

Original Research Article

The Effect of Water Additive Commercial (KimchiStoc®) on Natural Avian Influenza Virus Infection of Broiler Chickens: Pathological and Immunopathological Approach

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Abstract: Avian influenza virus (AIV) infection in chickens causes huge economic losses so that a solution is needed to overcome outbreak, namely through vaccination. However, administration of AIV vaccines can possibly initiate AIV subclinical infections in chickens. Therefore, another alternative approach is needed to prevent AIV infection, one of them is water additive commercial (KimchiStoc®). The purpose of the present study was to study effect of KimchiStoc® against natural AIV infection in broiler chickens on a commercial farm. In the present study, 55 DOC broiler chickens were used as experimental animals for 35 days. Before supplementation (Week-0), five DOCs were randomly selected, necropsied and the lungs were collected. Fifty remaining DOCs were divided randomly into 2 groups of 25 each. The first group was chickens without supplementation of KimchiStoc® (G1), while the second group was chickens supplemented with KimchiStoc® 0.2% with a dose of 5 days/week (G2). Observations were carried out for 5 weeks. Each week five chickens in each group were randomly necropsied. Lungs were collected and detected for AIV by using immunopathological immunohistochemical streptavidin biotin (IHC SB) The results showed that the spread of AIV in broiler chickens occurred in DOC until the fifth week. IHC SB test can be applied to detect AIV-infected broilers subclinically. The KimchiStoc® can prevent the natural infection of AIV in at least the third week after KimchiStoc® administration.

Keywords: broiler chickens, avian influenza virus, lungs, IHC SB, KimchiStoc®.

NOVELTY

KimchiStoc® is *novel herbal medicine* based on biotechnology modern approach that is capable of preventing for binding of avian influenza virus (AIV) with host receptor (sialic acid), increasing host immune system, activating T cell, anti antibacteria Gram + and Gram -, and anti toxin. The *novel herbal medicine* anti AIV could inactivate AIV that has infected and entered host cell. Therefore, KimchiStoc® is a novel herbal medicine of choice that should have been applied for overcoming AIV in Indonesia.

INTRODUCTION

The avian influenza virus (AIV) is easily mutated so that AIV is easily transformed from low pathogenic AIV (LPAI) to highly pathogenic AIV (HPAI). The AIV based on pathogenicity is

differentiated into LPAI and HPAI (Swayne DE and Pantin-Jackwood M, 2008). AIV subtype LPAI is known to cause <5% mortality and morbidity> 50% (Suarez DL, 2008). In Indonesia AIV subtype HPAI is H5 and H7 (Wasito R *et al.*, 2014a, b, c; Wasito R *et al.*, 2016). Until now, Indonesia has the status of endemic AIV subtype H5N1 (Wasito R *et al.*, 2016; Wasito R *et al.*, 2018a,b). In 2010, it was reported that AIV infected day old chick (DOC) broilers chickens (Setyawati S, 2010). Cases of AIV infection occurred due to the handling of AIV which has not been maximized and the vaccination strategy is weak (Martindah E *et al.*, 2007; OIE, 2014). This can cause subclinical AIV infection in chickens. Subclinical AIV infection can occur in chickens on poultry farms that implement vaccination systems in their poultry (Capua I *et al.*, 2003). This can happen because the AIV vaccine

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used only has a partial genetic similarity to the challenger AIV in the field. Chickens on poultry farms infected with subclinical AIV can be a continuous source of AIV disease transmission in other birds that are sensitive in the surrounding area (Martindah *E et al.*, 2007).

A diagnosis with the right method is needed, one of which is immunopathological immunohistochemistry (IHC), a qualitative test that is very accurate, effective, easy to do, affordable and environmentally friendly to detect viral antigens in the poultry respiratory tract, e.g AIV (Gu J *et al.*, 2007; Chamnanpood C *et al.*, 2011; Wasito R *et al.*, 2017). The IHC has high sensitivity and specificity in detecting the presence of pathogens (AIV) (Tjahyowati G, 2010; Spackman E *et al.*, 2008). The IHC test is performed to detect AIV subtype H5N1 in humans with acute respiratory symptoms (Gu *et al.*, 2007). The IHC method has also been successfully applied for the detection of H5N1 in organs of experimentally infected chickens (Chamnanpood C *et al.*, 2011). In Indonesia, the IHC method was applied for AIV H5N1 detection in comb skin, wattle, brain, trachea, lungs, heart, proventriculus, liver, kidney, spleen and ovaries in chickens infected by AIV in East Java and West Java (Damayanti R *et al.*, 2004). IHC method for detecting AIV that infected day old chick (DOC) broiler chickens was reported by Setyawati S, 2010. AIV organ target is the lungs (Wasito R *et al.*, 2017).

Due to the high AIV mutation rate, repeated occurrences of viral strains that are resistant to antiviral treatment and vaccination so that it is required the product development of a biotechnological modern approach capable of AIV eradication, including control and prevention. Flavonoid and glycosides, including quercetin and iso-quercetin are effective against AIV replication (Vaidya B *et al.*, 2016). Kimchi is a traditional fermented food that is consumed regularly in Korea. Kimchi is commercially available in more than 180 varieties and prepared from various ingredients. The main spices include red onion, garlic, ginger, salt, and red pepper powder. Red onion extract was reported to reduce lung lesions in layer chickens (Nataria RN *et al.*, 2019). Kimchi has antiviral and immunomodulatory abilities that are applied to various diseases including flu in humans and also livestock. At first, lactic acid bacteria (LAB) such as lactobacillus was used as probiotics (Ishibashi N and Yamazaki S, 2001). At present LAB is developed in livestock and poultry as an antiviral (Chun B-S *et al.*, 2007; Chon H *et al.*, 2008; Rather IA *et al.*, 2015).

Therefore, it is evident, that the studies of the effectiveness of Kimchi and its metabolites have been shown to have antiviral activity. The ability of water additive commercial (KimchiStoc®) for natural AIV infection in broilers chickens in Indonesia is unknown. Therefore, the present study will focus on the

effectiveness of KimchiStoc® as antiviral against AIV natural infection in broiler chickens with confirmation of diagnosis through pathologic lesions and immunopathological results.

MATERIAL AND METHODS

Location of Research

The present study was carried out at a commercial broiler chicken farm located in Yogyakarta, Indonesia. Making lung histopathological preparations and pathologic analysis was carried out at the Pathology Laboratory, Department of Pathology Faculty of Veterinary Medicine, Gadjah Mada University. Immunopathological immunohistochemical streptavidin biotin test was carried out at the Internal Medicine Laboratory, Faculty of Veterinary Medicine, Gadjah Mada University, Yogyakarta, Indonesia.

Materials and Tools

The samples used in the present study were 55 broiler chickens at one day old (DOC) that had been vaccinated against ND (Newcastle disease), IB-ND (infectious bronchitis-New disease) and IBD (infectious bursal disease) from the hatchery. The average DOC weight is ± 49.98 g. The physical condition of DOC has a relatively uniform size with a level of uniformity $\geq 80\%$, agile, responsive and color of the hair is not dull, dry umbilicus, no physical defects, and quickly adapt to changes in the environment.

The material used in the present study was prestarter commercial chicken feed B10 (age 1-14 days) and starter (aged 15-35 days), commercial water additive (KimchiStoc®, TRI-ON INTERNATIONAL CORP, KOREA), vitamins and drinking water. Feed consists of two types of feed, namely feed brooder and post brooder. The additive water used was given by mixing it with drinking water with a dose of 2 ml of water additive per liter of water (0.2%), 5-day intervals in a row per week, for 35 days of maintenance

The chemicals used in the present study consisted of chemicals for the manufacture of histopathological and immunopathological immunohistochemical preparations. The chemicals for making histopathological preparations include: 10% neutral buffer formalin, ethanol (80%, 95%, absolute), xylene, paraffin, and chemicals for immunopathological immunohistochemical streptavidin biotin staining.

The tools used in the present study include: cage, 5 kg capacity feed 2 pieces, 2 liters 4 pieces of drinking water, 3 kg digital portable scales (KP601-P70), 5 liter bucket, 2 liter measuring cup, 5 ml syringe Terumo brand, surgical instruments, 120 pieces of sample tubes, tools for making and analyzing histopathological preparations and immunopathological immunohistochemistry. The chicken cage with a length of 206 x width 120 x height 46 cm was partitioned into two units, one unit for the control group and one unit

for the treatment group. Each enclosure measuring 103 x 120 x 46 cm, thermohyrometer and brooder used at the age of 1-14 days were placed on the bulkhead between the two experimental cage units.

Broiler Chickens Preparation Procedure

Maintenance was carried out in 2 experimental cage units. Before used, the cage was disinfected first with a disinfectant. The food and drink place was cleaned with disinfectant, the husk used as a cage is sprayed with 10% neutral buffered formalin to inhibit and kill fungal and bacterial growth. Maintenance was carried out for 35 days. The vaccination program was carried out according to the health procedures applied by farmers, namely using the hatchery vaccination method, so that when the DOC arrived it had been vaccinated. The types of vaccines given at the hatchery included ND, IB-ND, and IBD. When chickens were at 1-14 days old, feeding was carried out four times a day, then at the age of 15-35 days was done three times a day, with a volume that was adjusted to the age of the chickens. The administration of drinking water was *ad libitum*, the addition of water additives commercial ((KimchiStoc®) in chickens in G2 group was mixed with drinking water as much as 2 ml / liter for 5 days per week.

Research Procedure

A total of 55 broiler chickens at one-day-old (DOC) were used in the present study. Five DOC aged 0 week (Week-0) were collected randomly for necropsy. Lung samples from five chicken in Week-0 were collected and histopathological preparations were made. Furthermore, the remaining 50 DOC, divided randomly into 2 groups (G1 and G2) of 25 each.. Chickens in G1 was not supplemented with 0.2% water additive commercial (KimchiStoc®), but chickens in G2 was supplemented with 0.2% KimchiStoc®.

Supplementation of 0.2% water additive commercial (KimchiStoc®) was obtained by mixing 4 ml of water additive into a place where chicken water had been filled with water up to a volume of 2 liters. The provision of drinking water is carried out by *ad libitum*, in the age of 1-14 days the frequency of giving 1 x a day (added after empty drinking water), and at the age of 14-35 days the frequency of giving 2x a day. Supplementing KimchiStoc® to the supplemented group is done each time adding chicken drinking water. The supplementation dose was for 5 consecutive days / week, during the maintenance period (\pm 5 weeks). In the fifth week, 5 chickens were collected from each treatment group to be necropsied, then the samples (lungs) were collected \pm 4 cm from each chicken. The lung organs were inserted into the sample pots which had contained 10% neutral buffer formalin and were labeled.

Histopathology and Immunopathological Immunohistochemistry Streptavidin Biotin

Before immunopathological immunohistochemical streptavidin biotin (IHC SB) test was carried out, lungs histopathological preparations were made. The first stage was made of lung histopathological preparations. After fixation of the lung samples with a 10% neutral buffer formalin, the lungs were cut to 4 mm thick. Furthermore, the process of dehydration, the lung tissues were put into a multilevel ethanol solution (80%, 95%, absolute) and cleaned with xylene dipped three times, each 1 hour with automatic histotechnicon tools. Next, the lungs tissues were infiltrated with liquid paraffin three times, two hours each. The lungs tissues were blocked by paraffin through rapid cooling. The lungs that has been blocked or paraffin printed, then cut with \pm 5 μ m thickness microtome. The cut off tissue was placed in a water bath and flattened parallel to the water surface. Furthermore, the lungs histopathological preparations were affixed to the glass object which had previously been given one drop of adhesive chemical in the form of glycerin on the glass surface of the glass object to be completely leveled, which had previously been cleaned with detergent.

Phase I. Histopathologic staining. At first, deparaffinization and rehydration were done. The lungs paraffin preparation of the glass object is removed by soaking it repeatedly in a solution of xylene I, II and III each of 2 minutes. Furthermore, rehydrated soaked ethanol 100%, 95% and 50%, 2 minutes respectively. Then the solution was put into a sterile I and II H2O solution 2 minutes, and soaked PBS pH 7.2 5 minutes. Then, the processed histopathologic lungs sections were then stained with hematoxylin and eosin.

Phase II. IHC SB staining. The lungs tissue preparations that had been depleted and rehabilitated in phase I, marked with pap-pen avoiding spilled IHC SB chemicals. The lungs preparations soaked 10 minutes with hydrogen peroxide are made from a mixture of 30% H2O2 (1 part) and absolute methanol (10 part). Then, washed PBS pH 7.2 3 times 2 minutes. Furthermore, IHC SB (Invitrogen, Histostain® SP product) was carried out. The lungs preparation was placed in the staining chamber treated with 2 drops (100 μ l) blocking solution and incubated for 10 minutes. After that, 2 drops (100 μ l) of primary antibody (anti-nucleoprotein AIV polyclonal antibody) were given and incubated 45 minutes, washed PBS pH 7.2 3 times each 2 minutes. Then the preparation was given 2 drops (100 μ l) of biotinylated secondary antibody and incubated 10 minutes washed with PBS pH 7.2 3 times each 2 minutes. The preparation was given 2 drops (100 μ l) of the conjugate enzyme (streptavidin-horseradish peroxidase) and incubated 10 minutes washed with PBS pH 7.2 as much as 3 times each for 2 minutes. The preparation was given 2 drops (100 μ l) of diaminobenzidine (DAB) chromogen. Preparations were given 2 drops (100 μ l) of hematoxylin as a base

dye and incubated for 1 minute, washed sterile H₂O, dipped in PBS pH 7.2 30 seconds. Then washed sterile H₂O and dehydrated it by dipping it in 50%, 95%, 100% ethanol and xylene solution for 1 minute each. The preparation was given 2 drops (100 µl) of mounting solution and closed by the cover glass, observed the digital camera system microscope.

Data Analysis

Data from the pathological and immunopathological results in the lungs were then analyzed using qualitative descriptive.

RESULTS AND DISCUSSION

In the present study, 0.2% water additive commercial (KimchiStoc®)'s ability to prevent natural AIV infection) was based on examination of anatomical pathological lesions, histopathology with routine hematoxylin-eosin staining (H and E) and immunopathological immunohistochemical streptavidin biotin (IHC SB).

All broilers chickens used in the present study did not show any clinical signs. They appeared normal (healthy). However, at the start of the present study, five chickens at one day old (DOC) as the initial indicator control (W-0) at necropsy had anatomical pathological lesions in the lungs. The lungs appear swollen, not transparent (opaque) and have severe and diffuse congestion and hemorrhages. The same anatomical pathological lesions were also seen in all chickens not supplemented with 0.2% KimchiStoc® (G1) that were necropsied in the first, second, third, fourth and fifth week after administration KimchiStoc® (Gambar 1). It was reported, that anatomical pathological lesions in the lungs as mentioned above are characterized by AIV infection in chickens (Wasito R *et al.*, 2016; Wasito R *et al.*, 2018b). Whereas, in all chickens supplemented with 0.2% KimchiStoc® (G2), hemorrhages were significantly reduced in the lung parenchymes starting to weeks 3 (Fig. 2). Water additive commercial 0.2% KimchiStoc® was proven to prevent hemorrhagic lesions in the lungs.



Fig. 1. Lung of a broiler chicken in G1 (not supplemented with 0.2% water additive commercial or KimchiStoc®) and was necropsied in the third week. There are visible anatomical pathological lesions in lung. The lung looks swollen, not transparent (opaque) and have severe and diffuse spotted and/or linear congestion and hemorrhages.



Fig.2. Lung of a broiler chicken in G2 (supplemented with 0.2% water additive commercial or KimchiStoc®) and was necropsied in the third week. The congestion and hemorrhages were significantly reduced in the lung parenchymes. The lung looks within the normal limits.

Histopathological lesions, such as severe and diffuse congestion and hemorrhages were seen in all chicken in G1 at the 3th week (Fig. 3), but were not seen in all chickens in G2 (Fig. 4). Likewise on the IHC SB examination, brownish-colored AIV nucleoprotein antigen deposits were seen in the parenchymal network of all chickens in G1 (Fig. 5), but were no longer seen in all chickens in G2 (Fig. 6).

Spotted and/or linear hemorrhages is anatomical pathological lesion that characterizes the chicken lungs infected with low pathogenic avian influenza (LPAI). Chickens infected with AIV show significant hemorrhagic lesions (Kamps BS *et al.*, 2006). Based on the pathogenesis of AIV, AIV is thought to cause viremia in the body of the host in the same way as other endotheliotropic viruses. Activation of polymorphonuclear and mononuclear inflammatory cells, and systemic cytokine excretion results in a predisposition to organ lesions (Kamps BS *et al.*, 2006).

In the present study, supplementation of 0.2% water additive commercial (KimchiStoc®) has positive effect on inhibiting hemorrhages in the lungs after administration in the third, fourth and fifth weeks. It is possible that KimchiStoc® has a preventive (and therapeutic) effect before the third week or after administration. It was reported, that the AIV subtype H5N1 induces reactive oxygen species or ROS that is capable of initiating lesions in the lungs (Lin X *et al.*, 2016). Lesions on tissue (lungs) due to ROS are called oxidative stress, while factors that prevent tissue from the ROS are called antioxidants. In Kimchi there are 3-(4'-hydroxyl-3', 5'-dimethoxyphenyl) propionic acid (HDMPPA) active antioxidant compounds. The HDMPPA compounds can have a preventive response to animal models of mice (apoE knockout mice) *in vivo*. HDMPPA compounds will increase NO production in the aorta which is accompanied by a decrease in ROS (Park K-Y *et al.*, 2014). Apparently, lactic acid bacteria (LAB) contained in KimchiStoc® can improve the response of adaptive immunity through increasing NK

(natural killer) cell cytotoxicity, activating APC (antigen presenting cell), and inducing the formation of anti-inflammatory compounds called cytokines (Park K-Y *et al.*, 2014).

It is possible that the lungs in chickens in G2 after the third-fifth week giving 0.2% KimchiStoc® has no histopathologic lesions because the KimchiStoc® contains anti-inflammatory compounds, anti-microbial, anti oxydative property and immune promotion (Park K-Y *et al.*, 2014) so as to prevent the occurrence of pathological lesions in tissues or organs, such as the lungs. The lungs are the main organs of breathing so that if a pathological lesion occurs in the lungs it can result in systemic disorders of the organ so that morbidity and mortality can occur which is quite significant (Kamps BS *et al.*, 2006). Congestion is the accumulation of blood fluid in the lumen of the blood vessels (veins) due to the inhibition of venous drainage. In general, congestion is a passive process that results in excess fluid in the cells, tissues or organs. Congestion can cause hypoxia and edema, which interfere with the normal functioning of the organ. Thus, in chickens infected with AIV HPAI subtypes, it can cause significant mortality in infected chickens due to congestion and hemorrhagic lungs so that lung function is severely disrupted. Severe congestion and hemorrhagic can occur in almost all lobes of chicken lungs infected with HPAI. According to Swayne DE and Pantin-Jackwood M (2008), HPAI AIV infection in poultry will cause significant pathological lesions in the form of hemorrhages and edema.

In the present study it was proven that broilers chickens ranging from DOC to ready-to-cut age by immunopathological immunohistochemistry streptavidin biotin test (IHC SB) were infected with the avian influenza virus (AIV). On observation with a light microscope, the AIV antigen which reacts with polyclonal antibody anti-nucleoprotein AIV (primary antibodies) appears in the form of brownish spots colorations that are focal or

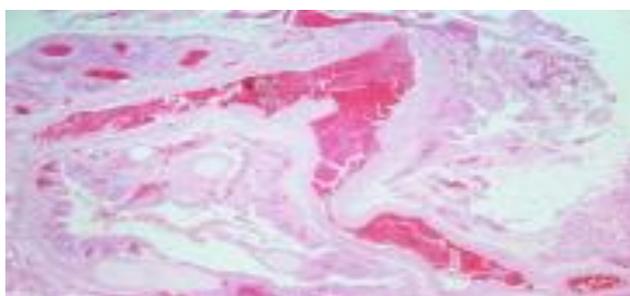


Fig. 3. Histopathological picture of the lung in a broiler chicken in G1 (not supplemented with 0.2% water additive commercial or KimchiStoc®) and was necropsied in the third week. There was severe and diffuse congestion and hemorrhages in the lung parenchymes (Hematoxylin eosin, 250x).

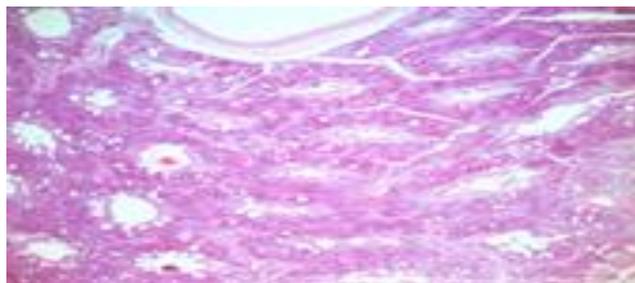


Fig.4. Histopathological picture of lung in a broiler chicken in G2 (supplemented with 0.2% water additive commercial or KimchiStoc®) and was necropsied in the third week. There is no visible congestion and hemorrhages the lung parenchyme. The lung looks within the normal limits (Hematoksilin eosin, 250x.).



Fig.5. Description of immunopathological immunohistochemical streptavidin biotin results of lung in broiler chickens in G1 (not supplemented with 0.2% water additive commercial or KimchiStoc®) and was necropsied in the third week. The lung is infected with avian influenza virus (AIV). Virions (AIV particles) are seen as brownish precipitation deposits coloration within lung parenchyme (Streptavidin biotin, 250x.).



Fig.6. Description of immunopathological immunohistochemical results of lungs in broiler chicken in G2 (supplemented with 0.2% water additive commercial or KimchiStoc®) and was necropsied in the third week. Lung looks within the normal limits (no obvious visible brownish precipitation deposits coloration of virion (AIV particles) comparing to that of the chicken in G1-Fig. 5) (Streptavidin biotin, 250x.).

Diffuse in the parenchymal tissue of the lungs. Secondary antibody is antibody labeled biotin anti primary antibody that acts as a link between primary antibody and streptavidin-horseradish peroxidase conjugate. Streptavidin binds biotin residues that are labeled to secondary antibody, forming a complex streptavidin biotin bond. Horseradish peroxidase will catalyze hydrogen peroxide on the substrate and release hydrogen ions. Furthermore, the hydrogen ion will bind to chromogen, 3',5'-diaminobenzidine (DAB) and form grains (precipitates) which if the tested tissue contains the target antigen (AIV) it will form a brownish coloration. Chromogen is a group of chemical compounds that functions to form colored compounds if they react with certain compounds. The interaction between antigens and antibodies is an invisible reaction.

The results of the present study indicate that AIV has a tendency to proliferate in ciliated epithelial cells of the respiratory tract that have necrosis and

lymphocyte infiltration. Intravenous AIV infection will cause the spread of the virus in the respiratory tract, which is the main target of AIV, namely respiratory tract epithelial cells that are sensitive to AIV infection. The sensitivity of the target cells to AIV is affected by specific receptors on the surface of the host cell. Receptors on the host cell surface play a specific role in AIV endocytosis, through attachment between viruses and host cell receptors. The AIV receptors are determinants of tropism, and hemagglutinin AIV will bind to galactose receptors that bind SA α -2,3-Gal and SA-2,6-Gal sialic acids on the host cell surface. SA SA-2,3-Gal and SA α -2,6-Gal receptors in chicken lungs are distributed in epithelial bronchial cells, parabronchi and vascular endothelial cells (Ramos I *et al.*, 2011) Organ that has a lot of vascularization, such as the lungs and heart will be affected quite fatally by AIV infection because endothelial cells of the blood vessels in these organs are the main place of AIV receptors. The avian influenza virus is of the endotheliotropic in nature.

Specific antibodies (polyclonal or monoclonal) that react with AIV proteins (antigens) have been developed for the IHC SB test, including antibodies to nucleoprotein (NP) and hemagglutinin (HA) proteins. AIV antigens with IHC SB are found in the nuclei and are often also present in the cell cytoplasm. If anti-nucleoprotein antibodies are used, then AIV is located inside the nucleus, whereas when antibodies to hemagglutinin are used, HA antigens are present in the cytoplasm and cell membrane. In the present study, the distribution of N antigens with the IHC SB test appears to be present in the nuclei, cytoplasm and cell membrane of parenchymal tissue of the lungs.

The broilers chickens used in the present field study had no clinical signs from the beginning to the end of the present study in Group I and Group II. However, in all chickens in Group I, there were pathognomonic anatomical pathological lesions in the lungs due to AIV infection. The lungs appears swollen, not transparent or opaque, have severe and diffuse spotted and/or striped congestion and hemorrhages. Supplementation of 0.2% water additive commercial or KimchiStoc® proved to prevent pathological lesions seen in the lungs at the third week after administration of KimchiStoc®. KimchiStoc® contains quercetin which can increase interferon γ production and lymphoid cell proliferation so that it has the potential to improve the body's immune system. Quercetin is reported to reduce AIV infection by restoring antioxidant potential, inhibiting signaling pathway (toll-like receptor / TLR), or inhibiting caspase-3 activity (Vaidya B *et al.*, 2016). These antioxidants play a role in coping with oxidative stress that occurs during AIV infection. In addition, the Kimchi contains sialic acid which functions immunologically to prevent interactions between pathogens (AIV) and AIV receptors in the host cell. Sialic acid is a negatively charged compound for attaching AIV to host cell receptors (Khatua B *et al.*, 2013). In addition, the presence of lactic acid bacteria (LAB) inside Kimchi could prevent the virus from replicating. Antiviral LAB occurs through inhibition of AIV attachment to receptors, endocytosis and immuno-modulation (Lee YM *et al.*, 2004).

CONCLUSIONS

- Supplementing commercially available 0.2% water additive commercial (KimchiStoc®) prevented (and / or treated) natural infection of AIV, at least starting in the third week after KimchiStoc® administration.
- Straptavidin biotin immunohistochemical immunological test is the right test for confirmation of the diagnosis of AIV in sub-clinical AIV-infected chickens, meaning that chickens appear normal (healthy) or show no clinical signs.
- The lungs that are swollen, not transparent (opaque), and have severe and diffuse spotted and /

or linear congestion and hemorrhages are pathognomonic pathological anatomical lesions in subclinical AIV-infected chickens.

- Subclinical AIV-infected chickens or LPAI (low pathogenic avian influenza virus) infection has occurred in chickens since the DOC to adult chickens.

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