

Enhancement of immune responses to infectious bursal disease vaccine by supplement of an extract made from *Momordica cochinchinensis* (Lour.) Spreng. seeds

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ABSTRACT The immunological effect of an extract from *Momordica cochinchinensis* seed (ECMS) on immune responses against infectious bursal disease (IBD) in chickens was evaluated. Fifty-two birds were equally divided into 4 groups and immunized with inactivated IBD vaccine alone (controls) or IBD vaccine emulsified with ECMS (20, 40, and 80 µg). Serum IgG antibody levels against IBD and BW were measured on 0, 7, 14, 21, 28, and 35 d after immunization. The ELISA results revealed that the chickens that received 20 µg of ECMS had significantly enhanced antibody levels on 14, 21,

28, and 35 d when compared with controls ($P < 0.05$). A significant increase in mitogenic stimulated lymphocyte proliferation was also recorded in all ECMS groups as compared with controls ($P < 0.05$; $P < 0.01$). No adverse effect of ECMS was noted on growth performance, although average weight gain was significantly higher in 20 µg (7, 14, 21, 28, and 35 d) and 40 or 80 µg (14 d) of ECMS groups as compared with controls ($P < 0.05$; $P < 0.01$). Further studies are suggested for the investigation of immunological effects of ECMS.

Key words: *Momordica cochinchinensis*, adjuvant, chicken, infectious bursal disease virus

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INTRODUCTION

Infectious bursal disease (IBD) caused by IBD virus (IBDV), is an acute and highly contagious immunosuppressive disease in young chickens. It causes high mortality and immunosuppression in susceptible birds, leading to a variety of secondary infections and a decreased response to vaccinations, which results in an important economic effect on the poultry industry worldwide (Lukert and Saif, 1997; Kim et al., 2004). The disease was found in 1962 and designated as Gumboro disease according to the geographic location of the first recorded outbreak (Müller et al., 2003). The economic effect of both clinical and subclinical diseases warrants the search for and the use of efficient vaccines (van den Berg, 2000a). Besides vigorous vaccination strategies, IBD continues to have a negative effect on the health status of chicken flocks all over the world (van den Berg et al., 2000b; Müller et al., 2003). Immu-

nization of chickens with attenuated live or inactivated IBDV vaccines is the principal method used for the control of disease. Live vaccines provide broad protection but also a proportional risk of reversion to virulence, whereas inactivated vaccines, although costly, are considered more safe and are used successfully (Box, 1989; Habib et al., 2006). Traditionally, available inactivated oil-adjuvanted viral vaccines have been shown to provoke poor cellular immune responses, which play an important role in combating viral infection (O'Hagan et al., 2001). A large number of potential adjuvants have been developed (Edelman and Tacket, 1990; Oda et al., 2000; Rajput et al., 2007a) or are under investigation in laboratory animals. Saponin-based adjuvants have the ability to stimulate the cell-mediated immune system as well as to enhance antibody production (Francis et al., 2002).

Our previous study showed that a supplement of saponin extracted from the bark of *Quilaja saponaria* Molina (Quil A) in vaccines significantly enhances the immune responses to foot-and-mouth disease vaccination (Xiao et al., 2007b). However, Quil A may be limited in veterinary use because of its hemolytic activity (Oda et al., 2000). Although adjuvant effect on im-

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immune responses elicited by ovalbumin with much lower hemolytic activity has been demonstrated (Xiao et al., 2007a), when an extract of the seed of *Momordica cochinchinensis* (Lour.) Spreng. (ECMS) was used as an adjuvant, ECMS proved to be effective for enhancement of immune responses to vaccination against avian influenza (H5N1) in chickens (Rajput et al., 2007b) and foot-and-mouth diseases in guinea pig or piglets (Xiao et al., 2007c). Another study has shown that the supplement of ECMS in Newcastle disease vaccine significantly enhances the immune responses against the disease in chickens (Xiao et al., 2009). The seed of *M. cochinchinensis* is a traditional Chinese medicine and is used against many diseases in animals and humans (Zheng et al., 1992; Gao, 2005; Rajput et al., 2007b). The medical use of the seeds was described in an ancient Chinese medical text, Kai Bao Materia Medica, in the Song Dynasty (793 AD; Gao, 2005) and the seeds are traditionally used for the treatment of inflammatory swelling, scrofula, tinea, diarrhea, as well as suppurative skin infections such as sores, carbuncles, furuncles, and boils in human beings and animals (Gao, 2005; Song and Hu, 2009). Currently, ECMS is included in both the Chinese pharmacopeia (Chinese Pharmaceutical Codex Evaluation Committee, 2005) and the Chinese veterinary pharmacopeia (Chinese Veterinary Pharmaceutical Codex Evaluation Committee, 2000).

In the present study, we have investigated the immune responses of chickens against inactivated IBD vaccine (G strain) combined with ECMS and the effect of ECMS on growth performance in chickens has also been evaluated.

MATERIALS AND METHODS

Experimental Birds

Fifty-two 1-d-old Hy-Line White layer chickens (male) were purchased from Hangzhou Layer Experimental Farm, Hangzhou, China. Different experimental groups were housed in separate wire cages in air-conditioned rooms at 37°C and lighted for 24 h at the beginning of the pretrial period. The temperature was gradually decreased to the room temperature and the light time to 12 h per day, which were kept constant in the following days. Chickens were fed with the commercial starter diet purchased from the same company mentioned above. The birds were given food and water ad libitum.

Preparation of ECMS

Dried *M. cochinchinensis* seeds were purchased from Traditional Chinese Material Medica, Anhui Province, China, and were appraised by the Zhejiang Institute of Veterinary Drug Control, Zhejiang, China. Seeds were extracted as described previously (Sheng et al., 2003;

Rajput et al., 2007b; Xiao et al., 2007c). In brief, the seeds were homogenized using a blender, and were then immersed in 50% ethanol for 24 h. The mixture was placed in a water bath at 90°C for 2 to 3 h and was filtered first through clean cloth then through filter paper (80 µm). After filtration, ethanol was removed using a R502B rotary evaporator (Shenko Tech Co. Ltd., Shanghai, China) and the condensed material was then dissolved in warm water. The solution of 101 Clarifier (Hua Xun Biochemistry Institute, Shanghai, China; 10% wt/vol) was added for overnight incubation at room temperature and centrifuged at 1,200 × *g* for 20 min. The collected upper layer of liquid was rotated with the help of a rotary evaporator and mixed with diethyl ether (anhydrous) at ratio of 1:2 for 2 h to dissolve the fat, color pigments, or some other substances. Diethyl ether was evaporated with the help of a rotary evaporator. It was then immersed in *n*-butanol for 3 h and the upper layer of liquid was collected. This was repeated 3 to 4 times and the liquid was rotated again for ethanol evaporation. The extracted fraction was dissolved in water (pH 6.5) and purified by passing through a chromatography column with resin D101A (Hai Guang Chemical Co. Ltd., Tianjin, China). The ECMS was obtained by evaporating the liquid eluted from the column.

A water-based solution of ECMS (1 mg/mL) was prepared and sterilized by passing through a 0.22-µm filter and was then analyzed for endotoxin level by a gel clot *Limulus* amoebocyte lysate assay (Zhejiang A and C Biological, Zhejiang, China). The endotoxin level was less than 0.5 endotoxin units/mL.

Vaccine

Inactivated IBD oil adjuvanted vaccine (G strain) purchased from Harbin Veterinary Research Institute, Harbin, China, was used to immunize the birds. The ECMS was dissolved in physiological saline solution to prepare the solution of various concentrations (20, 40, or 80 µg/100 µL), filtered (0.22 µm), and then emulsified in vaccine with the help of sterilized syringes as described previously (Huang et al., 1994).

Experimental Design

At 4 wk of age, chickens were randomly assigned to 4 groups of 13 individuals each. The birds were intramuscularly immunized with 0.2 mL of vaccine combined with 0.1 mL of saline (control) or emulsified with 0.1 mL of ECMS solution (20, 40, or 80 µg/100 µL). Blood samples were collected on 0, 7, 14, 21, 28, and 35 d postimmunization for determination of IBD-specific serum IgG. Lymphocytes were prepared from peripheral blood taken from the wing vein (5 birds per group) 5 wk after immunization for lymphocyte stimulation test. Body weight was measured every week throughout the study.

IBD-Specific IgG in Chicken

The IBD-specific serum IgG was analyzed by an ELISA kit (IDEXX Laboratories Inc., Westbrook, ME; Rajput et al., 2007b) according to the manufacturer's instruction. Briefly, serum samples or standard sera were diluted (1:500) in test tubes. The diluted serum samples (100 μ L) were dispensed in duplicate to IBDV antigen-coated microtiter plates and incubated for 30 min at room temperature. The plates were washed 6 times and 100 μ L of goat anti-chicken/turkey IgG horseradish peroxidase conjugate (IDEXX Laboratories) was added to the wells. After 30 min of incubation at room temperature, the plates were washed again and 100 μ L of 3,3',5,5'-tetramethylbenzidine substrate solution was added to each well for 15 min at room temperature. The reaction was stopped by adding 100 μ L of sulfuric acid and the optical density (OD) values at 630 nm were read with a microplate spectrophotometer (Dialab, GmbH, Wiener Neudorf, Austria).

Lymphocyte Proliferation Assay

Peripheral blood samples were collected into heparinized tubes from 5 birds in each group and kept on ice. Blood lymphocytes were separated as described previously (Finkelstein et al., 2003) with some modifications. Briefly, blood samples were diluted (1:1) with sterilized PBS and carefully layered onto the surface of Lymphocyte Separation Medium (Shanghai Hengxin Chemical, Shanghai, China) into a glass tube. After centrifugation (450 \times *g*) for 30 min at room temperature, buffy coat was transferred to a screw-capped V-shape glass tube containing PBS and centrifuged again at 450 \times *g* for 10 min at 4°C. The pelleted cells were washed 3 times in PBS and resuspended in complete medium (RPMI-1640, Gibco, Grand Island, NY) supplemented with 10% fetal calf serum (Hyclone, Tauranga, New Zealand) and 1% gentamicin. Cell numbers were counted with a hemocytometer by trypan blue dye exclusion technique. One hundred microliters of 5 \times 10⁶ live lymphocytes/mL (suspended in complete medium) were cultured in flat-bottomed 96-well microtiter plates (Costar, Shanghai, China) in triplicate wells with mitogens concanavalin A (**ConA**, Sigma, St. Louis, MO; 5 μ g/mL), lipopolysaccharide (**LPS**, Sigma; 10 μ g/mL), or phytohemagglutinin (**PHA**, Sigma; 10 μ g/mL) or no mitogen (non-stimulated). Plates were incubated at 41°C in 5% CO₂ for 48 h. To quantify cell proliferation, in the last 4 h of incubation, 50 μ L of 3,3',5,5'-tetramethylbenzidine substrate solution (2 mg/mL in PBS) was added to each well. After completion of the incubation period, the plates were centrifuged at 450 \times *g* for 20 min and untransformed 3,3',5,5'-tetramethylbenzidine substrate was removed carefully by inverting the plates on tissue papers. One hundred fifty microliters of dimethyl sulfoxide with 4% 1 *N* HCl was dispensed to each well to dissolve the formed formazan crystals and OD was

evaluated by microplate spectrophotometer (Dialab GmbH) at 630 nm after incubation at room temperature for 10 min in the dark. The stimulation index was calculated as the OD value of mitogen culture divided by the OD of nonstimulated cultures.

Effect on Growth Performance

Body weight was measured weekly (0, 7, 14, 21, 28, and 35 d) with the help of digital weight balance. Weight gain was calculated as the difference between the final and initial bird weight during each of the weighing periods.

Statistical Analysis

Statistical analysis was performed with the help of SPSS 10.0 software (SPSS Inc., Chicago, IL). Data are expressed as the mean \pm SD. Least significant difference and Tukey's multiple range tests were used to determine the difference among various groups. Difference between means was considered statistically significant at $P < 0.05$.

RESULTS

IBD-Specific Serum IgG Antibody Response

Serum samples collected from chickens were analyzed for IgG antibody level to IBDV using the ELISA test. The results are depicted in Figure 1. On d 7, no change in IgG antibody level between groups was observed after immunization. On d 14, 21, 28, and 35, antibody levels in birds receiving 20 μ g of ECMS were significantly higher than control ($P < 0.05$). Numerical enhancement of antibody level was recorded in the 40 and 80 μ g of ECMS groups from the second week of immunization to the end of the study when compared with control.

Effect of ECMS on Mitogen-Stimulated Lymphocyte Proliferation

The effects of ECMS on mitogen-stimulated lymphocyte proliferation in chickens immunized with IBDV are shown in Figure 2. Significantly higher lymphocyte proliferative response to ConA was recorded in birds treated with ECMS at all 3 doses (20 μ g, $P < 0.05$; 40 or 80 μ g, $P < 0.01$) or to LPS and PHA in birds treated with 40 μ g of ECMS ($P < 0.05$) than the control.

Effect on Growth Performance

To observe the effect of ECMS treatment on the growth performance in chickens, the BW gain was measured weekly throughout the study (Figure 3). Compared with the control, increased BW gain was found in the birds injected with IBD vaccine containing ECMS,

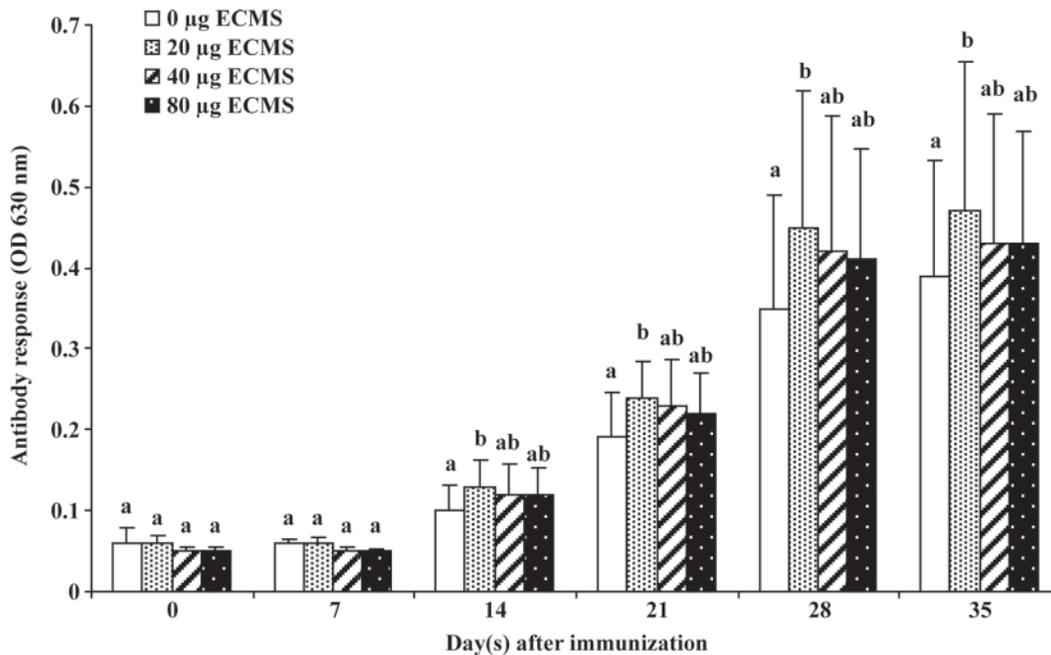


Figure 1. Infectious bursal disease virus-specific serum IgG antibody response in chickens ($n = 13$) immunized with 0.2 mL of infectious bursal disease virus inactivated vaccine alone (control) or combined with extract of *Momordica cochinchinensis* seed (ECMS), 20, 40, or 80 $\mu\text{g}/\text{dose}$. Blood samples were collected on 0, 7, 14, 21, 28, and 35 d postimmunization for ELISA assay. Bars with different letters at the same time point are significantly different ($P < 0.05$). OD = optical density.

with a significant difference ($P < 0.05$) found in the birds treated with ECMS at a dose of 20 μg .

DISCUSSION

Although much research has been carried out in searching for new vaccine adjuvants, the choice of adjuvants remains limited primarily because of the side effect caused at the injection site (Gupta and Siber, 1995). The traditional Chinese medicinal plants have

been used for enhancement of immunity in humans and animals for a long time (Wang et al., 2005; Yeh et al., 2007; Hsu et al., 2008; Hung et al., 2009). Recently, an extract made from a traditional Chinese medicine, *M. cochinchinensis* (Lour.) Spreng. seeds, has been proven to have adjuvant activity (Rajput et al., 2007b; Xiao et al., 2007a,c, 2009).

The present study shows that incorporation of ECMS into IBDV vaccine enhanced the serum IgG responses to IBD (Figure 1). Similar results are also found in oth-

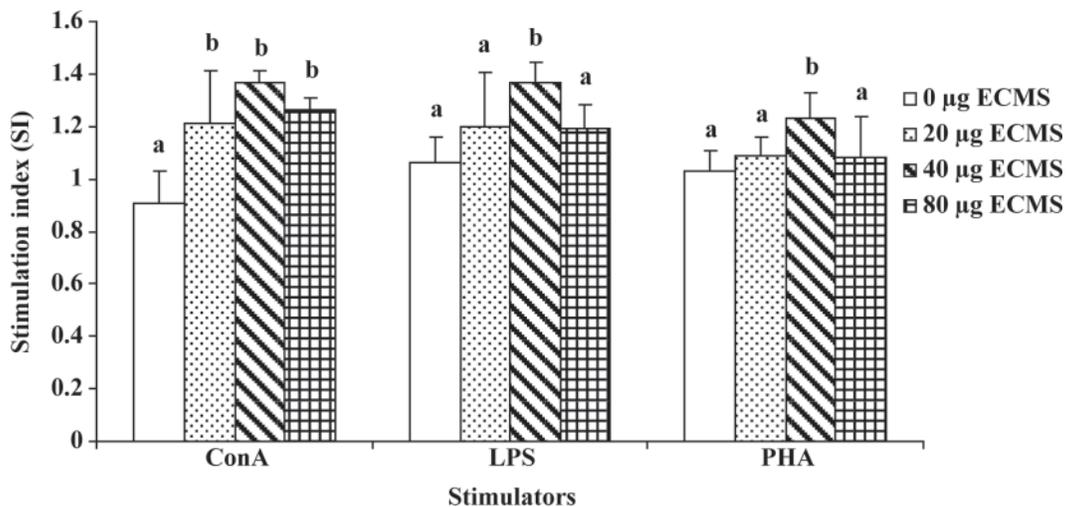


Figure 2. Mitogen-induced proliferative responses of lymphocytes isolated from peripheral blood in chickens ($n = 5$) immunized with 0.2 mL of infectious bursal disease virus inactivated vaccine alone (control) or combined with extract of *Momordica cochinchinensis* seed (ECMS), 20, 40, or 80 $\mu\text{g}/\text{dose}$. Heparinized blood samples were collected on 35 d postimmunization for lymphocyte proliferation test. Bars with different letters at the same time point are significantly different ($P < 0.05$). ConA = concanavalin; LPS = lipopolysaccharide; PHA = phytohemagglutinin.

er studies. For example, Xiao et al. (2007a,c) reported significantly enhanced IgG in guinea pigs and piglets immunized against foot-and-mouth disease or in mice immunized with ovalbumin when ECMS was incorporated into vaccines. In the present experiment, higher IgG levels were found in groups that received ECMS than the control, with significantly enhanced IgG levels detected in birds that received 20 µg of ECMS. Among the ECMS-treated groups, the group treated with 20 µg of ECMS had a higher IgG level than the others. This finding is in agreement with our previous study (Rajput et al., 2007b). When chickens were immunized with inactivated influenza (H5N1) in oil emulsion supplemented with different doses of ECMS, significantly increased IgG levels were found in the 10 or 20 µg of ECMS-treated groups as compared with control. However, in another study, the optimal dose of ECMS as an adjuvant in Newcastle disease vaccination was 80 µg/dose (Xiao et al., 2009). The main active constituents of ECMS are considered to be saponins (Xiao et al., 2007a,c). The mode of action of saponin adjuvants is clear. It has been shown that saponins and protein antigens in the presence of cholesterol and phospholipid are able to form nanoparticles, which can be rapidly transported from the injection site throughout the body and retained for longer periods to facilitate the antigen presentation and enhance the immune responses (Watson et al., 1989). It cannot be ruled out that ECMS components and antigens form complexes in the body after injection. Therefore, we assume that the optimal dose of ECMS might be different antigens when different vaccine antigens are used.

Lymphocyte proliferation assay is widely used to evaluate cell-mediated immune responses in normal

and disease states in chickens (Martin et al., 1994; Miyamoto et al., 2002). Stimulated lymphocyte proliferation depends on the mitogen used. Concanavalin A and PHA primarily stimulate T-cell division, whereas LPS stimulates B cells (Toivanen and Toivanen, 1973; Lee, 1974; Weber, 1975; Lassila et al., 1976; Hovi et al., 1978; Sharma and Tizard, 1984; Grasman, 2002). A significantly higher lymphoproliferation response to ConA or PHA was recorded in birds treated with ECMS at all 3 doses (ConA) or at a dose of 40 µg (PHA) than the control, suggesting that T lymphocytes were activated when IBDV vaccine was used with ECMS. Significantly higher lymphoproliferation response to LPS was recorded in the 40 µg of ECMS-treated group, suggesting that B cells were activated. Traditional IBD vaccines have focused on stimulating the humoral immunity, whereas studies have shown that the cell-mediated immunity is also important in IBDV infections (Rautenschlein et al., 2002; Williams and Davison, 2005; Hung et al., 2009). The present data have revealed that cellular immunity is activated when ECMS is incorporated into IBDV vaccine. However, no cellular response of ECMS was recorded in an earlier study (Xiao et al., 2009) when the birds were immunized with Newcastle disease vaccine. This difference may be attributed to the difference in the nature of antigens present in the inoculated vaccines.

To observe the effect of ECMS treatment on the growth performance in chickens, BW gain was measured weekly throughout the study. Compared with the control, increased BW gain was found in the birds injected with IBD vaccine containing ECMS, with a significant difference ($P < 0.05$) found in the birds treated with ECMS at a dose of 20 µg. No significant change

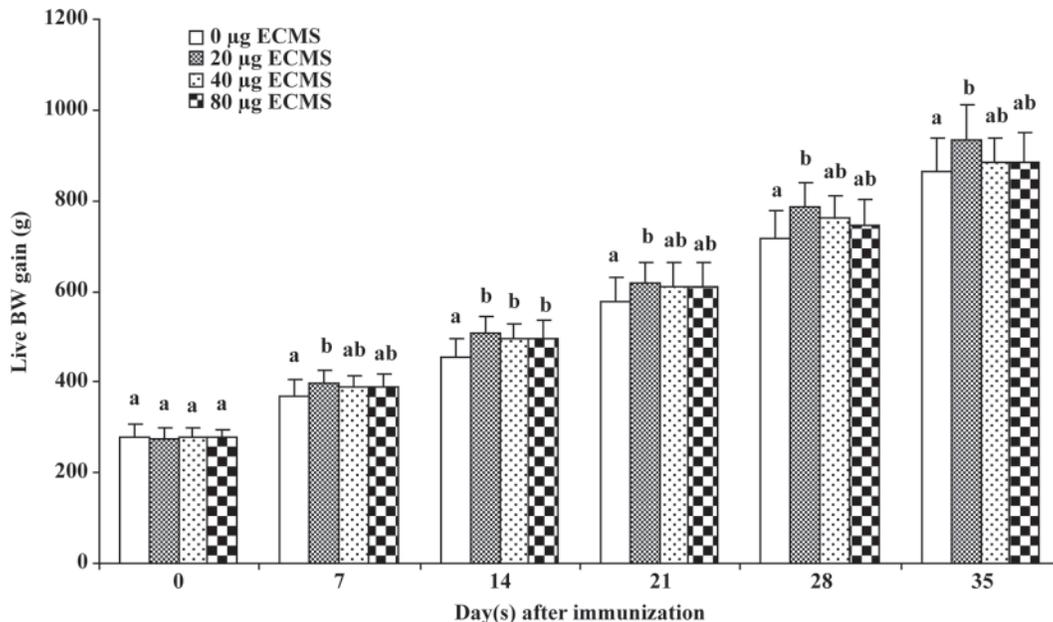


Figure 3. Growth performance of chickens. Body weight of each bird (n = 13) was measured on 0, 7, 14, 21, 28, and 35 d postimmunization and compared among the groups. Bars with different letters at the same time point are significantly different ($P < 0.05$). ECMS = extract of *Momordica cochinchinensis* seed.

was reported when chickens were immunized with Newcastle disease vaccine plus ECMS in the study by Xiao et al. (2009). Such difference may be due to incorporation of ECMS into different antigens. Positive or negative effects of dietary saponins have been well documented (Ishaaya et al., 1969; Sim et al., 1984; Mader and Brumm, 1987; Jenkins and Atwal, 1994; Makkar and Becker, 1996; Wang et al., 2000a,b). In ruminants and other domestic animals including chickens, the dietary saponins have significant effects on all phases of metabolism (Cheeke, 1996).

Based on our observations of the adjuvant activities and the effects on growth performance, ECMS deserves further study to improve bird vaccines.

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