# Effects of dietary egg yolk antibody powder on growth performance, intestinal *Escherichia coli* colonization, and immunocompetence of challenged broiler chicks

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**ABSTRACT** The present study aimed to investigate the effects of dietary supplementation of different levels of specific IgY (sIgY) and nonspecific IgY (nsIgY) egg yolk antibody powder on growth performance, immune functions, and intestinal morphology of *Escherichia coli* O78:K80-challenged broiler chicks. Lyophilized antibody isolated by the water-dilution method was obtained from the eggs of laying hens hyperimmunized with *E. coli* O78:K80. A total of 392 broiler chicks were randomly assigned to 7 dietary treatments with 4 replicates of 14 chicks (7 males and 7 females) each. Before offering the experimental diets, 7-d-old broiler chicks (except the negative control) were challenged orally with 0.5 mL ($10^9$ cfu/mL) of *E. coli* O78:K80 suspension. The challenge was continued for an additional 7 d from d 14 to 21 with 1.0 mL of a late log phase culture ($10^9$ cfu/mL) until the level of *E. coli* in feces reached $10^5$ cfu/g. The 6 challenged groups received a basal diet supplemented with 0 (as positive control), 0.1, 0.2, or 0.4% (wt/wt) sIgY from eggs of immunized hens or levels of 0.2 or 0.4% (wt/wt) nsIgY from eggs laid by the nonimmunized hens. The negative control group was fed with the same unsupplemented diet. Oral infection caused an increase in ileal *E. coli* enumeration, total blood leukocytes, heterophil:lymphocyte ratio, the concentration of serum and intestinal secretory IgA, and the numbers of jejunal goblet cells and lamina propria lymphatic follicles. After 3 wk of feeding, the levels of 0.2 and 0.4% sIgY and 0.4% nsIgY had the most suppressive effects ($P < 0.01$) on the ileal *E. coli* enumeration and secretory IgA concentration. However, serum IgA concentration was slightly decreased only at the presence of 0.4% sIgY and nsIgY. Dietary supplementation with at least 0.2% sIgY decreased ($P < 0.05$) the circulating heterophil:lymphocyte ratio. Inclusion of both sIgY and nsIgY increased the villus height:crypt depth ratio and decreased the jejunal goblet cells and lamina propria lymphatic follicle numbers, with the most pronounced effects assigned to sIgY-supplemented groups. The best feed conversion ratio was obtained when the dietary inclusion of at least 0.2% sIgY continued for 3 wk. The present results indicate that dietary administration of at least 0.2% sIgY for 3 wk improved the intestinal health indices and immunological responses of broiler chicks orally challenged by *E. coli* O78:K80.

**Key words:** broiler chick, egg yolk antibody (immunoglobulin Y) powder, *Escherichia coli* O78:K80, oral passive immunity, mucosal morphology

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## INTRODUCTION

During the last years, the extended research works developed the approaches to prevent and to control bacterial infections in the poultry industry. These methods include improved hygienic methods, vaccine applications, use of competitive exclusion products, and antimicrobial chemotherapy (with increased resistance to antimicrobial agents), possessing limited success (Gomis et al., 2003; La Ragione et al., 2004; Knezevic and Petrovic, 2008). Therefore, there is an increasing inclination in the use of novel protective strategies such as oral passive immunotherapy. One of the major strategies to achieve public health in human and domestic animal species is the efficient, noninvasive, and cost-effective production of specific antibodies against various antigens that can be applied in medicine, veterinary medicine, and research (Stockwin and Holmes, 2003). Chicken egg yolk antibody (IgY) has received special attention in recent years because it can be easily produced in a high quantity and is both feasible and safe (Gassmann et al., 1990).


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Administration of IgY to control the enteric pathogens has been shown to be effective for the inhibition of several enteric pathogens including enteropathogenic *Escherichia coli* in pigs (Girard et al., 2006), enterotoxigenic *E. coli* in early weaned pigs (Owusu-Asiedu et al., 2003), *Salmonella* spp. in neonatal calves (Yokoyama et al., 1998), *Helicobacter pylori* in Mongolian gerbils (Nomura et al., 2005), *Vibrio anguillarum* in rainbow trout (Arasteh et al., 2004), *Candida albicans* in mice (Ibrahim et al., 2008), and parvovirus in dogs (Van Nguyen et al., 2006). Compared with these positive results, Wilkie et al. (2006) suggested that oral administration of IgY did not suppress the intestinal *Clostridium perfringens* colonization in the challenged birds during the 72-h experimental period. Similarly, Fulton et al. (2002) observed that the ducks supplemented with anti-*Salmonella* Enteritidis antibodies in drinking water were only partially protected against *Salmonella* Enteritidis challenge; however, full protection was observed only with the co-administration of a probiotic. Although the positive results have been obtained for some pathogens in animal species, however, there are limited reports published on the ability of IgY to reduce or eliminate enteric pathogens in poultry.

On the other hand, strains of *E. coli* serotype O78 have been found to cause the numerous extra- and intraintestinal clinical symptoms in various hosts (Adiri et al., 2003). In the poultry industry, colibacillosis caused by avian pathogenic *E. coli* (**APEC**), especially serogroups O1, O2, and O78, is the primary cause of morbidity, mortality, and condemnation of carcasses worldwide (Gomis et al., 2001; Ewers et al., 2004). Interestingly, the results of many studies have indicated that *E. coli* strains isolated from the majority of the flocks belonged almost exclusively to the O78 serotype (Adiri et al., 2003; McPeake et al., 2005; Zhao et al., 2005). Therefore, *E. coli* O78:K80 can be a suitable model for investigation of specific IgY (**sIgY**) inhibitory effects on experimentally challenged birds.

Because there are limited data concerning the ability of IgY to reduce enteric pathogens in poultry species, the present study was designed to evaluate the efficacy of dietary supplementation of IgY powder for protection of *E. coli* O78:K80-challenged birds as a model and to investigate the effects of dietary antibody administration on performance and immunological responses of broiler chicks.

## MATERIALS AND METHODS

### Antigen Preparation

The bacterial strain of *E. coli* O78:K80 was obtained from Razi Vaccine and Serum Research Institute (Karaj, Iran). To ensure viability of the culture, bacteria was grown in tryptic soy broth (Merck, Darmstadt, Germany) for 6 h (until late log phase) at 37°C with shaking at 180 rpm. Cells were harvested by centrifugation at 5,000 × *g* for 15 min at 4°C (Sigma 6K15 laboratory centrifuge, Osterode am Harz, Germany). The pellet was washed 3 times with PBS (pH 7.2) and resuspended in a quantity of PBS to achieve a concentration of $2 \times 10^9$ cfu/mL. To prepare the killed vaccine, 100 mL of the bacterial suspension was sonicated in an ice bath using a microtip (Ultraschallprozessor, Dr. Hielscher GmbH, Stuttgart, Germany) at maximum amplitude for 15 min and the lysate was filtered through a 0.45-μm sterile filter (Acrodisc Gelman Sciences, Mississauga, Ontario, Canada). Sterility of the filtrate was confirmed by plating it on tryptic soy agar. The prepared vaccine was stored at −20°C before the immunization of laying hens could be performed.

### Antibody Preparation

Forty 36-wk-old Single Comb White Leghorn hens were immunized intramuscularly at 2 different sites (0.5 mL per site) of breast muscle. Antigen was produced (as described above) as cell-containing or free suspensions as the control. The bacteria was emulsified with an equal volume of Freund's complete adjuvant (CF112, Biogen, Mashhad, Iran) for the first immunization, Freund's incomplete adjuvant (CF111, Biogen) for booster immunization at the second week, and was then administered without adjuvant at the fourth week after the initial immunization, as described by Ruan et al. (2005). The immunization dose was approximately $1.0 \times 10^9$ cfu of *E. coli* O78:K80 in a volume of 1.0 mL equally divided between 2 injection sites. Eggs were collected daily for 12 wk, starting from the day of initial immunization, stored at 4°C, and processed maximally within 7 d after laying. For antibody preparation, the water-soluble fraction (**WSF**) of egg yolk, as a resource of IgY, was isolated as described by Akita and Nakai (1992) with minor modifications. Briefly, egg yolk was separated from egg white and the yolk membrane was punched. The membrane-free yolk was transferred into a graduated cylinder and mixed with 6 volumes of cold acidified distilled water (pH 2.5 adjusted by 0.1 *M* HCl). The mixture was then adjusted to a pH value of 5.0 to 5.2 and incubated at 4°C for 12 h. After centrifugation at 12,000 × *g* and 4°C for 20 min, the supernatant was considered as a WSF and was then lyophilized (freeze dryer, DW 8030, Heto Holten, Allerød, Denmark) and stored at −20°C until further use.

### Protein Assay

Total protein concentration of IgY powder was determined by the Bradford (1976) method. Bovine serum albumin (Sigma Chemical Co., St. Louis, MO) was used as the reference protein.

### ELISA

Antibody-specific activities of laying hen's sera and WSF were measured by ELISA as described by Sunwoo et al. (2002) with minor modifications. A 96-well micro-

titer plate (Nunc MaxiSorp C bottom well modules and frames, 445101, Bethyl Laboratories Inc., Montgomery, TX) was coated overnight at 4°C with 200 μL of $10^6$ cfu sonicated *E. coli* O78:K80 in carbonate-bicarbonate buffer (0.05 *M*, pH 9.6; Sigma Chemical Co., St. Louis, MO). The plate was washed 3 times with washing solution containing 50 m*M* Tris buffered saline at the pH value of 8.0 and 0.05% Tween 20 (**TBS-T**; Sigma Chemical Co.). After washing, 200 μL of TBS-T was added to each well and incubated at 37°C for 30 min as the blocking step. Thereafter, the plate was washed with TBS-T and samples (100 μL) were added. Sera were diluted 1,000× and the WSF, obtained by 6× dilution of yolk (see above), was further (167 times) diluted to obtain 1,000× dilution. Diluted samples were added to the wells and incubated at 37°C for 90 min. After incubation, the plate was washed 3 times with TBS-T and 100 μL of goat anti-chicken IgG conjugated with horseradish peroxidase (A30-104P-25, Bethyl Laboratories Inc.), diluted $10^{-5}$ with TBS-T, was added and incubated at 37°C for 90 min. The plate was then washed with TBS-T and incubated for an additional 30 min with 100 μL of substrate solution (tetramethylbenzidine:$H_2O_2$, 1:1 by vol/vol; Kirkegaard and Perry Co., Gaithersburg, MD). The reaction was stopped with the addition of 100 μL of 2 *M* $H_2SO_4$ and absorbance was measured at 450 nm using an automated spectrophotometer (Ultramicroplate Reader, ELx808, Bio-Tek Instruments Inc., Winooski, VT). Titers were calculated as mean ± SE of the $\log_{10}$ of the dilution multiple in the ratio of sample optical density to control (nonimmunized hen's serum or WSF) optical density.

To measure the total IgY concentration of IgY powder, ELISA was performed as described above with chicken IgG ELISA quantitation kit (E30-104, Bethyl Laboratories Inc.; Schuijffel et al., 2005) except that the microtiter plate was coated with 100 μL of goat anti-chicken IgG at a final concentration of 10 μg/mL.

## Chicks, Diets, and Experimental Design

For the prevention study, broiler chicks (Ross 308) were challenged orally with 0.5 mL of *E. coli* O78:K80 suspension at d 7 and then for additional 7 consecutive days from d 14 to 21 with 1.0 mL of a late log phase culture ($10^9$ cfu/mL) until the level of *E. coli* in feces reached to $10^5$ cfu/g (measured using spread plate method as described below). In vivo experiment was conducted using 392 broiler chicks in a completely randomized design with 7 dietary treatments (6 challenged groups and 1 unchallenged group as negative control) and 4 replicates of 14 birds (7 males and 7 females). The 6 challenged groups received normal isocaloric-isonitrogenous corn-soybean meal-based diets (Table 1) supplemented with 0 (as positive control), 0.1, 0.2, or 0.4% (wt/wt) sIgY from eggs of immunized hens or with 0.2 or 0.4% (wt/wt) nonspecific IgY powder (**nsIgY**) from eggs laid by the nonimmunized hens. The negative (unchallenged) control group received the same basal diet without antibody fortification. All experimental diets were formulated to meet or exceed NRC (1994) nutrient recommendations for broiler chicks during the grower period. Chicks had free access to water and to the experimental diets at all times. Body weight and feed consumption were measured weekly and the average daily weight gain and daily feed intake as well as feed conversion ratio were determined over 4 age periods (21 to 28, 29 to 35, 36 to 42, and 21 to 42 d of age).

## Intestinal E. coli Enumeration

On d 21, 28, and 42, the entire contents of the ileum from Meckel's diverticulum to the ileocecal junction were carefully removed from 2 birds per replicate. The collected contents were then mixed thoroughly, placed in the sterile tubes, and kept in an ice-covered box, then immediately transferred to the laboratory. A volume containing 1 g of feces was serially (1:10) diluted to $10^{-6}$ using 0.85% NaCl, and 0.1 mL of each dilution was plated in duplicate onto Eosin Methylene Blue agar plates (Merck). The inoculated plates were incubated at 37°C for 24 h and *E. coli* colonies were confirmed using biochemical tests of indole, methyl red, Voges-Proskauer, and citrate reactions. The number of colony-forming units per plate was counted by spread

**Table 1.** The composition and nutrient content of basal diet (21 to 42 d of age)

| Item | Amount |
|---|---|
| Ingredient (%) | |
| Yellow corn | 67.00 |
| Soybean meal[1] | 29.00 |
| Oyster shell | 1.60 |
| Dicalcium phosphate | 1.20 |
| Common salt | 0.20 |
| Vitamin premix[2] | 0.25 |
| Mineral premix[3] | 0.25 |
| DL-Methionine | 0.06 |
| Na bicarbonate | 0.44 |
| Calculated analysis | |
| ME (kcal/kg) | 2,893 |
| CP (%) | 18.15 |
| Methionine (%) | 0.36 |
| Methionine + cysteine (%) | 0.67 |
| Lysine (%) | 0.95 |
| Calcium (%) | 0.84 |
| Available phosphorus (%) | 0.35 |
| Na (%) | 0.2 |
| K (%) | 0.78 |
| Cl (%) | 0.16 |
| Electrolyte balance (mEq/kg) | 249.3 |

[1]Immunoglobulin Y powder in all experimental diets was substituted with the equal quantity of soybean meal.

[2]Vitamin premix provided the following per kilogram of diet: vitamin A (from vitamin A acetate), 9,800 IU; cholecalciferol, 2,100 IU; vitamin E (from DL-α-tocopheryl acetate), 22 IU; riboflavin, 4.4 mg; nicotinamide, 40 mg; calcium pantothenate, 35 mg; menadione, 1.50 mg; folic acid, 0.80 mg; thiamine, 3 mg; pyridoxine, 10 mg; biotin, 1 mg; choline chloride, 560 mg; and ethoxyquin, 125 mg.

[3]Mineral premix provided the following per kilogram of diet: Mn, 65 mg; Zn, 55 mg; Fe, 50 mg; Cu, 8 mg; I [from Ca $(IO_3)_2 \cdot H_2O$], 1.8 mg; and Se, 0.30 mg.

plate method to determine the total number of bacterial colony-forming units per gram of feces.

### Measurement of Serum and Intestinal Mucosal Secretory IgA

On d 21 and 42, two chicks per replicate were randomly killed to examine the concentration of serum and secretory IgA (**sIgA**) with chicken IgA ELISA quantitation kit (E30-103, Bethyl Laboratories Inc.; Schuijffel et al., 2005). Blood samples were collected from the jugular vein and sera were collected by centrifugation. Sera samples were harvested and stored (−20°C) before analysis. To measure the sIgA, the 2-cm segments of jejunal region anterior to Meckel's diverticulum were excised and then exposed. The mucus was collected by scraping the mucosal surface of the intestine and weighed. Thereafter, the mucus from each chick was suspended in a 4-fold volume of PBS (wt/wt), vortexed thoroughly, and centrifuged at 5,000 × *g* for 30 min at 4°C. After centrifugation, the supernatant was removed and stored at −80°C for further analysis. Before measurement of the intestinal antibody response, total protein content of intestinal washings was measured using the Bradford (1976) method (as described above). The total protein was used as the source of primary antibody after the protein content of the initial dilution was kept constant across all groups. An ELISA was performed as described above for IgY, except that the microtiter plate was coated with 100 μL of goat anti-chicken IgA and goat anti-chicken IgA conjugated with horseradish peroxidase (A30-103P-27, Bethyl Laboratories Inc.) diluted 1/75,000 with TBS-T.

### Measurement of Circulating Differential Leukocyte Count

At d 42, the EDTA-containing blood samples of 2 random chicks per cage were taken to determine the differential leukocyte count. Total blood cells were counted by Automated Hematology Analyzer (KX21N, Sysmex, Kobe, Japan). Total and differential counts of leukocytes were made by screening a Gimsa-stained slide. The different subpopulations of leukocytes were counted and the heterophil:lymphocyte ratios were calculated as described by Stedman et al. (2001).

### Measurement of Jejunal Histological Changes

At the end of the trial, 2 chicks were randomly killed from each replicate to determine the effects of dietary treatments and oral *E. coli* O78:K80 challenge on intestinal cell morphology. Tissue samples were collected and a 2-cm segment of jejunal region anterior to Meckel's diverticulum was excised for light microscopic observations. The histological sections were immediately fixed in 10% formaldehyde solution and the fixed samples were embedded in paraffin. Transverse and longitudinal sections that were 5 μm thick were prepared using microtome, then stained with hematoxylin-eosin (HE) and examined under the light microscope (Sun et al., 2005).

### Statistical Analysis

All data were analyzed using the GLM procedure of SAS software (SAS Institute, 2001). The following model was assumed in the analysis of all studied traits: $Y_{ij} = \mu + A_i + e_{ij}$, where $Y_{ij}$ = observed value for a particular character; $\mu$ = overall mean; $A_i$ = effect of the ith treatment; and $e_{ij}$ = random error associated with the ijth recording. Least squares method was used to identify the significant differences between *E. coli* O78:K80-challenged groups at the 0.05 significance levels. Single degree of freedom contrasts were made among treatment means to compare negative versus positive control group, positive control versus sIgY-fed groups, and also positive control versus nsIgY-fed ones.

## RESULTS AND DISCUSSION

Intensification of poultry production systems accompanied by consumer fears over antibiotic residues and resistance have increased the need to find alternatives for antibiotic in animal feed. One of the highly fascinating and efficacious alternative approaches to control the intestinal pathogens is the dietary administration of immunoglobulins, particularly IgY. The advantage of feeding antibodies is that they provide a continuous control for potential pathogens and do not result in the development and establishment of antibiotic-resistant strains of microorganisms. Although the beneficial effects of IgY have been demonstrated for some pathogens in the animal models (Yokoyama et al., 1998; Owusu-Asiedu et al., 2003; Arasteh et al., 2004; Nomura et al., 2005; Girard et al., 2006; Van Nguyen et al., 2006; Ibrahim et al., 2008), few studies with various results have been done with poultry species.

### IgY Powder Properties

In our preliminary experiences (unpublished data), after the first immunization of laying hens, serum and egg yolk antibody-specific activities increased 4-fold and remained at the highest levels from d 7 and 14, respectively, and this priority remained constant throughout the experimental period (up to 12 wk). Initial evaluations of crude antibody preparation of nonimmunized hens indicated that the specific antibody activity against *E. coli* O78:K80 in WSF was only about one-fourth as activity in WSF obtained from eggs of immunized hens. In addition, our in vitro trial showed that the lyophilized antibody powder had considerable activity against *E. coli* O78:K80 culture (data not shown). Although total IgY concentration and IgY purity (total IgY in protein) of sIgY were approximately 30% higher

**Table 2.** Effect of supplemental specific and nonspecific IgY powder in broiler diets on ileal *Escherichia coli* enumeration

| | Count[1] ($\log_{10}$ cfu/g) | | |
|---|---|---|---|
| Treatment | Day 21 | Day 28 | Day 42 |
| Negative control (NC)[2] | 3.64 | 4.43 | 5.07 |
| Positive control (PC)[2] | 5.17 | 5.87 | 6.87[a] |
| sIgY[3] (%) | | | |
| 0.1 | 5.44 | 6.1 | 6.71[ab] |
| 0.2 | 5.36 | 5.65 | 5.97[c] |
| 0.4 | 5.53 | 5.89 | 6.14[c] |
| nsIgY[3] (%) | | | |
| 0.2 | 5.24 | 6.02 | 6.9[a] |
| 0.4 | 5.6 | 5.76 | 6.63[b] |
| | | *P*-value | |
| Treatments[4] | 0.77 | 0.36 | 0.01 |
| Contrasts | | | |
| NC vs. PC | 0.01 | 0.01 | 0.01 |
| PC vs. sIgY | 0.31 | 0.92 | 0.01 |
| PC vs. nsIgY | 0.39 | 0.91 | 0.25 |
| sIgY vs. nsIgY | 0.91 | 0.44 | 0.01 |
| SEM | 0.23 | 0.15 | 0.08 |

[a–c]Means with no common superscript within each column are significantly ($P < 0.05$) different.

[1]The number of *E. coli* was calculated as $\log_{10}$ cfu per gram of ileal content.

[2]Negative and positive controls consisted of unchallenged and challenged birds, respectively, without dietary IgY powder supplementation.

[3]sIgY = specific IgY powder; nsIgY: nonspecific IgY powder; the different levels of specific and nonspecific IgY powder were supplemented in the diets of *E. coli* O78:K80-challenged birds during d 21 to 42.

[4]*P*-value was calculated for studied variables on *E. coli* O78:K80-challenged birds.

($P < 0.05$) than nsIgY, the protein concentrations of sIgY and nsIgY were similar and about 470 mg/g of powder. Based on these observations, the sIgY were prepared from the eggs of immunized laying hens from d 21 to the end of the experimental period (12 wk).

## Enumeration of Ileal E. coli in Broiler Chicks

After challenge of broiler chicks for 7 consecutive days (d 14 to 21), the number of *E. coli* in the ileal content was increased significantly compared with the negative control group and reached about $10^5$ cfu/g of feces (Table 2). The mean $\log_{10}$ number of *E. coli* in the ileal samples of challenged birds was $5.39 \pm 0.09$ cfu/g ($\log_{10}$ cfu/g $\pm$ SE) at d 21. The ileal *E. coli* enumeration of challenged birds remained significantly ($P < 0.01$) higher than that of the negative control throughout the trial. Adherence is a necessary prerequisite for *E. coli* colonization and pathogenesis. Although the role of fimbriae and adhesion in APEC infections has mainly been studied in relation to their possible function in bacterial colonization of the chicken respiratory tract (Dho-Moulin and Fairbrother, 1999), other interesting studies (Harry and Hemsley, 1965; La Ragione et al., 2000; Edelman et al., 2003) have indicated that these bacteria could bind to intestinal epithelium and colonize effectively. The initial study reported that 10 to 15% of the isolated *E. coli* from intestinal flora of healthy chickens may belong to an O-serotype associated with colibacillosis lesions, indicating that intestinal colonies may act as a reservoir for virulent *E. coli* (Harry and Hemsley, 1965). Edelman et al. (2003) found that the *E. coli* O78-type 1 fimbriae adhered efficiently to the crop and intestinal epithelial cells. They also indicated that AC/I fimbriae could bind to goblet cells. Similarly, the study by La Ragione et al. (2000) conclusively approved the essential role of flagella and type 1 and curli fimbriae for persistent intestinal colonization by *E. coli* O78:K80. Our results reinforce the previous reports that *E. coli* O78:K80, as one of the APEC serotypes, may bind to the intestinal surface, proliferate, and remain in intestinal contents for a long period.

Although administration of different sIgY and nsIgY levels showed no significant effect in light of ileal *E. coli* count of challenged birds at d 28, dietary supplementation with IgY caused marked ($P < 0.01$) differences in enumeration of ileal *E. coli* in challenged birds at d 42. The effective doses for reduction of ileal *E. coli* enumerations were 0.2 and 0.4% sIgY and 0.4% nsIgY, respectively. This finding is consistent with previous reports, demonstrating that sIgY administration is capable of controlling enteric pathogens in animal models (Yokoyama et al., 1998; Owusu-Asiedu et al., 2003; Arasteh et al., 2004; Van Nguyen et al., 2006; Ibrahim et al., 2008). In contrast to these positive results, Wilkie et al. (2006) reported that although the antibody-specific activity was detectable after consuming IgY-amended feed throughout the intestine, oral supplementation of IgY did not reduce intestinal *C. perfringens* in challenged birds during 72 h. Although the differences in the bacterial species may be an effective factor in the response to IgY administration, it is probable that short-time administration of IgY for bac-

terial inhibition might be a reason for ineffectual IgY inclusion. For effective oral IgY administration, it is of great importance to diagnosis the most suitable virulence factors that should be used to immunize laying hens. It is demonstrated that virulence factor-specific antibodies appear to be the most effective when they target specific virulence factors such as attachment fimbriae or outer membrane proteins of pathogens (Yokoyama et al., 1998; Kobayashi et al., 2004). In this experiment, we used the sonicated bacteria for the immunization of laying hens, which contained the mixture of virulence factors of *E. coli* O78:K80. Kariyawasam et al. (2004) demonstrated that the performance of the mixtures of antibodies seemed to reflect an additive effect of the component antibodies and tended to be intermediate compared with the best and worst single specificity IgY. Table 2 indicates that the dynamics of antibody administration can be time-dependent, and the constant administration of antibody would be more effective in inhibition of pathogens for prophylaxis or therapy of enteric infection. Overall, these results suggest that the properties of immunizing antigen for IgY production, and also the period and dosage of IgY administration for passive immunity, are very important for effective oral IgY inclusion.

## Serum and Intestinal Mucosal sIgA

After oral infection, the concentration of serum IgA was increased and remained significantly higher ($P < 0.01$) compared with the negative control throughout the experimental period (Table 3). The challenged birds had serum IgA that had been decreased slightly in the presence of 0.4% sIgY and nsIgY at 42 d of age. This response indicated that *E. coli* O78:K80 (as an enteropathogenic bacterium) might strongly induce the generation and secretion of serum and mucosal IgA. It has been demonstrated that the mucosal immunity response is very sensitive. On the other hand, not only the enteropathogenic bacteria (Sheela et al., 2003; Kulkarni et al., 2007) but also the commensal bacteria such as probiotics (Perdigon et al., 1991) may be capable of inducing IgA secretion. Our data suggest that IgA response was time-dependent and the concentrations of serum and sIgA were enhanced with age. Similar to a previous report (Sheela et al., 2003), it suggests that the mucosal immunity matures gradually with the age. Hedge et al. (1982) argued that young birds are less exposed to environmental antigens, and this makes their germinal centers less developed and consequently not as immunocompetent as older birds.

After challenge, the concentration of jejunal sIgA was significantly increased ($P < 0.01$) and remained at the high level to the end of trial compared with the negative control group. In challenged birds, the concentration of sIgA was overshadowed by administration of different sIgY and nsIgY levels at d 42. Although the concentration of sIgA was decreased in the presence of the highest sIgY or nsIgY levels, the level of 0.4% sIgY had the most suppressive effect on sIgA concentration in challenged birds. These findings are parallel with the results of ileal bacterial enumeration. As described,

**Table 3.** Effect of dietary specific and nonspecific IgY powder supplementation on serum immunoglobulin A (IgA) and intestinal mucosal secretory IgA (sIgA) in broiler chickens

| | IgA (μg/mL) | | sIgA[1] (μg/mg of protein) | |
|---|---|---|---|---|
| Treatment | Day 21 | Day 42 | Day 21 | Day 42 |
| Negative control (NC)[2] | 316.1 | 463.6 | 12.81 | 18.07 |
| Positive control (PC)[2] | 496.7 | 721.7 | 17.44 | 29.28[a] |
| sIgY[3] (%) | | | | |
| 0.1 | 476.9 | 673.6 | 15.87 | 26.52[ab] |
| 0.2 | 512.6 | 663.5 | 16.84 | 23.81[bc] |
| 0.4 | 466.4 | 558.0 | 16.87 | 20.01[c] |
| nsIgY[3] (%) | | | | |
| 0.2 | 546.4 | 734.5 | 17.24 | 29.45[a] |
| 0.4 | 465.9 | 593.7 | 14.46 | 24.19[bc] |
| | | | *P*-value | |
| Treatments[4] | 0.83 | 0.36 | 0.63 | 0.01 |
| Contrasts | | | | |
| NC vs. PC | 0.01 | 0.01 | 0.02 | 0.01 |
| PC vs. sIgY | 0.83 | 0.21 | 0.58 | 0.01 |
| PC vs. nsIgY | 0.87 | 0.45 | 0.36 | 0.24 |
| sIgY vs. nsIgY | 0.73 | 0.39 | 0.48 | 0.01 |
| SEM | 48.43 | 65.85 | 1.34 | 1.73 |

[a–c]Means with no common superscript within each column are significantly ($P < 0.05$) different.

[1]The concentration of sIgA was expressed as micrograms of IgA per milligram of intestinal mucosal washing protein.

[2]Negative and positive controls consisted of unchallenged and challenged birds, respectively, without dietary IgY powder supplementation.

[3]sIgY = specific IgY powder; nsIgY = nonspecific IgY powder; the different levels of specific and nonspecific IgY powder were supplemented in the diets of *E. coli* O78:K80-challenged birds during d 21 to 42.

[4]*P*-value was calculated for studied variables on *E. coli* O78:K80-challenged birds.

supplementation of diets with 2 higher levels of sIgY or the highest level of nsIgY caused a significant ($P < 0.01$) decrease in ileal *E. coli* count of challenged birds on d 42. Therefore, it could be logical that with decrease in intestinal pathogens, the mucosal IgA generation and secretion would reduce.

After using a polyclonal (outcome from whole bacterial cells) antibody, it may be more possible to raise specific antibodies against cross-reacting antigens (Lee et al., 2002). The cross-reactivity of IgY may provide an additional advantage for food application of IgY due to its antibacterial effects on other pathogenic bacteria as well as target bacteria for prophylaxis or therapeutic use (Sunwoo et al., 2002). Nevertheless, the results of Lee et al. (2002), Sunwoo et al. (2002), and Amaral et al. (2008) showed that the most reactivity and growth inhibitory effects of IgY were only toward the homologous strain. The comparison of equal levels of sIgY and nsIgY suggests that not only the concentration and specific activity of *E. coli* O78:K80-sIgY was higher in sIgY than nsIgY (as described above), but also it is probable that the reduced ileal *E. coli* enumeration after feeding sIgY is mainly related to reduction of O78:K80 and a range of homologous serotypes in the intestinal contents.

The concentration of serum IgA was decreased slightly ($P = 0.21$) in the presence of 0.4% sIgY and nsIgY at d 42 of age. These data suggest that the trend in IgA antibody response at the intestinal site is mirrored in serum IgA concentration, which is consistent with the results of Muir et al. (2002). Overall, the present findings indicate that the dietary administration of 0.4% sIgY, as the most effective level, could reduce the concentration of sIgA via inhibition of intestinal bacterial proliferation in challenged birds and also may reduce ($P = 0.21$) serum IgA concentration as a sIgA-dependent variable.

## Differential Circulating Leukocyte Count

The numbers of total leukocytes and heterophils as well as the heterophil:lymphocyte ratio were significantly ($P < 0.01$) raised after oral infection with *E. coli* O78:K80 (Table 4). This response was mainly due to the increase ($P < 0.01$) in heterophil counts. Although the numbers of lymphocytes, monocytes, and eosinophils were increased, these increases were not statistically significant. Edelman et al. (2003) indicated that *E. coli* O78:K80-type 1 fimbriae in addition to intestinal epithelium cells could bind to follicle-associated epithelium in the ileum, which is recognized as an important site for invasion for enteric pathogens. It stimulates the migration of leukocytes (located in almost every mucosal tissue) for an increased chance to encounter specific antigens (Bimczok and Rothkötter, 2006). It has been shown that the avian heterophils, as the predominant phagocyte polymorphonuclear leukocyte, form the first line of avian cellular defense against bacterial infection. Heterophil activity would restrict bacterial proliferation to a level permitting more efficient elimination of bacteria by the subsequent host defenses (Mellata et al., 2003).

Inclusion of 0.2 and 0.4% sIgY had the most modulatory effect on absolute count of heterophils and the heterophil:lymphocyte ratio. Dietary IgY powder supplementation was not observed to have any significant effect on the enumeration of lymphocytes, monocytes, or eosinophils. Heterophil:lymphocyte ratio may be a good quantitative indicator for stress and bacterial endotoxin, so that bacterial lipopolysaccharide could

**Table 4.** Effects of different levels of specific and nonspecific IgY powder on total circulating leukocytes (TL), lymphocytes, heterophils, monocytes, eosinophils, and heterophil:lymphocyte ratios

| Treatment | TL ($\times 10^3/\mu L$) | Lymphocytes ($\times 10^3/\mu L$) | Heterophils ($\times 10^3/\mu L$) | Monocytes ($\times 10^3/\mu L$) | Eosinophils ($\times 10^3/\mu L$) | Heterophil: lymphocyte |
|---|---|---|---|---|---|---|
| Negative control (NC)[1] | 13.9 | 9.17 | 4.23 | 0.38 | 0.14 | 0.47 |
| Positive control (PC)[1] | 20 | 10.8 | 8.25[ab] | 0.65 | 0.31 | 0.79[a] |
| sIgY[2] (%) | | | | | | |
| 0.1 | 19.7 | 10.1 | 8.62[a] | 0.73 | 0.27 | 0.87[a] |
| 0.2 | 17.5 | 11.0 | 5.66[c] | 0.5 | 0.32 | 0.53[b] |
| 0.4 | 18.7 | 11.4 | 6.39[bc] | 0.58 | 0.38 | 0.63[ab] |
| nsIgY[2] (%) | | | | | | |
| 0.2 | 19.3 | 10.8 | 7.44[abc] | 0.72 | 0.36 | 0.72[ab] |
| 0.4 | 21.1 | 11.5 | 8.66[a] | 0.65 | 0.29 | 0.79[a] |
| | | | *P*-value | | | |
| Treatments[3] | 0.59 | 0.94 | 0.02 | 0.81 | 0.97 | 0.05 |
| Contrasts | | | | | | |
| NC vs. PC | 0.01 | 0.24 | 0.01 | 0.13 | 0.18 | 0.01 |
| PC vs. sIgY | 0.38 | 0.98 | 0.08 | 0.74 | 0.90 | 0.20 |
| PC vs. nsIgY | 0.90 | 0.77 | 0.81 | 0.82 | 0.86 | 0.74 |
| sIgY vs. nsIgY | 0.12 | 0.98 | 0.01 | 0.24 | 0.82 | 0.02 |
| SEM | 1.43 | 1.03 | 0.70 | 0.13 | 0.1 | 0.08 |

[a–c]Means with no common superscript within each column are significantly ($P < 0.05$) different.

[1]Negative and positive controls consisted of unchallenged and challenged birds, respectively, without dietary IgY powder supplementation.

[2]sIgY = specific IgY powder; nsIgY = nonspecific IgY powder; the different levels of specific and nonspecific IgY powder were supplemented in the diets of *Escherichia coli* O78:K80-challenged birds during d 21 to 42.

[3]*P*-value was calculated for studied variables on *E. coli* O78:K80-challenged birds.

**Table 5.** Effect of dietary specific and nonspecific IgY powder supplementation on histological changes of jejunum in broiler chickens

| Treatment | Villus height (μm) | Crypt depth (μm) | VCR[1] | GCN[1] | LLF[1] |
|---|---|---|---|---|---|
| Negative control (NC)[2] | 1,004 | 148 | 6.78 | 1+[3] | 1+ |
| Positive control (PC)[2] | 858[c] | 166 | 5.17[b] | 5+ | 5+ |
| sIgY[4] (%) | | | | | |
| 0.2 | 1,008[a] | 154 | 6.55[a] | 3+ | 2+ |
| 0.4 | 993[a] | 151 | 6.58[a] | 3+ | 2+ |
| nsIgY[4] (%) | | | | | |
| 0.4 | 961[b] | 161 | 5.97[a] | 3+ | 4+ |
| | | *P*-value | | | |
| Treatments[5] | 0.01 | 0.19 | 0.01 | | |
| Contrasts | | | | | |
| NC vs. PC | 0.01 | 0.02 | 0.01 | | |
| PC vs. sIgY | 0.01 | 0.04 | 0.01 | | |
| PC vs. nsIgY | 0.01 | 0.48 | 0.01 | | |
| sIgY vs. nsIgY | 0.15 | 0.19 | 0.16 | | |
| SEM | 11.15 | 5.33 | 0.22 | | |

[a–c]Means with no common superscript within each column are significantly ($P < 0.05$) different.

[1]VCR = villus height:crypt depth ratio; GCN: goblet cell numbers; LLF: lamina propria lymphatic follicles.

[2]Negative and positive controls consisted of unchallenged and challenged birds, respectively, without dietary IgY powder supplementation.

[3]Number of +'s indicates severity of the histological changes.

[4]sIgY = specific IgY powder; nsIgY = nonspecific IgY powder; the different levels of specific and nonspecific IgY powder were supplemented in the diets of *Escherichia coli* O78:K80-challenged birds during d 21 to 42.

[5]*P*-value was calculated for studied variables on *E. coli* O78:K80-challenged birds.

increase this proportion (Shini et al., 2008). In the presence of 0.2 and 0.4% sIgY, the ratio of heterophils to lymphocytes was significantly decreased in challenged birds. We postulate 2 mechanisms for this depression. First, the significant decrease in ileal *E. coli* enumeration after using these sIgY levels might reduce the pathogen stimulatory effects on the leukocyte migration and heterophil proliferation. Otherwise, in these levels, the concentrations of sIgA and thereby serum IgA were decreased (Table 3). It has been demonstrated that although sIgA exerts the first line of defense by limiting invasion of pathogens, serum IgA may act as a second defensive line (Otten and van Egmond, 2004). In contrast to sIgA, serum IgA has been defined as an inflammatory antibody that induces local inflammatory reactions via the activation of the complement system. These reactions include the influx of polymorphonuclear leukocytes and the release of cytokines (Snoeck et al., 2006). Therefore, it is possible that with the reduction of serum IgA concentration and its inflammatory effects, the frequency of heterophils would decrease. Consequently, the present findings suggest that although after oral infection the heterophil:lymphocyte ratio was increased, dietary administration of 0.2 and 0.4% sIgY could reduce this parameter by direct depression of local pathogens as well as an indirect decrease in inflammatory reactions induced by serum IgA.

## *Jejunal Histological Changes*

As shown in Table 5, the ratio of villus height to crypt depth was decreased significantly after oral infection. This observation resulted from the decrease ($P < 0.01$) in villus height and concomitant increase ($P = 0.02$) in crypt depth. However, the severity of depression was decreased after dietary administration of 0.2 or 0.4% sIgY or 0.4% nsIgY for 3 wk. After bacterial challenge, the increase of goblet cell numbers and proliferation of lamina propria lymphatic follicles were motivated, but utilizing 0.2 or 0.4% sIgY caused a remarkable decrease in the proliferation of lamina propria lymphatic follicles. These findings reinforce the previous observations that demonstrated that after exposure to pathogenic microbes, remarkable architectural and functional changes, such as increased permeability, increased production of enzymes and immunoglobulins, and decreased villus height:crypt depth ratio, occurred in the intestine (Mcfall-Ngai, 1998). Furthermore, recent studies indicated that bacterial adaptations of the villus have direct effects on the goblet cell dynamics and the mucus blanket via the local release of bioactive factors, or indirectly via the release of host proinflammatory cytokines triggered in response to pathogens (Van Kessel et al., 2004; Shirkey et al., 2006). These endogenous and exogenous immunomodulatory factors can induce an immune response to antigens, mediated by the gut-associated lymphoid tissue (**GALT**) as one of the most important immunological compartments of the body (Schat and Myers, 1991). In the present study, the significant increase in sIgA secretion by GALT after oral infection is a manifest witness for this response. Therefore, the results could suggest that *E. coli* O78:K80, as a pathogenic bacterium, could induce widespread structural and functional alterations in the intestine of broiler chickens.

Dietary administration of 0.2 or 0.4% sIgY or 0.4% nsIgY for 3 wk could improve the intestinal health indicators with more positive effects assigned to sIgY.

Specific IgY from hens immunized with plural bacterial antigens has several inhibitory effects on bacterial growth, toxin production, and adhesion to the intestinal cells (Sugita-Konishi et al., 1996), whereby it could increase the ratio of villus height to crypt depth and decrease the goblet cell numbers. The reduction of lamina propria lymphatic follicle proliferation, in addition to local effects of sIgY on mucosal surfaces, might be because IgY can be taken up by pinocytosis from the intestinal lumen and subsequently transported into the lamina propria, then passed into the lymph fluid without inducing an inflammatory response (Swarbrick et al., 1979).

## Growth Performance

The present results showed that the ADFI was not influenced by oral *E. coli* O78:K80 challenge or supplemental dietary IgY powder (Table 6). However, the bacterial challenge had a significant influence ($P < 0.01$) on feed conversion ratio after 2 wk. Although dietary administration of sIgY could improve ($P = 0.02$) the feed conversion ratio after 2 wk, the best results were obtained when the inclusion of at least 0.2% sIgY continued for 3 wk ($P < 0.01$). This improvement was largely due to the increase in average daily weight gain. Our findings indicated that although the best performance in challenged birds was observed after dietary sIgY supplementation (sIgY vs. nsIgY), the long-term (3 wk or longer) application of high levels of nsIgY could also improve ($P = 0.04$) the feed conversion ratio. It seems that the beneficial effects of sIgY on inhibition of bacteria and development of intestinal morphology such as increase in absorptive surface area and decrease in mucosal inflammation improve the health status of the gastrointestinal tract and subsequent growth performance.

As explained previously, using 2 higher levels of sIgY could increase the villus height:crypt depth ratio. The crypts of Lieberkuhn can be assumed to be the villus manufactory. New epithelial cells are produced by the stem cells that reside at the bottom of the crypts and migrate along with the villi to the top (Schat and Myers, 1991). A high proportion of villus height to crypt depth may indicate slower tissue turnover, suggesting that a lower demand is required to compensate for normal sloughing or atrophy of villus due to inflammation from pathogens. Therefore, lower energy would be required to support slower tissue turnover. Taller villi indicate more mature epithelia and enhanced absorptive functions due to increased absorptive area of the villus. Greater villi height increases the enzyme activities secreted from the tips of the villi (Hampson, 1986), resulting in improved digestibility. Otherwise, it is evidenced that the GALT contains more immunocytes than any other tissue in the body (Brandtzaeg, 1977). In the present study, it was demonstrated that administrating 0.2 or 0.4% sIgY in addition to reduction of lamina propria lymphatic follicles proliferation and sIgA secretion decreased the ratio of heterophils to lymphocytes. It suggests that sIgY in addition to local antiinflammatory effects have several indirect effects on systemic inflammation. It is demonstrated that there is a negative relationship between growth performance and stimulation of immune system (Siegel et al., 1984). It may be due to the cachectin activities of interleu-

**Table 6.** Effects of different levels of specific and nonspecific IgY powder on broiler chicks performance

| | ADG (g/d per bird) | | | | ADFI (g/d per bird) | | | | FCR[1] (feed:gain) | | | |
|---|---|---|---|---|---|---|---|---|---|---|---|---|
| Treatment | 21 to 28 d | 29 to 35 d | 36 to 42 d | 21 to 42 d | 21 to 28 d | 29 to 35 d | 36 to 42 d | 21 to 42 d | 21 to 28 d | 29 to 35 d | 36 to 42 d | 21 to 42 d |
| Negative control (NC)[2] | 51.0 | 67.7 | 76.6 | 65.1 | 115.5 | 169.0 | 184.2 | 156.2 | 2.27 | 2.50 | 2.41 | 2.40 |
| Positive control (PC)[2] | 48.6 | 62.8 | 65.2 | 58.9 | 111.7 | 167.0 | 175.7 | 151.5 | 2.30 | 2.66 | 2.70[a] | 2.57[a] |
| sIgY[3] (%) | | | | | | | | | | | | |
| 0.1 | 48.8 | 66.0 | 72.9 | 62.6 | 110.8 | 171.7 | 182.5 | 155.0 | 2.27 | 2.60 | 2.51[bc] | 2.48[bc] |
| 0.2 | 49.4 | 67.8 | 74.9 | 64.0 | 112.2 | 174.0 | 180.0 | 155.4 | 2.27 | 2.57 | 2.41[c] | 2.43[c] |
| 0.4 | 50.1 | 68.1 | 76.4 | 64.9 | 114.0 | 175.0 | 183.7 | 157.7 | 2.28 | 2.57 | 2.40[c] | 2.43[c] |
| nsIgY[3] (%) | | | | | | | | | | | | |
| 0.2 | 51.0 | 65.4 | 68.0 | 61.5 | 119.0 | 172.0 | 182.0 | 157.8 | 2.33 | 2.63 | 2.68[a] | 2.57[a] |
| 0.4 | 52.1 | 63.5 | 67.8 | 61.8 | 119.0 | 164.0 | 177.2 | 153.7 | 2.29 | 2.60 | 2.54[b] | 2.49[b] |
| | *P*-value | | | | | | | | | | | |
| Treatments[4] | 0.75 | 0.32 | 0.1 | 0.14 | 0.48 | 0.63 | 0.90 | 0.70 | 0.34 | 0.21 | 0.01 | 0.01 |
| Contrasts | | | | | | | | | | | | |
| NC vs. PC | 0.35 | 0.07 | 0.01 | 0.01 | 0.47 | 0.76 | 0.30 | 0.26 | 0.28 | 0.01 | 0.01 | 0.01 |
| PC vs. sIgY | 0.67 | 0.05 | 0.01 | 0.01 | 0.89 | 0.22 | 0.34 | 0.19 | 0.27 | 0.02 | 0.01 | 0.01 |
| PC vs. nsIgY | 0.18 | 0.47 | 0.30 | 0.13 | 0.11 | 0.79 | 0.58 | 0.25 | 0.79 | 0.20 | 0.08 | 0.04 |
| sIgY vs. nsIgY | 0.32 | 0.07 | 0.02 | 0.06 | 0.12 | 0.20 | 0.69 | 0.78 | 0.13 | 0.13 | 0.01 | 0.01 |
| SEM | 1.86 | 1.91 | 2.92 | 1.53 | 3.80 | 4.91 | 6.00 | 3.10 | 0.02 | 0.03 | 0.03 | 0.02 |

[a–c]Means with no common superscript within each column are significantly ($P < 0.05$) different.

[1]FCR = feed conversion ratio.

[2]Negative and positive controls consisted of unchallenged and challenged birds, respectively, without dietary IgY powder supplementation.

[3]sIgY = specific IgY powder; nsIgY = nonspecific IgY powder; the different levels of specific and nonspecific IgY powder were supplemented in the diets of *Escherichia coli* O78:K80-challenged birds during d 21 to 42.

[4]*P*-value was calculated for studied variables on *E. coli* O78:K80-challenged birds.

kin-1, interleukin-6, or tumor necrosis factor-α (Klasing et al., 1987) and loss of appetite caused by acute phase proteins (Grimble, 1994). Therefore, it is probable that downregulatory effects of sIgY on inflammatory mechanisms could improve the broiler chick performance. The alteration of growth performance was initiated after 2 wk and reached the best results after 3 wk of dietary sIgY administration. Based on these observations, we can speculate that the direct and indirect effects of sIgY, as an antiinflammatory agent, on both local and systemic responses have been improved broiler performance.

In conclusion, the present results indicated that *E. coli* O78:K80 oral challenge had subversive pathogenesis effects on intestinal morphology and broiler growth performance by exciting the local and systemic immune system. Dietary administration of at least 0.2% sIgY for 3 wk could improve the intestinal health indicators and growth performance of broiler chickens, probably via direct and indirect modification of immune system functions.

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