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Studies on effect of prebiotic on immune response of broiler chicken to ND -AI combined inactivated vaccine

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Abstract : Effect of prebiotic on immune response to companied inactivated ND-AI vaccine in presence of bacterial infection were studied, 160 day old Cobb broiler chicks were divided into 4 equal groups; 40 chicks in each. Group 1 negative control non vaccinated while group 2 received lysozyme while groups 3 received Betaine, finally group 4 were kept as positive vaccinated control positive group. Chickens group were vaccinated subcutaneously with the recommended dose of inactivated ND-AI combined vaccine. *E.coli* O78 K80 H11 strains in phosphate buffered saline (PBS) was used as a bacterial challenge strain and was used by oral infection, each chick was given 0.5ml containing 1 x 10^4 viable microorganism/ml. Blood samples were collected weekly for haemagllutination inhibition (HI) test , bursa and liver were collected for histopathological examination.

Results of HI test against ND revealed that best mean antibody titer was 6.14±0.69 in birds received Betaine (gr 3), followed by 6.00 ±0.95 in those received lysozyme (gr 2) , followed by that of group 4 (positive vaccinated group infected with E.coli) which showed 4.00±1.89 , then finally 4.00 ±0.58 in negative control group. Results of AI haemagllutination inhibition (HI) antibodies was the best in group 2 that received betaine which was (4.86±0.69) followed by 4.71± 0.95 in lysozyme (gr. 2) , followed by group (4) positive vaccinated group which was 4.43± 0.54 and finally 3.43±0.79 in the negative control group1. Also it was noticed that group (4) vaccinated infected with *E.coli* control positive showing clinical signs of E.coli infection in the form of diarrhea and subcutaneous inflammation (cellulitis) mortality rate was 30%.

Concerning histopathological findings the examined bursa, and liver stained sections of control negative group show no detectable pathological lesions until the end of the experiment, on the other hand it was found that Bursa of Fibricus of group (3) which received Betain showing moderate hyperplasic activity of the lymphoid follicle which later on become more obvious by the end of the experiment, concerning liver sections group (4) infected vaccinated non treated group showed severe hydropic degeneration other field showed severe congestion of the central vein while groups (2) and (3) were less affected as they showed mild congestion of portal vein.

In conclusion building immune foundation against respiratory virus is crucial and can be fulfilled by proper vaccination programme and can be enhanced by prebiotics which could improve antibody titers of inactivated respiratory vaccine , unfortunately this need further investigations specially of studding aspects of celluler immune response with used prebiotics.

Key words: broiler vaccination, prebiotic, *E.coli*, compined inactivated AI and ND, lysozyme, betaine.

Introduction

Building immune foundation against most important viral poultry disease such as Newcastle disease (ND) and avian influenza (AI) viruses is of great value in modern poultry production. ND virus is one of respiratory viral poultry diseases that causes severe economic losses in poultry industry worldwide [1], the disease causing high mortality and morbidity rate and affecting poultry performance[2], also avian influenza virus causing severe economic losses including most prominent subtypes low pathogenic avian influenza strain (LPAI) H9N2 strain or highly pathogenic avian influenza strain (HPAI) H5N1[3] and represent threat to public health [4]. prevention of this respiratory virus disease by main two ways, strict biosecurity together with proper vaccination schadual. Improvement of immune response against viral respiratory vaacine is of great value specially under our field condition which poultry farms under continues challenge. Nowadays prebiotics known to have beneficial outcome in poultry industry either by improving performance[5] or improves immune response against newcastle virus vaccines[6,7,8] together with influenza vaccine[9,10,11]. One of widely used prebiotics nowadays is Betaine as it was found that at an appropriate dose of betaine may spare some quantity of dl-methionine and dietary energy to support the growth process of heat-stressed broilers resulting in improve live weight and feed intake[12] , more over [13] noticed that diatry supplementation of Betaine resulted in significant higher Lymphocytes (L) count in Domyati Duckling, similar results was found by [14] who noticed that betaine containing diet stimulate lymphocyte proliferation in broilers chickens also Betaine supplementation substantially increased antibody titre against influenza virus vaccine together with increase bursa of fabricus weight [15], which considered betaine promoting immune system of broiler chickens. It is postulated that reduction in the concentration of dietary methionine affect immunity in chickens therefore, sparing dietary methionine with betaine may influence the immune responses[14]**.** Not only betaine prebiotic has a great role in poultry industry but also lysozymes were supported by many researchers puplications. Lysozyme is a 1,4-β-acetylmuramidase that hydrolyzes the glycosidic bond between *N*-acetylmuramic acid and *N*-acetylglucosamine [16]. Hydrolysis products include murimyl dipeptide, a potent adjuvant capable of enhancing immunoglobulin A (IgA) secretion, macrophage activation and rapid clearance of bacterial pathogens in vivo[17]. Moreover lysozyme is also capable of binding to the lipid A portion of bacterial endotoxin[18] thus preventing hazard effect of *C.perfringens* toxins ,also lysozyme-lipid A binding results in a conformational change that keeps endotoxin from interacting with macrophage receptors and dampens the release of the proinflammatory cytokines interleukin-1 (IL-1), interleukin-6 (IL-6) and tumor necrosis factor-α (TNF-α)[19,20]. Lysozyme could activate systemic immune response as when it administrated orally and absorbed in the gut to increase plasma levels of Lysozyme, which could produce systemic effects and potentiate the activity of monocytes and macrophages [21]. At the intestinal level, lysozyme has been reported to interact with intestinal bacteria to liberate immune-modulating peptidoglycans and Peyer's patches, and intraepithelial lymphocytes to activate the host's immune system[22,23]. Therefore, it is speculated that Lysozyme could activate systemic and intestinal immune responses.

From the above mentioned data our research designated in order to investigate effect of both Betaine and Lysozyme prebiotic on immune response of inactivated ND-AI inactivated vaccine in broiler chickens.

Material and methods

1- Experimental Chicks:

One hundred and sixty, 1- old Cobb broiler chicks were divided into 4 equal groups 40 chicks in each as shown in table (1). Group 1 negative control non vaccinated while group 2 received Lysozyme while groups 3 received Betaine, finally group 4 were kept as positive vaccinated control positive group. Chickens group were vaccinated with inactivated ND-AI combined vaccine (CEVA) with recommended dose according to manufactured directions.

2. Ration

Commercial starter and grower broiler chicken ration were given till 21 and 32 days of age, respectively. The used commercial balanced ration based on yellow corn or soyabean that met the National Research Council (NRC) (1984) broiler chicken requirements. The starter ration contained crude protein-not less than 21%, crude fat-not less than 2.94%, crude fibers-not less than 2.35%, metabolizing energy-not less than 3054 Kcal/kg ration and used for the first 3 weeks of age. The grower ration contained crude protein-not

less than 17.15%, crude fat-not less than 2.5%, metabolizing energy-not less than 3020 Kcal/kg ration and used for the remaining of the experimental period. The ration contained coccidiostate (Semiduramicin) while no antibiotics were added to it

3- Vaccine Strains

Newcastle disease (ND) vaccine strains

a) Hitchiner Bı live vaccine, each vial contain virus titre of 10^9 EID_{50} was used after titeration[24] for vaccination of experimental chicks via eye instillation route.
b) Inactivated ND-AI combined vaccine (CEVA) with recommended dose according manufacturer directions.

4- prebiotic:

a- Lysozyme10%:

produced by Nanchang lifeng Industry and Trading Co.,Ltd., Batch no. 20131030 , exp. date : October 2016 .

Dosage 0.5 gm/L drinking water.

b- Betaine Anhydrous 98% :

produced by Nanchang lifeng Industry and Trading Co., Ltd., Batch no. 20131106, exp. Date : june 2016.

Dosage 1 gm/L drinking water.

5- vaccination time and application methods:

Live vaccines applied against Newcastle disease (ND) using live Hitchner B1 at 5 days of age by eye drops instillation methods while against infectious bronchitis (IB) disease using live H 120 strain at one day old by coarse spray and against infectious bursal disease (IBD) using live intermediate plus strain (Bursine plus®) at 14 days of age by eye drops instillation while inactivated vaccine against avian influenza (AI-ND) disease CEVA® at 7 days old of age through subcutaneous route at the back of the neck.

6-Bacterial strain :

E.coli strain (O78 K80 H11) used were orally inoculated with infected groups in rate of 1 ml of saline containing 10^8 colony forming unit (CFU*) E. coli*/ ml [25]

7- Embryonated chicken eggs (ECEs):

Specific pathogen frees (SPF) obtained from Kom Oshim, El-Fayoum, Egypt. ECEs were used for virus isolation and propagation of isolated virus vaccine and HI antigen.

8- Egg inoculation:

The procedures of sample preparation and egg inoculation were done according to[26,27]. Slide HA was applied on allantoic fluid of inoculated chicken embryos to detect. Estimation of 50% end point was carried out according to.[28]

9- Serum samples:

Blood samples for serum were collected weekly for haemagllutination test, bursa, liver and spleen were collected for histopathological examination.

10- Hemagglutnation antigens:

AI haemagllutination antigen was kindly obtained from Animal Health Research Institute, Dokki, Giza; while ND haemagglutnating antigens were prepared by propagating of Lasota life vaccine was on embryonated

chicken eggs (ECE) and used as HH antigen . Both used antigens were titrated to 4 HA units using HA test according to[29].

11- Haemagllutination inhibition (HI) test:

Serum samples were tested to evaluate the antibodies titer against ND and AI, using the standard HI method The test was carried out according to the standard procedure described by [30] the end point were estimated according to scheme described by [31]

12- Histopathological Studies:

Tissue specimens from liver and intestine of experimental birds of each group chicks were fixed in 10% neutral formalin solution and the specimens were routinely processed in paraffin embedding method ,sectioned and stained with Haematoxylin and Eosin (H&E) for light microscopic examination according to[32]**.**

13- Experimental design:

One hundred and sixty, day old Cobb broiler chicks were divided into 4 equal groups 40 chicks in each as shown in table (1). Group 1 negative control non vaccinated while group 2 received lysozyme while groups 3 received Betaine, finally group 4 were kept as positive vaccinated control positive group. Chickens group were vaccinated with inactivated ND-AI combined vaccine (CEVA) with recommended dose according manufacture directions. E.coli bacterial challenge strain were used by oral infection with 0.5ml of E coli O78 K80 H11 strains containing 1 x 10^4 viable microorganism /ml phosphate buffered saline (PBS). Blood samples were collected weekly for HI test , bursa, thymus and liver were collected for histopathological examination.

Table (1): Treatment of chicken groups vaccinated with AI-ND inactivated vaccine

Group	Infection	Vaccination with AI-ND inactivated vaccine	Type of treatment
1	-	-	Negative control
2	+	+	lysozyme
3	+	+	betaine
4	+	+	Positive control

Results and Discussion

Improvement of antibody titers against most economic important viral disease is of great value specially in our Egyptian field which birds under continues challenge, viral threshold is very high and improper vaccination schedule. Many prebiotics play role in improving antibody titer of inactivated viral vaccine together with other beneficial effects that improves productivity and poultry performance. Therefore our experiment is designated in order to study the effects two prebiotics on immune response under Egyptian field condition.

Results of HI against ND are shown in table (2) which revealed that best mean antibody titer was group (3) received Betaine which was 6.14±0.69, followed by group (2) received Lysozyme which was 6.00 ±0.95 , followed by group (4) positive vaccinated with inactivated vaccine which was 4.00±1.89, then finally negative control group received live vaccine only which was 4.00 ±0.58.

Table (2):Main HI titers against Newcastle disease vaccines in broiler chicken groups

Gr No	Treatment	Infection	Weeks post vaccination	ND-HI log $_2$ titre range	Mean ± SD
1	Negative control received live vaccine only		0	0-4	2.67 1.23
			1	2-3	2.43 0.53
			2	3-5	4.00 0.58
2	Lysozyme	+	0	0-4	2.67 1.23
			1	2-3	2.83 0.49
			2	4-7	6.00 0.95
3	Betain	+	0	0-4	2.67 1.23
			1	3-4	3.70 0.49
			2	5-7	6.14 0.69
4	Positive vaccinated group	+	0	0-4	2.67 1.23
			1	2-4	3.50 1.30
			2	4-5	4.00 1.89

Table (3): Main HI titers against *Avian Influenza* H5N1 vaccines broiler chicken groups.

Gr No	Treatment	Infection	Weeks post vaccination	AI- HI log $_2$ titre range	Mean ± SD
1	Negative control		0	0	0.00 0.00
			1	1- 2	1.86 0.38
			2	3- 4	3.86 0.69
2	Lysozyme	+	1	3- 4	3.43 0.79
			2	4-6	4.71 0.95
3	Betain	+	1	1- 4	3.00 1.16
			2	4 - 6	4.86 0.69
4	Positive vaccinated group	+	1	2- 3	2.57 0.55
			2	4-5	4.43 0.54

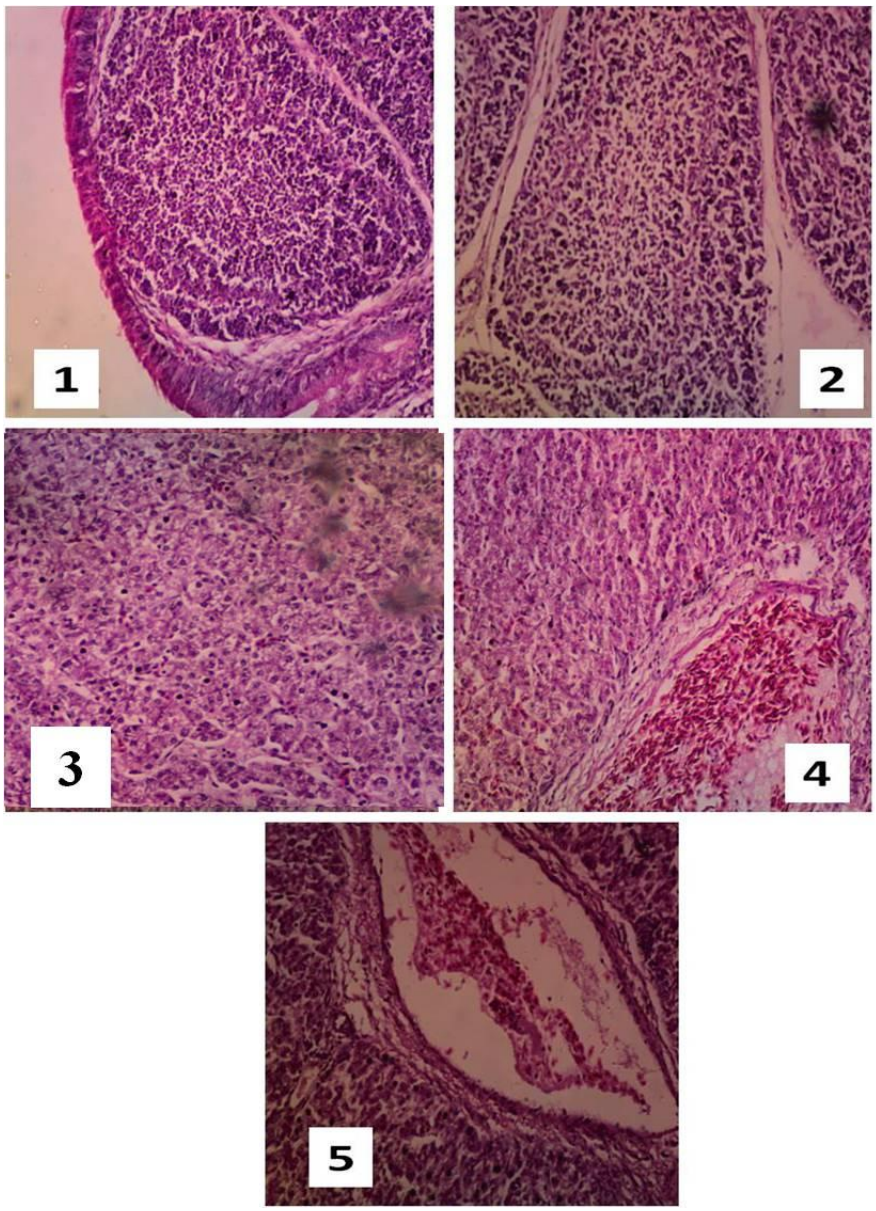

Fig (1-5):Bursa and Liver stained sections of Chicken groups vaccinated against AI and ND using combined inactivated vaccine given Lysozyme or Betain and infected with *E. coli* chickens (H&E X 200).

Fig (1) Chicken bursa group 3 showing moderate hyperplasic activity of the lymphoid follicle. Fig (2) Chicken Bursa group 2 showing hyper plastic activity in lymphoid follicle. Fig (3) Chicken liver showing sever hydropic degeneration of the cytoplasm in the hepatocyte with disorganization of the hepatic cord. Fig (4). Chicken liver showing severe congestion of the central vein. Fig (5):Chicken liver showing mild congestion of the portal vein

Results of HI test against Avian Influenza are shown in table (3) which revealed that best was group (2) received betaine which was 4.86±0.69 followed by group (2) which received lysozyme which was 4.71± 0.95 followed by group (4) positive vaccinated group which was 4.43± 0.54 and finally group (1) negative control group which was 3.43±0.79.highest immune response against both Newcastle disease virus inactivated vaccine and Avian Influenza vaccine was in group 3 receive betaine. Inspite of that this results was not matched with [14] who stated that Betaine supplementation has no role on antibody titer for ND vaccine, on the other hand it was found that broilers fed diet containing betaine has higher bursa of fabricius weight and higher humeral immune response against respiratory virus inactivated vaccine [15] this due to sparing dietary methionine with betaine as it was found that reduction in the concentration of dietary methionine affect immunity in chickens [14], other researchers assist our results as they suggested that betaine promoted immune system of broiler

chickens[33,34]. also immune response was improved in group received lysozyme when compared with vaccinated non treated groups, this results was matched with[21] who noticed that lysozyme could activate systemic immune response as when it administrated orally and absorbed in the gut to increase plasma levels of lysozyme, which could produce systemic effects and potentiate the activity of monocytes and macrophages. moreover dietary lysozymes enhancing immunoglobulin A (IgA) secretion, macrophage activation [17] that resulting in activate systemic and intestinal immune responses.

Also it was noticed that group (4) vaccinated infected with *E.coli* control positive showing clinical signs of E.coli infection in the form of diarrhea and subcutaneous inflammation (cellulitis) mortality rate was 30%, this results was matched with many researchers as[35] who noticed that avian pathogenic *E.coli* infection cause severe cellulitis and high mortalities with 15% survivals, also diarrhea was reported by[36], other explained that cause of diarrhea maybe due to enterotoxogenic strains avian pathogenic *E.coli* (APEC)[37].

Concerning histopathological findings control negative group show no pathological lesions, on the other hand it was found that Bursa of Fibricus of Betain received group 3) which showing moderate hyperplasic activity of the lymphoid follicle (fig 1) which later on become more obvious by the end of the experiment (fig 2) this activity maybe due to that betaine improve bursa of Fabricus weight resulting in improves its activity[15] which was clear in betaine group when compared to other groups including control negative. Liver sections group (4) infected vaccinated non treated group showed severe hydropic degeneration (fig 3) other field showed severe congestion of the central vein (fig 4) while groups (2) and (3) were less affected as they showed mild congestion of portal vein (fig 5). This results indicate that Lysozyme and Betaine has a protective activity for liver tissue when compard with positive infected group with E.coli this explained by[38] who reported that Lysozymes decrease population of APEC in the intestine thus decrease infectious dose diluting pathological changes, while [39]stated that betaine provides antioxidant capacity for attenuating the hepatocyte necrosis by CCl4, and so considered a potent nutritional or therapeutic factor for reducing liver fibrosis.

From the above mentioned data it could be concluded that building immune foundation against respiratory virus is crucial and can be fulfilled by proper vaccination programme and can be supported by prebiotics (Lysozyme and Betaine) which could improve antibody titers of inactivated vaccine, unfortunately this need further investigations specially of studding aspects of celluler and humeral immune response with used prebiotics.

References

1. Sachin Kumar and Monika Koul (2016): Newcastle disease virus: A constant threat to the poultry industry in India. Vaccine 34. 597-598.
2. Aminu Shittu ;Abdullahi Abdullahi Raji; Shuaibu A Madugu; Akinola Waheed Hassan; and Folorunso Oludayo Fasina (2014): Predictors of death and production performance of layer chickens in opened and sealed pens in a tropical savannah environment. BMC Vet Res ; 10:214. doi: 10.1186/s12917-014-0214-7.
3. Chen H. (2009):H5N1 avian influenza in China. Science in China (Series C:Life Sciences);(05): 41927.
4. Butt KM, Smith GJ, Chen H, Zhang LJ, Leung YH, Xu KM, Lim W, Webster RG, Yuen KY, Peiris JS, Guan Y.(2005a): Human infection with an avian H9N2 influenza A virus in Hong Kong in 2003. J Clin Microbiol, 2005a; 43(11): 5760-7.
5. Abudabos A.M. ; Al-Batshan H.A.; Murshed M.A. (2015): Effects of prebiotics and probiotics on the performance and bacterial colonization of broiler chickens. S. Afr. j. anim. sci. vol.45 n.4 Pretoria.
6. Murarolli VDA ; Burbarelli MFC ; Polycarpo GV ; Ribeiro PAP ; Moro MEG ; Albuquerque R (2014): Prebiotic, probiotic and symbiotic as alternative to Antibiotics on the Performance and Immune Response of Broiler Chickens. Rev. Bras. Cienc. Avic. vol.16 no.3 Campinas http://dx.doi.org/10.1590/1516-635x1603279-284.
7. Xiao , C ; G. Bao ,G; and Hu, S. (2009): enhancement of immune responses to Newcastle disease vaccine by a supplement of extract of Momordica cochinchinensis (Lour.) Spreng. Seeds. Poultry Science 88 :2293–2297 doi: 10.3382/ps.2009-00059.
8. Ahmed Hegazi, Amr M. Abdou and Fyrouz Abd Allah (2013): Influence of Honey on Immune Response Against Newcastle Disease Vaccine. International Journal of Basic and Applied Virology 2(1): 01-05, ISSN 2222-1298 © IDOSI Publications, DOI: 10.5829/idosi.ijbav.2013.2.1.8154.

9. Landy N, Ghalamkari GH, Toghyani M (2012): Evaluation of St John's Wort (Hypericum perforatum L.) as an antibiotic growth promoter substitution on performance, carcass characteristics, some of the immune responses, and serum biochemical parameters of broiler chicks. Journal of Medicinal Plants Research 6:510-515.
10. Zhai L, Li Y, Wang W, Hu S (2011) :Enhancement of humoral immune responses to inactivated Newcastle disease and avian influenza vaccines by oral administration of ginseng stem-and-leaf saponins in chickens. Poult Sci 90:1955-1959.
11. Alireza Talebi ; Amir Amani ; Masoud Pourmahmod; Poya Saghaei; and Reza Rezaie (2015): Synbiotic enhances immune responses against infectious bronchitis, infectious bursal disease, Newcastle disease and avian influenza in broiler chickens. Veterinary Research Forum ; 6(3) 191-197.
12. Singh AK, Ghosh TK, Creswell DC, Haldar S (2015): Effects of supplementation of betaine hydrochloride on physiological performances of broilers exposed to thermal stress. Open Access Animal Physiology Volume 7.pages 111-120. DOI https://dx.doi.org/10.2147/OAAP.S83190.
13. Awad A.L. ; Ibrahim A.F.; Fahim H.N. and M.M. Beshara (2014): Effect of dietary Betaine supplementation on growth performance and carcass traits of DOMYATI Duckling under Summer conditions. Egypt. Poult. Sci. Vol (34) (IV): (1019-1038).
14. Rao SVR, Raju MVLN, Panda AK, Saharia P, Sunder GS (2011) :Effect of Supplementing Betaine on Performance, Carcass Traits and Immune Responses in Broiler Chicken Fed Diets Containing Different Concentrations of Methionine. Asian-Aust J Anim Sci 24, 662-669.
15. Masoud Alahgholi, Sayed Ali Tabeidian, Majid Toghyani and Sayed Sadra Ale Saheb Fosoul (2014): Effect of betaine as an osmolyte on broiler chickens exposed to different levels of water salinity. Archiv Tierzucht 57 .4, 1-12. doi: 10.7482/0003-9438-57-004.
16. Proctor, V. A., Cunningham, F. E. & Fung, D.Y.C. (1988) The chemistry of lysozyme and its use as a food preservative and a pharmaceutical. Crit. Rev. Food Sci. Nutr. 26:359-395.
17. Kawano, M., Namba, Y. & Hanaoka, M. (1981) Regulatory factors of lymphocyte-lymphocyte interaction. I. Con A-induced mitogenic factor acts on the late G1 stage of T-cell proliferation. Microbiol. Immunol. 25:505-515.
18. Brandenburg, K., Koch, M.H.J. & Seydel, U. (1998) :Biophysical characterisation of lysozyme binding to LPS Re and lipid A. European J. Biochem. 258:686-695.
19. Takada, K., Ohno, N. & Yadomae, T. (1994) :Binding of lysozyme to lipopolysaccharide suppresses tumor necrosis factor production in vivo. Infect. Immun. 62:1171-1175.
20. Reusens-Billen, B., De Clercq, L., Barreira, V. I., Hanotier, C. J., Remacle, C. & Hoet, J. J. (1994): Prevention of the cytotoxic effect of IL-1 by human lysozyme on isolated rat islets. Diabetes Res. Clin. Pract. 23:85-94.
21. Seno, S., Inuoe, S., Akita, M., Setsu, K., Tsugaru, Y. & Furuhata, H. (1998):Intestinal absorption of lysozyme molecules and their destination, an immunohistochemical study on rat. Acta Histochemica et Cytochemica, 31, 329 - 334.
22. Jolle`s, P. (1976): A possible physiological function of lysozyme. Biomedicine, 25, 275-276.
23. Namba, Y., Hidaka, Y., Taki, K. & Morimoto, T. (1981): Effect of oral administration of lysozyme or digested bacterial cell walls on immunostimulation in guinea pigs. Infection and Immunity, 31, 580-583.
24. Reed, L.J., & Muench, H. (1938): A simple method of estimating fifty percent endpoints. Am. J. Hygiene, 27, 493-497
25. El-Boushy, M.E.; Awad; Sanaa, S., Hanfey, A. (2006): Immunological, hematological and biochemical studies on pefloxacin in broilers infected with E. coli. 8th Conference, Vet. Med. Zag., pp. 503-515.
26. Allan, W. H. (1981): Diagnostic Procedures-Response, In the Proc of the 1st Internat.Symp on Avian Influenza, Beltsville, Maryland, USA. U.S. Animal Health Ass. 167-171.
27. Pearson, J. E. and Senne, D. A. (1981): Avian Influenza Diagnostic Procedures in the United States, In Proc, of the 1st International Symposium on Avian Influenza, Beltsville, Maryland, USA. U.S, Animal Health Association. 157-167.
28. World Organisation For Animal Health (OIE) (2012). Manual of Diagnostic Tests and Vaccines for Terrestrial. Seventh Edition.
29. Reed, L. J. and Muench, H. (1938): Simple method of estimating 50 per cent end point", Am. J. Hyg. , 27: 493-499.
30. Majiyagbe,K.A.,and S.B.Hitchner (1977):Antibody Response to strain combination of NDV,as measured by HI., Av.Dis.21(4) 576-584.

31. Kaleta,E.F. and Siegmann,O.(1971): Comparativestudies on the demonstration of haemagglutinating inhibiting and virus neutralizating antibodies after vaccination against NewCastle disease. Arch. Grefugelk, 35, 79.
32. Bancroft, J.D. and M. Gamble, (2008): Theory and Practice of Histological Techniques. 6th Ed.,Churchill Livingstone, Elsevier, China.
33. Hamidi H, Jahanian R, Pourreza J (2010): Effect of Dietary Betaine on Performance, Immunocompetence and Gut Contents Osmolarity of Broilers Challenged With a Mixed Coccidial Infection. Asian J Anim Vet Adv 5,193-201.
34. Klasing KC, Adler KL, Remus JC, Calvert CC (2002): Dietary Betaine Increases Intraepithelial Lymphocytes in the Duodenum of Coccidia-Infected Chicks and Increases Functional Properties of Phagocytes. J Nutr 132, 2274-2282.
35. Susantha Gomis; Lorne Babiuk; Dale L. Godson; Brenda Allan; Tannis Thrush; Hugh Townsend; Philip Willson; Edwin Waters; Rolf Hecker; and Andrew Potter (2003): Protection of Chickens against Escherichia coli Infections by DNA Containing CpG Motifs. Infect. Immun.vol. 71 no. 2 857-863. doi: 10.1128/IAI.71.2.857-863.
36. Lutful Kabir (2010): Avian Colibacillosis and Salmonellosis: A Closer Look at Epidemiology, Pathogenesis, Diagnosis, Control and Public Health Concerns. Int J Environ Res Public Health .7(1): 89–1. doi: 10.3390/ijerph7010089.
37. Dho-Moulin M, Fairbrother JM. (1999): Avian pathogenic Escherichia coli (APEC) Vet. Res. 1999;30:299–316.
38. Gong M., Anderson D., Rathgeber B., Maclsaac J. (2016): The effect of dietary lysozyme with EDTA on growth performance and intestinal microbiota of broiler chickens in each period of the growth cycle. J Appl Poult Res doi: 10.3382/japr/pfw041.
39. Meng-Tsz Tsai,Ching-Yi Chen, Yu-Hui Pan, Siou-Huei Wang, Harry J. Mersmann, and Shih-Torng Ding (2015): Alleviation of Carbon-Tetrachloride-Induced Liver Injury and Fibrosis by Betaine Supplementation in Chickens. Evidence-Based Complementary and Alternative Medicine Volume 2015 (2015), Article ID 725379, 12 pages http://dx.doi.org/10.1155/2015/725379.

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