Table (5): Mean number of CD3A positive cells in the thymus and CD79A positive cells in bursa of Fabricius and spleen in broiler chicken in all experimental groups.

Positive Cells	Group 1 (Control)	Group 2 (Probiotic)	Group 3 (*Salmonella*)	Group 5 (*Salmonella* + P)	Group 4 (*E Coli*)	Group 6 (*E Coli* + P)
CD3A in Thymus	112.6±13.55	48.2±9.73	96.4±11.10	77±5.47	61.8±4.43	34.4±8.11
P * Value	**0.043**		**0.042**		**0.043**	
CD79A in Bursa	78.6±3.97	65.6±9.81	83.8±6.64	53±4.96	21.6±3.64	13.6±2.51
P * Value	**0.043**		**0.059**		**0.068**	
CD 79A in Spleen	58.25±2.36	90.4±4.03	51.5±7.85	34.25±4.11	32.5±9.32	49±9.20
P * Value	**0.059**		**0.068**		**0.144**	

*P value by Mann-Whitney U Test

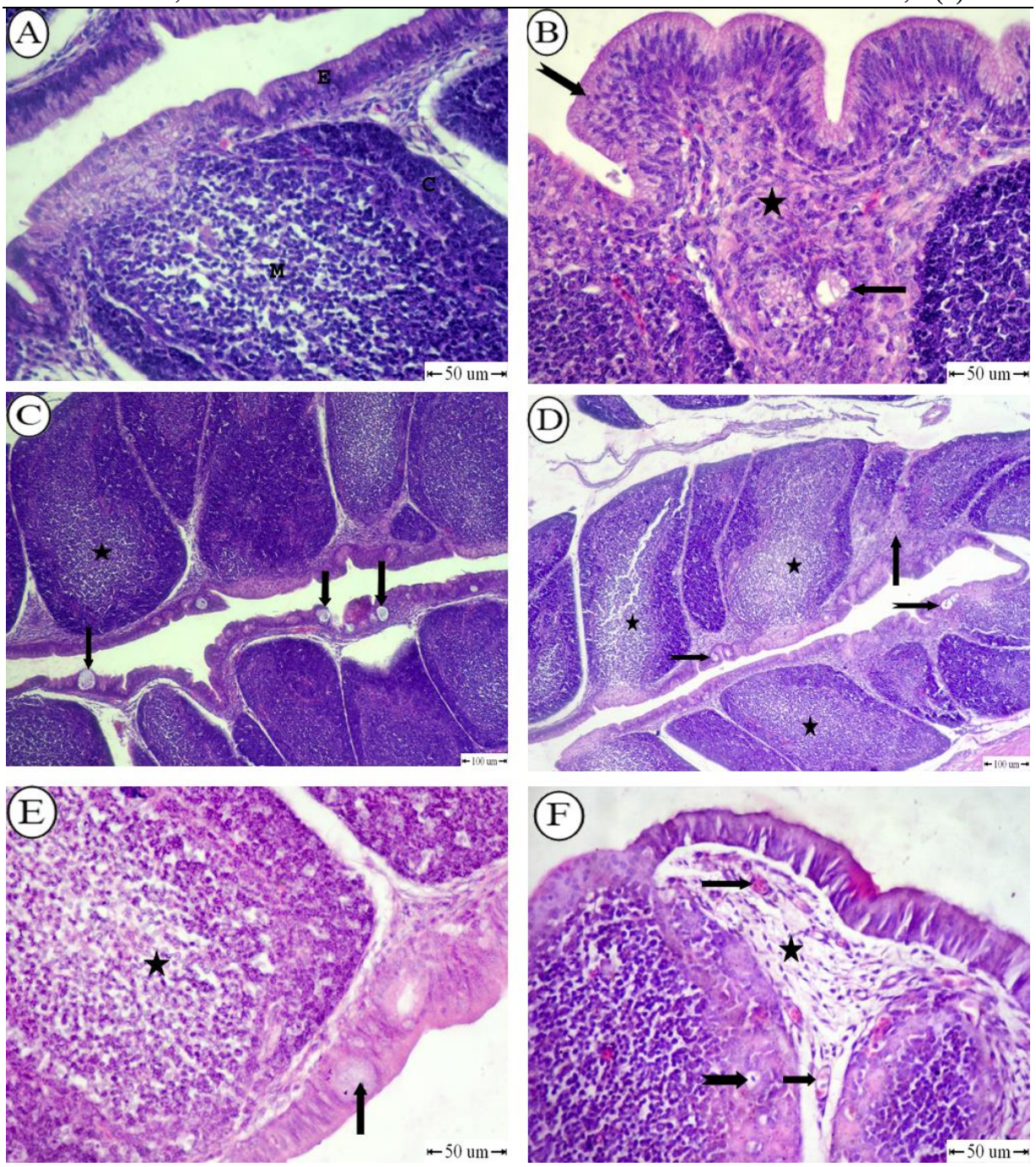

Fig. 5. Histopathological examination of bursa of Fabricius in (A) (control group) showing bursal follicles consist of the cortex of the follicle (C), medulla (M) of the follicle and inter-follicular surface epithelium. bar=50. **(B) Probiotic-treated group** showing hyperplasia of epithelium (notched arrow), thickening of subepithelial connective tissue stroma (star) and formation of small cysts(arrow), bar=50. **(C) (Salmonella-infected group)** Thirty days post-infection showing mild depletion of lymphoid follicles (star) formation epithelial cysts (arrow). bar=100. **(D) (*Salmonella*-infected group and challenged with probiotics)** Bursa was showing moderate depletion of lymphoid cells of bursal follicles (star) and formation of multiple small epithelial cysts (arrow), bar=100. **(E) (*E coli* infected group)** Thirty days post-infection showing severe depletion and lysis of bursal follicles (star) and presence of epithelial cysts (arrow), bar=50. **(F) (E-coli infected group and challenged with probiotics)** showing congestion of blood vessels (arrow) and subepithelial oedema in connective tissue stroma (star) with the formation of epithelial cysts (notched arrow), bar=50 H & E.

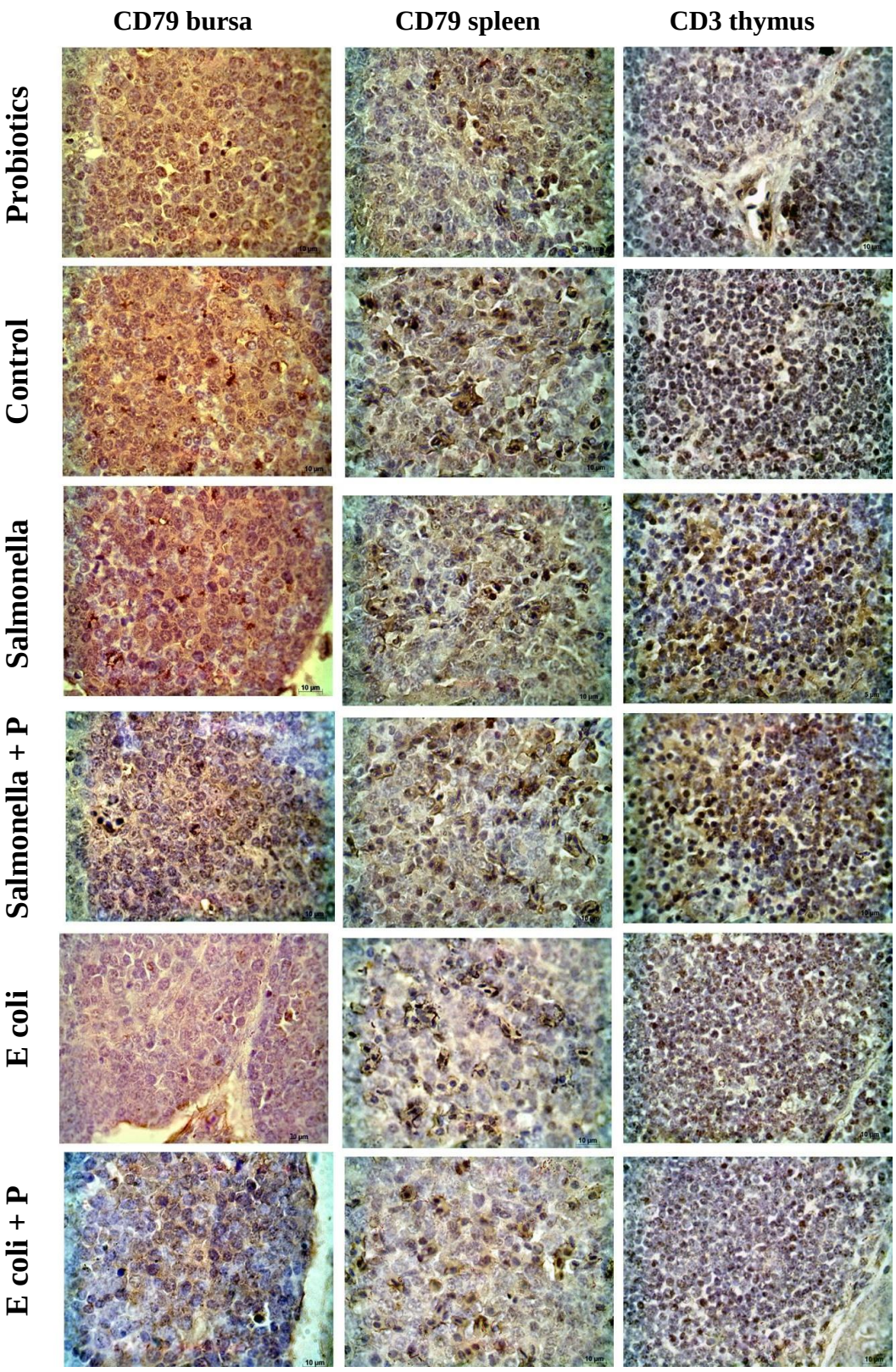

Fig. 6. Immuno-stained sections of broiler chickens in different groups showing CD 3 immune-positive cells in the thymus and CD 79 immune positive cells in lymphoid follicles of bursa of Fabricius and white pulp of spleen. Bar = 10.

Discussion

A maximum increase in the body weight was demonstrated in the group receiving probiotics in addition to the standard diet (group 2) compared to the control (group 1) and the challenged groups with or without probiotic supplementation. The group challenged with *Salmonella enteritidis* recorded the least weight gain among all experimental groups. The current improved weight gain in the probiotic-treated group is concurrent with the observation reported by Chiang and Hsieh (1995), Omprakash et al. (1996) and Owosibo et al. (2013). This maximum improvement of the weight gain in the probiotic-treated group might be due to maintaining healthy intestinal flora by competitive exclusion and antagonism, increasing digestive enzyme activities and promoting digestion rate of energy nutrient as it has been stated by Owosibo et al. (2013).

Similar to the results of Shibat El-hamd and Mohamed (2016), probiotic supplementation improved local intestinal immunity in broiler chickens and caused a decrease in the CFU of *Salmonella enteritidis* and *E coli (O2: H45)* challenged groups. The probiotics-containing Lactic acid produces an unfavorable pH for growth of *Salmonella* (Alkoms et al., 2000; Rolfe, 2000 and Johansen et al., 2004). Also, the decrease in CFU could be attributed to the competitive exclusion of lactobacilli to the enteric bacteria (Heres et al., 2003). Unlikely, Andino et al. (2014) mentioned that the probiotic did not afford protection from infection with *Salmonella* in an in vivo experiment in mice.

The data of the haematological parameters of broiler chickens' blood profiles were comparable. Treatment with probiotics had no significant effect on most measured blood values (RBCs, WBCs and Hb), a result that agrees with previous studies reported by Djouvinov et al. (2005), Alkhalf et al. (2010), Owosibo et al. (2013) and Abudabos et al. (2016). On the other hand, the differential leucocytic counts were significantly ($P<0.05$) influenced by dietary treatment with probiotics, where the probiotic-treated group (Group 2) showed a highly significant decrease in lymphocyte percentage. This result is consistent with those of Lillehoj and Chung (1992), Kamruzzaman et al. (2005) and Owosibo et al. (2013), but it disagrees with the findings of Shibat El-hamd and Mohamed (2016) who observed a significant increase of lymphocytes count in probiotic-treated broiler chicken. The constant intake of lactobacilli probiotics has been mentioned to induce a local immune-stimulant effect on the intestinal mucosa that attracts lymphocytes to the intestinal lamina propria causing a decrease in lymphocyte percentage in the blood (Lillehoj and Chung, 1992). The current highly significant reduction in lymphocyte percentage in challenged groups (3, 4, 5 and 6) compared to the control group simulates the findings of Kokosharov (2002) and Shibat El-hamd and Mohamed (2016).

The current study showed a highly significant ($P<0.05$) increase in granulocytes percentage in probiotic-supplemented and *E coli* + Probiotic treated groups when compared with control group. These results agree with that reported by Shibat El-hamd and Mohamed (2016) but disagree with the experimental model of Kokosharov (2002) who indicated that a peak level of myelocytes and young granulocytes production were enhanced from bone marrow one hour after a single injection of *salmonella gallinarum* endotoxin in mature birds because of its essential role as phagocytic cells.

The histopathological examination of a liver of broilers in the group supplemented with probiotics showed mild vacuolar degeneration of hepatocytes. The intestine, however, showed hyperplasia of the lining epithelium and abundance of goblet cells. These results partly simulate the findings of Ghalib et al. (2018) who mentioned that probiotic alone causes mild vacuolar degeneration when compared with the control group. In this concern, Caspary (1992) indicated that the increased height of the intestinal villi increases the intestinal surface area which could improve the absorption of available nutrients. Also, Langhout et al. (1999) and Shamoto and Yamauchi (2000) suggested that increasing villus height in the intestine may indicate an enhanced function of the intestinal villi. Moreover, Brahmankar et al. (2011) reported that an increase in the mucus production which may be due to increasing the activity of intestinal gland and he suggested that this enhances dietary absorption which may explain the apparently improved weight gain in the group supplemented with probiotics in the current study.

Although probiotic supplementation, in the present study, improved local intestinal immunity in broiler chickens and caused a decrease in the colony forming unit of *Salmonella enteritidis* and *E coli* (O2: H45) in challenged groups, these groups showed severe vacuolar degeneration of hepatocytes, congestion of splenic red pulp and mild depletion of bursal follicles. These findings may indicate that probiotics had only a local beneficial effect on the intestine and this effect did not extend to other organs in the body. This suggestion was supported by the current findings that the cecum of group supplemented with probiotics showed intact epithelium. In the same concern, it has been supposed that probiotics reduce intestinal colonization of *salmonella* by competing for iron (Deriu et al., 2013).

The current immunohistochemical findings on the surface antigens (CD3A) in the thymus and CD79A in the bursa and spleen indicated that probiotics seem to have no effect on activating the two immune organs. However, Andino et al. (2014) believe that probiotics could ameliorate immune response but enough time between probiotic administration and *Salmonella* infection may be crucial to allow the immune system to enhance protection against disease.

Conclusions

Probiotics obviously improved the growth performance and local immune response in the intestine, however there is no clear evidence of improving general immune status of the experimental birds.

Conflict of interest statement

The authors declare that they have no conflict of interest.

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