EFFECT OF *MYCOPLASMA GALLISEPTICUM* INFECTION ON ND VACCINE IN BROILER CHICKENS UNDER VARIOUS TREATMENTS

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ABSTRACT

This study aimed to investigate the effect of *Mycoplasma gallisepticum* infection on Immune organs and Immune response of ND vaccine in broiler chickens .210 day- old Broiler chicks were randomly assigned to seven equal groups as following G1: control was not received any treatment, G2: only *M. gallisepticum* infection, G3: only ND vaccine, G4 ND vaccine and *M. gallisepticum* infection , G5: ND vaccine and *M. gallisepticum* infection and treated with probiotic G6: ND vaccine and *M. gallisepticum* infection and treated with Glycyrrhizic Acid by drinking water, G7 : ND vaccine and *M. gallisepticum* infection and treated with tylosin by drinking water. Blood samples were collected at 1st, 5th, 10th, 16th, 25th, 27th and 35th day for immunological tests and histopathological examination of spleen and bursa of fabricious at the result revealed the highest level of the ND antibody titers in G5, G6, G7,G1 and G3 respectively. Histopathological analysis of the spleen organ revealed necrosis in G4, white pulp hyperplasia in G6 and G7 while the lesions of bursa indicate that severe hyperplasia in *M. gallisepticum* infection group. The study concluded that probiotic and glycyrrhizic acid reduce effect of *M. gallisepticum* infection on immune response and immune organs.

Keywords: Poultry, vaccine, molicutes, bursa of fabrecious, herbal
***Part of M.Sc. Thesis of the 1st author**

تأثير *Mycoplasma gallisepticum* في لقاح مرض نيوكاسل في فروج اللحم باستعمال معاملات مختلفة

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المستخلص

هدفت هذه الدراسة إلى بيان تأثير عدوى الميكوبلازما غاليسبتيكوم MG في الأعضاء المناعية والاستجابة المناعية للقاح الكثافة النويسية في فروج اللحم. تم توزيع 210 من افراخ فروج اللحم البالغة من العمر يوما عشوائيا على سبع مجموعات متساوية على النحو التالي :G1 مجموعة سيطرة لم يتم تلقي أي علاج، G2عدوى MG فقط، :G3 لقاح ND فقط، لقاحG4 ND وعدوى MG، :G5 لقاح NDوعدوى MG ومعالجتها بالمعزز الحيوي :G6 ND وعدوى MG ومعالجتها بحامض الكلسريريزيك عن طريق مياه الشرب ، :G7 لقاح ND وعدوى MG ومعالجتها بالتايلوسين بمياه الشرب. تم جمع عينات الدم في اليوم 1 و 5 و 10 و 16 و 25 و 27 و 35 للفحوصات المناعية الفحص النسيجي المرضي للطحال والجراب في النتيجة كشفت عن أعلى مستوى من عيار الأجسام المضادة ND في G5 و G6 و G7 و G1 و G3 على التوالي. كشف التحليل النسيجي المرضي لعضو الطحال عن نخر في G4 ، تضخم اللب الأبيض في G6 و G7 بينما تشير آفات الجراب إلى تضخم شديد في مجموعة عدوى MG. يمكن الاستنتاج أن المعزز الحيوي وحامض الكلسريريزيك يقلل من تأثير عدوى MG في الاستجابة المناعية والجهاز المناعي.

الكلمات المفتاحية: دواجن ، لقاح ، الرخويات ، جراب فابريشيا ,اعشاب.
جزء من رسالة ماجستير للباحث الاول.

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INTRODUCTION

Mycoplasma gallisepticum infection is a prominent poultry disease that has caused serious economic losses for the poultry industry (38), decreasing the quality of the carcass and placing sick birds at risk for new infections (14). Then the pathogen was classified as a pleuropneumonia-like organism (PPLO) and called *M. gallisepticum* (33). *Mycoplasma gallisepticum* virulence is associated with ability to change surface features and escape from the immune system (7, 20, 16). *Mycoplasma gallisepticum* varied lipoprotein haemagglutinin is essential for adhesion and antigenic diversity formation (8). Mycoplasma pathogenicity appears to be dependent largely on ciliary function (18). Newcastle disease virus (NDV) frequently associated with respiratory diseases, and mycoplasmal co- infection may increase the signs and symptoms (25). *Mycoplasma gallisepticum* mostly infects the respiratory system, it also infects the reproductive system, brain, and eyes (19, 29)**.** Mycoplasma infected broiler chicks showed depression, acute conjunctivitis, ruffled feathers, sneezing, coughing, and dyspnea (3, 32). *Mycoplasma gallisepticum* can cause immunosuppressive impacts by and the immune system damage and impairment with development B and T cells, and chicken immune system (11). Other study reported that the chickens vaccinated with *M. gallisepticum* and ND vaccines produced weak protection, but better than chickens non-vaccinated with ND because of ND vaccine can cause immunosuppression (6, 13). Other finding supports the negative correlation between inactivated ND vaccine and *M. gallisepticum* vaccines, when vaccinating with the ND vaccine only, the mortality in birds were low, no pathologica lesions or clinical signs (12, 26). Glycyrrhizic acid (GA) is used to treat many respiratory diseases. Glycyrrhizic acid increased resistance to *M. gallisepticum* infection through inhibited inflammatory factors (36). Probiotics as *Lactobacillus salivarius* are commonly used to increase the growth and resistance of the diseases in chickens, probiotics were high availability and low (34). *Bacillus subtilis* reduced lung injury by decreasing *M. gallisepticum* colonization, and reduced from pro-inflammatory cytokines production (9).LISA kits are commercially available, performed, and used as research serologic tests and screening tests for *Mycoplasma gallisepticum* from various samples, such as egg yolks, respiratory secretions, and serum (4, 5, 37). This study aimed to focusing on the study of the activities of the Newcastle vaccine in *Mycoplasma gallisepticum* infection of broilers by estimating the antibodies by ELISA kits, as well as, this study focused to identify the roles of probiotics, antibiotics, and glycyrrhizic acid with ND vaccine combinations in the enhance the immune response and infection control.

MATERIALS AND METHODS

Experimental animals: The present study was conducted on Broiler chicks (No. 210) of the breed (Ross 308 Broiler chickens) from the Al-Halafawi hatchery- Babil Al-Mahawil were used in this experiment and 35 days was duration of this experimental study.

The experimental groups of the study include:

Group1 (CN1): control negative group was not received any treatment.

Group2 (*M. gallisepticum*): Control Positive A included 30 chicks were infected by *M. gallisepticum* strain at dose 0.2 ml of 106 CFU of *M. gallisepticum* source by intra tracheal route at 5^{th} day.

Group3 (CN2-NDV): Control positive B included 30 chicks were vaccinated with ND vaccine (Nobilis®, live vaccine) at dose 6.0 $\log^{11}$ EID50 by eye drop route at 1^{st} day.

Group4 (*M. gallisepticum* -NDV): 30 chicks were received ND vaccine at dose 6.0 $\log^{11}$ EID50 by eye drop route at 1^{st} day with *M. gallisepticum* infection at dose 0.2 ml from 10^6 CFU at 5^{th} day.

Group5 (*M. gallisepticum* -NDV-Pro.): 30 chicks were received ND vaccine at dose 6.0 $\log^{11}$ EID50 by eye drop route at 1^{st} day and *M. gallisepticum* infection at dose 0.2 ml from 10^6 CFU at 5^{th} day with probiotic (Poultry star®) at dose $2*10^{11}$ CFU / kg from 5^{th}-15^{th} day.

Group6 (*M. gallisepticum* -NDV-GA): 30 chicks were received ND vaccine at dose 6.0 $\log^{11}$ EID50 by eye drop route at 1^{st} day and *M. gallisepticum* infection at dose 0.2 ml from 10^6 CFU at 5^{th} day with Glycyrrhizic Acid

(CG-Herbs®) at dose 1 ml/ L orally by drinking water from 5^{th}-15^{th} day.

Group7 (*M. gallisepticum* -NDV-TYLO): 30 chicks were received ND vaccine at dose 6.0 log^{11} EID50 by eye drop route at 1^{st} day and *M. gallisepticum* infection at dose 0.2 ml from 10^6 CFU at 5^{th} day with tylosin (Tylodad ®) at dose 1 gm/ 2 litter of D.W- orally by drinking water from 5^{th}-15^{th} day. Blood samples were collected from wing vein of all groups at 5^{th}, 16^{th}, and 26^{th} day old for immunological tests.

Histopathological examination: At 35 day histological examination of organs (bursa of fabricius and spleen) from chickens of all groups were placed in formalin 10% for fixation, then the samples were prepared for histological examination by passing them with different concentration of ethyl alcohol, xylene and paraffin. they worked them waxy molds and cut them with a shredder of five microns and fixed on glass slides and dyed Hematoxylin and Eosin (23).

Immunological Tests: Blood samples left to clot at room temperature. This normally takes between 10 and 20 minutes. After centrifuging the samples for 20 minutes at 3000 rpm to obtain the serum, the serum stored the serum samples at -20 °C until utilized them for commercial ELISA kits testing. The specific antibody titer of ND were measured by ELISA kits and carried out according to the manufacturer's assay protocols (SunLong Biotech, China). This study evaluated Newcastle disease (ND) antibody titers in the seven chicken groups at the 5^{th}, 16^{th}, and 26^{th} days from the experiment for estimating the development of antibody titers through the experiment.

Statistical analysis: Data analysis was carried out statistically using SPSS (Statistical Analysis System - version 20). The one-way ANOVA and Least Significant Differences (LSD) post hoc test were performed to evaluate if there were statistically significant differences between the means. $P \leq 0.05$ was used to determine statistical significance.

RESULTS AND DISCUSSION

At 5^{th} and 16^{th} days from experiment, the results not recorded any significant differences in the ND antibody titers of the experimental groups. While at 26th day group G3 (Control negative with ND vaccine) recorded highest level of the ND antibody titers, also group 5 (*M. gallisepticum* infection with ND vaccine and probiotic), 6 (*M. gallisepticum* infection with ND vaccine and Glycyrrhizic acid), and 7 (*M. gallisepticum* infection with ND vaccine and Tylosin) were exhibited a significantly moderate ND titer levels between group 1 (Control negative) and group 3 (Control negative with ND vaccine) and (Table 1).

Table 1. Comparisons of ND antibody titer among different chicken groups at 5, 16^{th}, and 26^{th} day of the experimental study.

Group	5^{th} Day	16^{th} Day	26^{th} Day
G1 (CN1)	10818.5± 1885.09A	1987.5 ± 591.02 A	323 ± 126.6 C
G2 (*M. gallisepticum*)	11190 ± 581.2 A	1832.3 ± 305.3 A	206.25 ± 39.2 C
G3 (CN2-NDV)	11668.5 ± 459.5 A	2875.25 ± 666.3 A	6963 ± 1417.7 A
G4 (*M. gallisepticum* -NDV)	12265 ± 948.04 A	2287 ± 674.6 A	3202.2 ± 1201.8BC
G5(*M. gallisepticum* -NDV-Pro.)	13007.5 ± 1480.6 A	2077.25 ± 266.01A	5605.5 ± 1070.8AB
G6 (*M. gallisepticum* -NDV-GA)	12056.2 ± 2252.2 A	2327 ± 287.8 A	4357.5± 1756 AB
G7(*M. gallisepticum* -NDV-TYLO)	9558.75 ± 2353.2 A	2675.75 ± 934.6 A	3645.75 ± 230.8AB
LSD	3449	1043	3317.25

The differences in uppercase letters vertically indicates on significant differences at $P \leq 0.05$.

The results showed a drop in antibody titers in the end of the experiment groups of control negative at 26^{th} day of experiment (G1: 323 ± 126.6) and *M. gallisepticum* infection (G2: 206.25 ± 39.2) in chickens. While the results indicated that treatment groups with Tylosin and especially with probiotic and Glycyrrhizin acid maintained the highest levels of ND antibody titers and immune response across the experiment periods. The group treated with probiotic maintained the level of the antibodies of ND until 26^{th} day (G5 5605.5 ± 1070.8). Probiotic as *Bacillus subtilis* is contributed in the maintain the immunity and prevent the severity of the respiratory infection of the chickens (39), and increases the resistance against the *M. gallisepticum* infection (35). The results were discussed the positive role Glycyrrhizic acid in the body against the *M. gallisepticum* infection in the group G6, and maintain the level of antibodies of ND at 26^{th} day (G6: 4357.5± 1756). The role of the probiotic in the *M. gallisepticum* infection through in the decrease the lung injury by

reducing *M. gallisepticum* colonization and pro-inflammatory cytokines and improving the role of microbiota in the *M. gallisepticum* infection (9). Glycyrrhizic acid have pharmacological properties as hepatoprotective, anti-asthmatic, antioxidative, immunoregulatory effects, antimicrobial and anti-inflammatory (24). Glycyrrhizic acid is used to treat many respiratory diseases, GA in case *M. gallisepticum* infection inhibited inflammatory factors, and treated lung damage (36). The importance use of these supplements with the *M. gallisepticum* infection, because of *M. gallisepticum* can cause immunosuppressive impacts as a result of immune system damage and impairment of development B and T cells, and chicken immune system (11). The study reported that the chickens infected with *M. gallisepticum* and vaccinated with ND vaccine in the groups (G4-G7) produced protection, better than chickens non-vaccinated with ND in the groups (G1 and G2), although considered weak response when compared to the group was received ND vaccine without infection (G3), this result corresponding with Awad et al., (6). Other finding supports the negative correlation between inactivated ND vaccine and *M. gallisepticum*, when vaccinating with the ND vaccine only, the mortality in birds were low, no PM lesions or clinical signs, decrease virus shedding (12). When chickens infected with ND virus and *Mycoplasma*, showed increase ND virus shedding and significant decrease in interferon and immune responses (27). The results showed that the immunoglobulin titer to the ND was elevated 5^{th}, and declined at 16^{th}, then moderate increase at 26^{th} day, the ND vaccine at 5th day activated high level of early antibody production, primarily IgM. This initial response to the vaccine antigen by IgM response was short-lived and deceased at 16th day, the immunity shifts from an IgM response to IgG response, which provides longer-lasting immune response around 26th day from experiment. it generally indicates a normal immune response to ND vaccination (28). Histopathological analysis of the lesion of the bursa of fabricius and spleen because of these organs related to the immune system to these organs in chickens. The G2 group (*M. gallisepticum* infection) showed that spleen tissue affected by necrosis, severe hemorrhage and depleted lymphoid follicles, the bursa in group two chicks reflected tissue lesions that were hyperplasia, lymphocytic and macrophage infiltration, epithelial vacuolation, and thickened of submucosa (Figure 1).

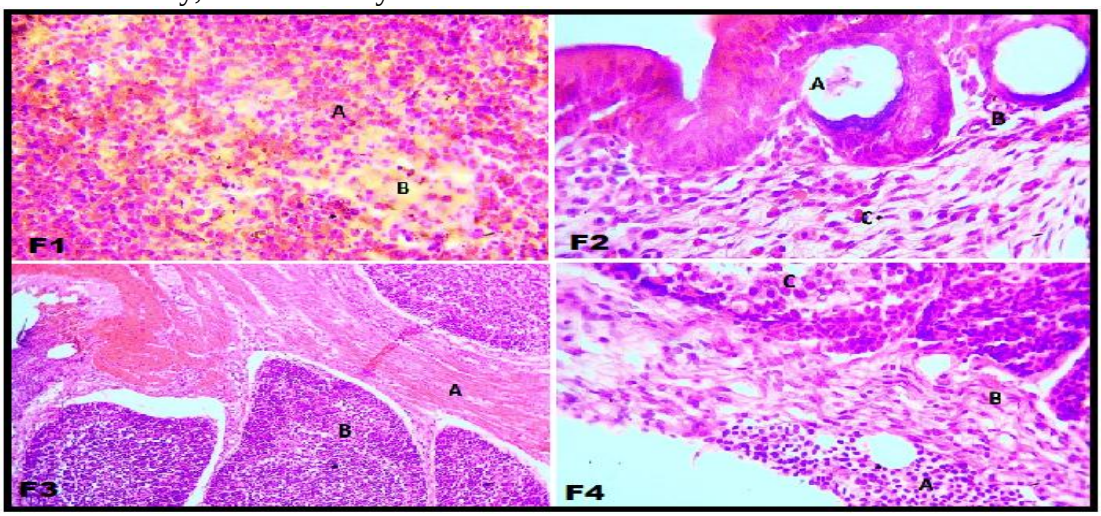

Figure 1. Histopathological findings of the G2 chicks group (*M. gallisepticum* infection); F1: micrograph of the spleen characterized by depleted white pulp with necrotic red pulp (A), and oedema (B), 200× magnification. F2: micrograph of the bursa characterized by hyperplasia of mucosa with large vacuoles (A), apoptotic cells (B), and thick submucosa with mononuclear cells and eosinophiles infiltration (C), 200× magnification. F3: micrograph of the bursa characterized by increase fibromuscular layer with mononuclear cells infiltration (A), and hemorrhage in follicles (B), 400× magnification. F4: micrograph of the bursa characterized by sever lymphocytic infiltration in mucosa (A), mononuclear cells infiltration mostly macrophage in submucosal (B), and depleted and necrotic follicles (C), 400× magnification.

Histopathological analysis of organ tissues of G3 group (Control negative with ND vaccine) showed numerous lesions inside bursa of fabricius as follows: lymphocyte infiltration in

mucosa, in the bursa, hyperplasia of lymphoid follicles, and epithelial vacuolation (Figure 2). In the G4 group (*M. gallisepticum* infection with ND vaccine), the spleen sections revealed into necrosis of white/red pulp with hemorrhage, and amyloid-like infiltration. The bursa sections included increase in the epithelial thickening, and lymphoid depletion (Figure 3). Histopathological findings of bursa revealed that variable-sized vacuoles with hyperplasia of mucosa of bursa of the chicks (Figure 4). According to the histopathological changes of the bursa of fabricius and spleen specimens of the chicks in G6 group (*M. gallisepticum* infection with ND vaccine and Glycyrrhizic acid) hyperplasia of white pulp were noticeable in the spleen and variable epithelial vacuoles in the bursa (Figure 5). Histopathological changes of bursa of fabricius of G7 group (*M. gallisepticum* infection with ND vaccine and Glycyrrhizic acid) were revealed the presence lymphoid follicle hyperplasia in the bursa sections (Figure 6).

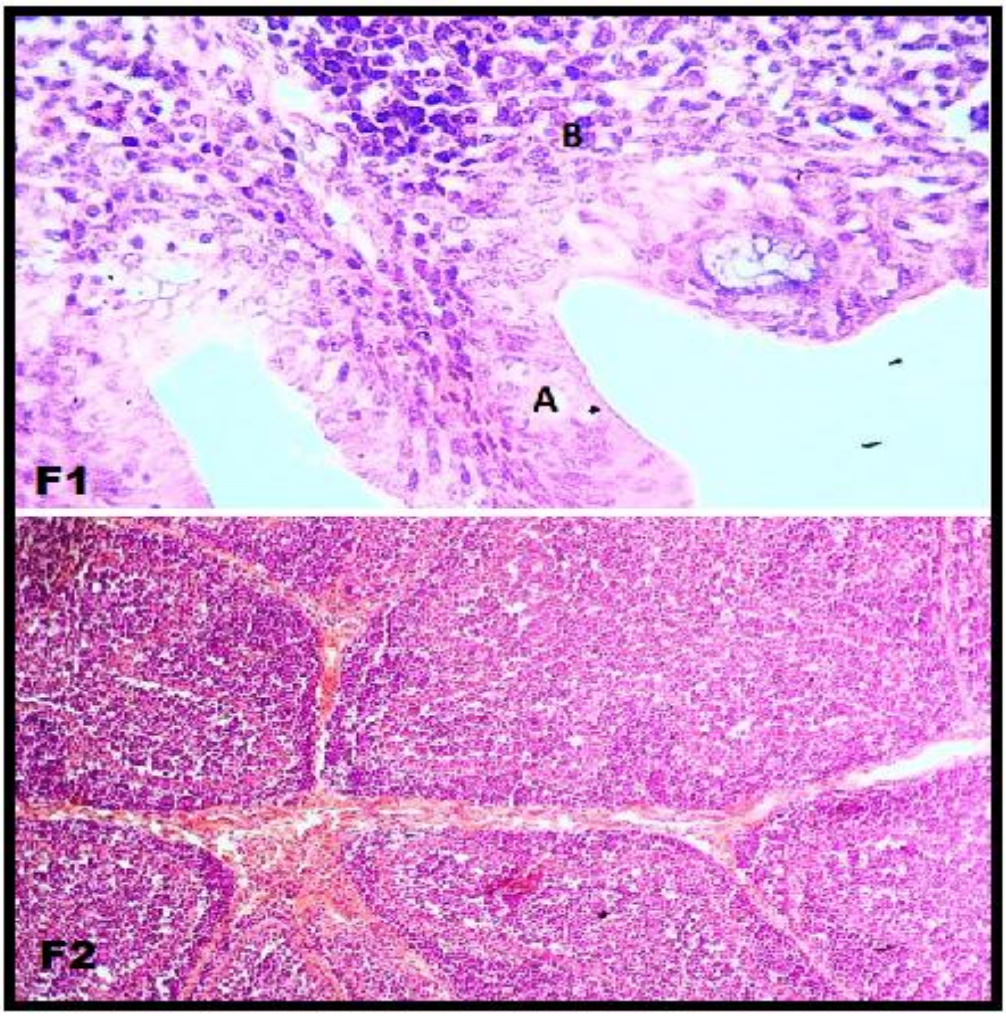

Figure 2. Histopathological findings of the G3 chicks group (Control negative with ND vaccine); F1: micrograph of the bursa characterized by epithelia layer contain vacuoles with mucin (A), infiltration of lymphocyte in the subepithelial layer (B), 400× magnification. F2: micrograph of the bursa characterized by hyperplasia of lymphoid follicle, 100× magnification.

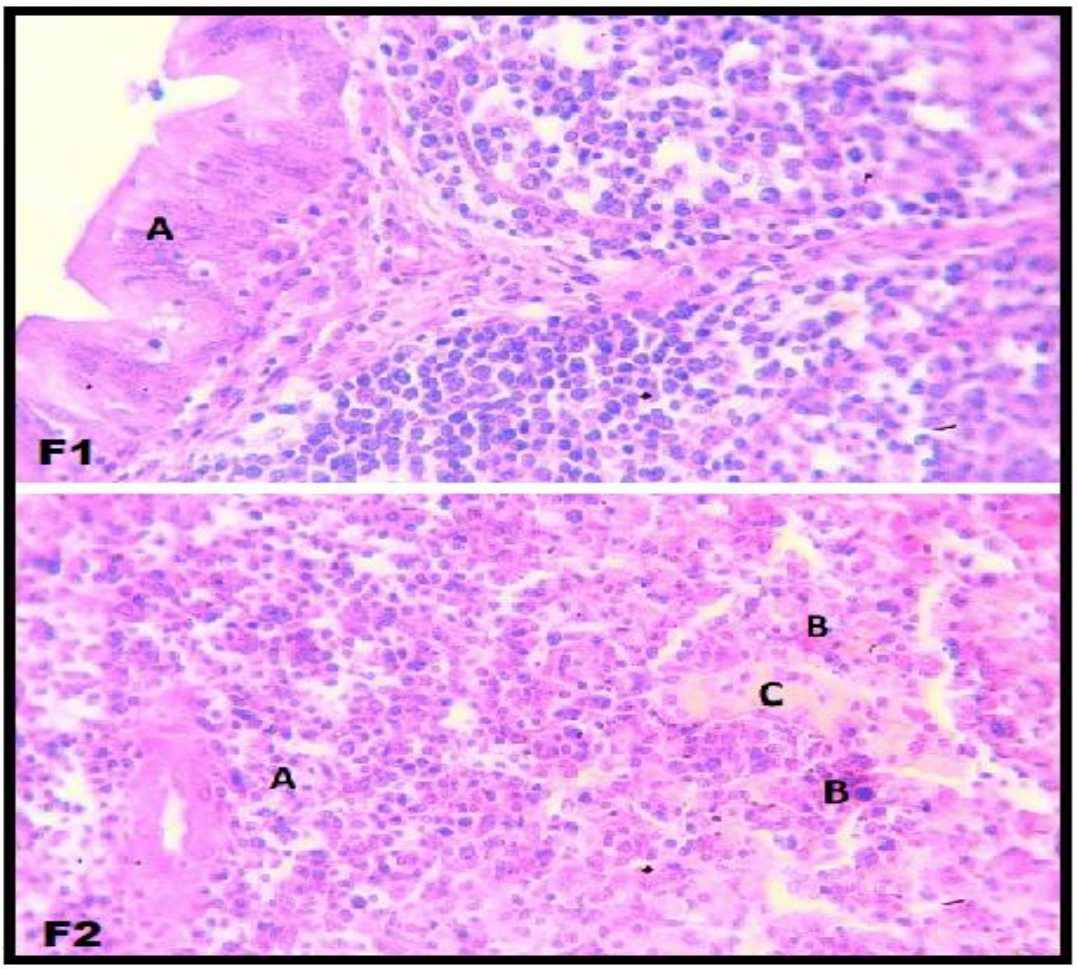

Figure 3. Histopathological findings of the G4 chicks' group (*M. gallisepticum* infection with ND vaccine); F1: micrograph of the bursa characterized by increase in the epithelium thickening with and variable and multiple vacuoles (A), atrophied and depleted lymphoid follicle (B), 400× magnification. F2: micrograph of the spleen characterized by depletion and necrotic of white and red pulp with lymphocytes (A), interstitial hemorrhage (B), and eosinophilic, amorphous, acellular substances (amyloid) like infiltration (Baccon spleen) (C), 200× magnification.

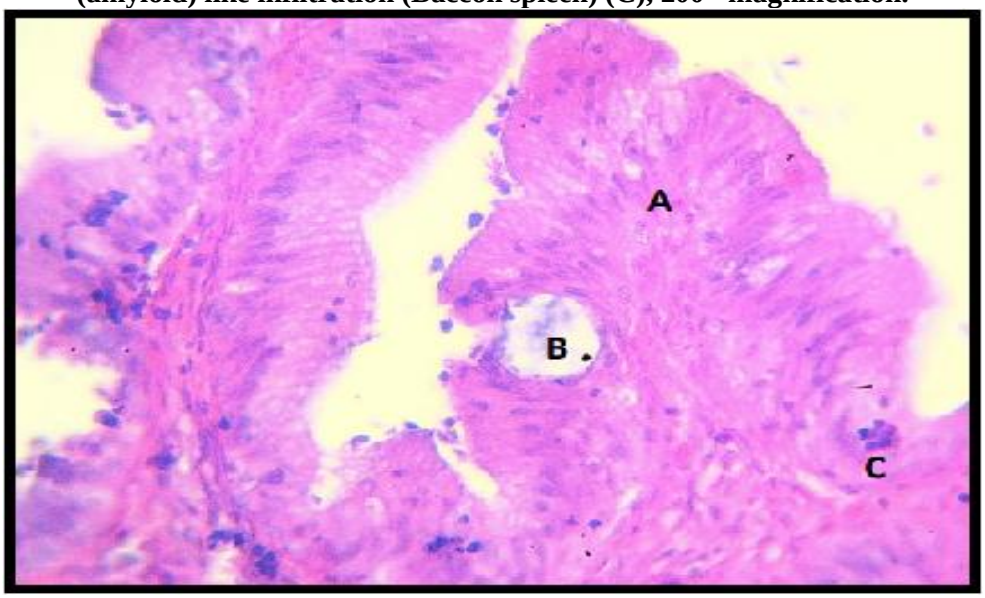

Figure 4. Histopathological findings of the G5 chicks' group (*M. gallisepticum* infection with ND vaccine and probiotic); micrograph of the bursa characterized by hyperplasia of mucosal layer as papillae (A), variable size of vacuoles with mucin in the epithelial layer (B), and aggregation of mononuclear cells (C), 400× magnification.

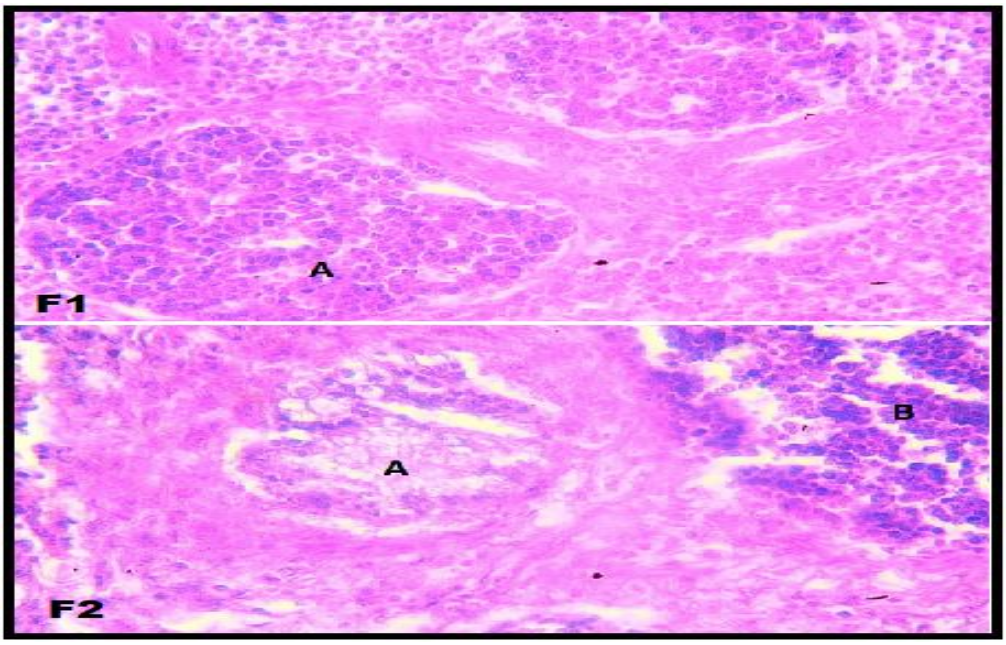

Figure 5. Histopathological findings of the G6 chicks' group (*M. gallisepticum* infection with ND vaccine and Glycyrrhizic acid); F1: micrograph of the spleen characterized by hyperplasia of white pulp (A), 400× magnification. F2: micrograph of the bursa characterized by hyperplasia of mucosal layer with variable vacuoles (A), hyperplasia of lymphoid follicle (B), 400× magnification.

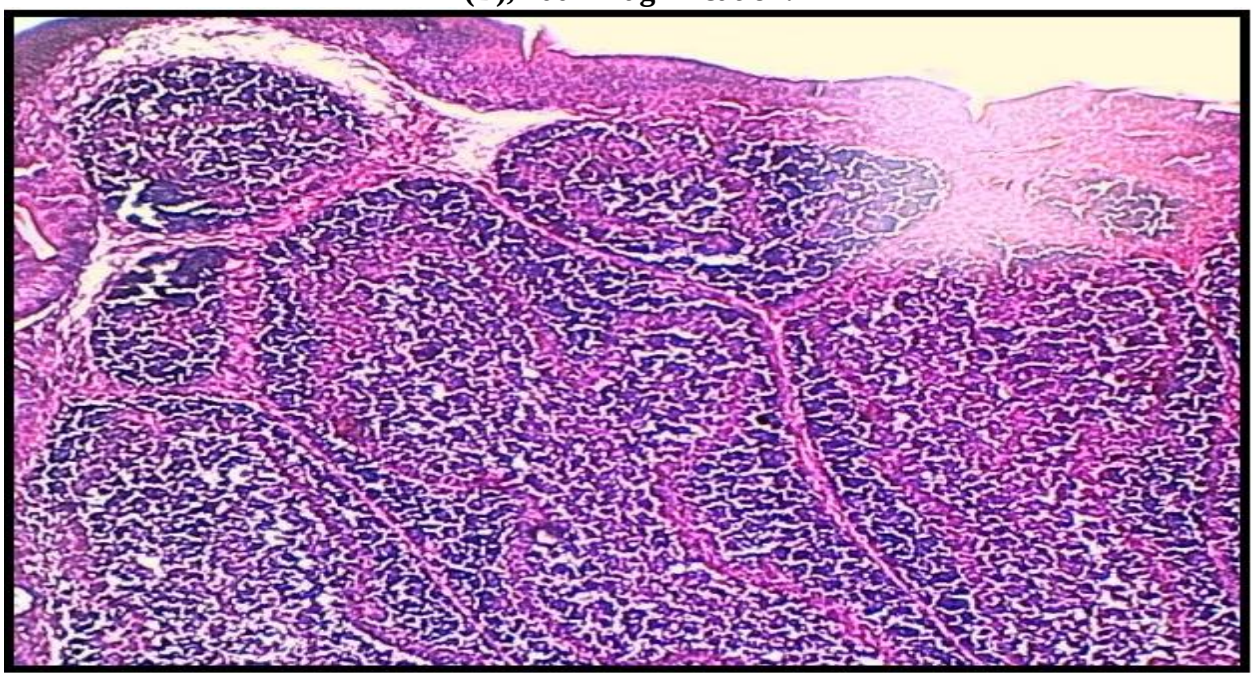

Figure 6. Histopathological findings of the G7 chicks' group (*M. gallisepticum* infection with ND vaccine and Glycyrrhizic acid); micrograph of the bursa characterized by hyperplasia of mucosal layer (A), hyperplasia of lymphoid follicle (B), 400× magnification.

Histopathological analysis of the spleen organ revealed that main lesion necrosis in *M. gallisepticum* -NDV, white pulp hyperplasia in *M. gallisepticum* -NDV-GA and *M. gallisepticum* -NDV-TYLO, severe lymphoid depletion in *M. gallisepticum* infection only group. The infection often causes depletion of lymphoid cells in the white pulp of the spleen, indicating immune suppression, the hyperplasia of lymphoid follicles is observed due to attempt of the immune system to respond to the *M. gallisepticum* infection, while in the severe *M. gallisepticum* infections may lead to necrotic foci in the spleen parenchyma because of extensive tissue inflammation and damage (21).

The histopathological lesions of bursa indicate that severe hyperplasia in *M. gallisepticum*

infection group, and mild hyperplasia in CN2-NDV, and presence of epithelial thickening and atrophied with depleted lymphoid follicle in *M. gallisepticum* -NDV group. *M. gallisepticum* infection often causes atrophy and depletion of lymphoid follicle in the bursal, and leading to compromised immunity. The impact of Newcastle disease vaccine on histological changes is temporary histological in various organs of broiler chickens due to host's immune and resolve quickly, the effects of vaccine include, mild inflammatory cell infiltration, transient epithelial desquamation of trachea, focal interstitial pneumonia, transient lymphoid hyperplasia of bursa, and mild hepatocyte swelling (17, 31, 30). Many studies occurred in Iraq on the *Mycoplasma gallisepticum* infection in broilers (2, 27, 15), and important role of the Newcastle vaccine or feed additives on the production stage of hens (1, 22), because of control on the infectious diseases is one important reason to enhance poultry production (10, 40), further studies on this pathogen are required.

In conclusion, the probiotic and Glycyrrhizic acid main role is the preventing the *M. gallisepticum* infection and decline the damage which caused by *M. gallisepticum* infection. The vaccine of ND had negative role *M. gallisepticum* infection and could decrease immune responses for infection.

CONFLICT OF INTEREST

The authors declare that they have no conflicts of interest.

DECLARATION OF FUND

The authors declare that they have not received a fund.

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