IRANIAN
JOURNAL
OF
VETERINARY
RESEARCH

Effect of methionine deficiency on duodenal and jejunal IgA+ B cell count and immunoglobulin level of broilers

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Summary

Dietary methionine (met) is reported to enhance antibody production and boost cell-mediated immunity in chickens. Methionine deficiency has been shown to affect the development of the lymphoid organs and the generation of antibodies in chickens. This study is designed to investigate the effects of met deficiency on IgA+ B cells and immunoglobulins (sIgA, IgA, IgG and IgM) for a 6 week period in the duodenum and jejunum of Cobb broiler chicken using immunohistochemistry and enzyme-linked immunosorbent assay (ELISA) techniques. The results of the study showed that the IgA+ B cell count reduced significantly in the met deficiency group compared to the control group ($P<0.05$ or $P<0.01$). The contents of sIgA, IgA, IgG and IgM in the met deficiency group were significantly decreased ($P<0.05$ or $P<0.01$), especially at 28 and 42 days of age. It can be concluded that met deficiency exerts significant effects on the humoral immune system of intestinal mucosa. This study has provided valuable experimental insight which could be useful for future studies on the function of met in the intestine of humans and other animals.

Key words: Cobb broiler, Duodenum, IgA+ B cell, Jejunum, Methionine deficiency

Introduction

As the first-limiting amino acid in poultry diets, methionine (met) affects poultry production parameters such as body weight (BW) gain, feed conversion ratio and carcass quality. Furthermore, met plays many important metabolic functions and can therefore be classified as a functional amino acid. Methionine is essential for various physiological functions (Yang *et al*., 2004; Oz *et al*., 2008) such as body growth promotion (Yen *et al*., 2002; Mirzaaghatabar *et al*., 2011; Zhang *et al*., 2015), neonatal intestinal growth promotion (Zhong *et al*., 2016), detoxification (Kim *et al*., 2006; Tavare *et al*., 2016), antitumor and anticancer functions (Horvat *et al*., 2006; Li *et al*., 2009), resistance for coccidium infection (Rama *et al*., 2003), methyl transfer (Stadtman *et al*., 2002; Waterland *et al*., 2006; Sanchez-Roman *et al*., 2011), synthesis of proteins (Brosnan, 2006; Tesseraud *et al*., 2011; Li *et al*., 2014) and so on. In addition, met is found to be closely related to immune functioning (Konashi *et al*., 2000; Swain and Johri, 2000; Guerrero and Reiter, 2002; Zhang and Guo, 2008; Elmada *et al*., 2016) and influences the protein synthesis and catabolism of the immune system (Grimble, 2006).

The intestine plays a key role in digestion and absorption of electrolytes, nutrients and water from the lumen as well as being essential for the secretion of enzymes or transporters (Wu *et al*., 2013). In addition, the intestine affects the host defense through the mucosal immune system (Hecht, 2003; Peterson and Artis, 2014). The intestine represents the largest compartment of the immune system (Mowat and Agace, 2014) which is directly influenced by dietary bioactive molecules. Methionine which is eventually transformed into bioactive molecules affecting intestinal functions such as immunity, is an essential amino acid for animals, particularly for poultry. Past studies have demonstrated that met deficiency can alter the relative proportion of T lymphocyte subsets (Wu *et al*., 2012) and inhibit the proliferation and differentiation of bursal lymphocytes (mainly B cells) (Wu *et al*., 2013). This study aims to investigate the effect of met deficiency on the IgA+ B cells and sIgA, IgA, IgG and IgM contents in the duodenum and jejunum of Cobb broiler chicken up to 42 days of age using immunohistochemistry and enzyme-linked immunosorbent assay (ELISA). Moreover, from this study, new experimental evidence is produced regarding the effects of met deficiency on intestinal functioning which could provide insightful information for future studies on humans or other animals.

Materials and Methods

Chickens and diets

Seventy two one-day-old healthy Cobb broiler chickens were randomly allotted by BW to 2 groups with 6 replicates each and 6 broiler chickens in each replicate. A control diet and the same diet with met deficiency was given to chickens in the control and met deficient groups respectively, for 42 days. The experimental diet was formulated by NRC (1994). The met content of the met deficient diet was measured by HITACHI L-8800

automatic Amino Acid analyzer (Japan). The results showed that the met content in the starter diet was 0.26%, and 0.28% in the grower diet, whereas the met deficient diet had no supplementary met added. Based on the met deficient diet, 0.24% and 0.12% met was added to produce the control diet (starter diet, met 0.50%; grower diet, met 0.40%) (Table 1). Broilers were fed on either a control or a met deficient diet, and given water and aforementioned diets *ad libitum* as well. The broilers were kept in cages with electrically heated units. The environment and facilities provided to the laboratory animals were in line with national standards (Chinese, GB4925-2010). This research complied following China West Normal University Animal Care Committee guidelines.

Immunohistochemical examination for IgA⁺ B cells in the duodenum and jejunum

Six broiler chickens at 14, 28 and 42 days of age in each group were humanely sacrificed, followed by gross examination. Duodenum (ascending part) and jejunum (middle part) were collected from six chickens of each replicate and fixed in 10% neutral buffered formalin after postmortem examination, processed and trimmed, and embedded in paraffin. IgA⁺ B cells were detected in the duodenum and jejunum crypts of the broilers by immunohistochemistry. Immunohistochemical staining and counting were performed as described by Liu *et al.* (2013). Briefly, slices were dewaxed in xylene, rehydrated through a graded series of ethanol, washed in distilled water and phosphate buffer saline (PBS) and then blocked for endogenous peroxidase by incubation with 3% H_2O_2 in methanol for 15 min. To retrieve

antigens, the tissue sections were microwaved in a 0.01 M sodium citrate pH = 6.0 buffer. Another wash in PBS was then conducted, followed by a 30-min incubation at 37°C in 10% normal goat serum. The slices were incubated overnight at 4°C with the diluted (1:100) primary antibodies. The antibodies used were polyclonal mouse anti-chicken IgA heavy chains (SouthernBiotech 8330-01). To set up negative controls, PBS was applied to slices instead of the primary antibody. After the PBS wash, the slices were incubated in 1% biotinylated secondary antibody goat anti-mouse IgG (ZSGB-BIO SP Kit) for 1 h at 37°C and were further incubated with HRP-streptavidin (ZSGB-BIO SP Kit) for 30 min at 37°C. To visualize the immunoreaction, sections were submerged in diaminobenzidine hydrochloride (DAB). The slices were monitored under the microscope and the reactions were immediately terminated by immersion in distilled water after the slices were stained brown. Slices were then lightly counterstained with hematoxylin, dehydrated in ethanol, cleared in xylene and mounted.

Cell counts of IgA⁺ B cells were derived using a computer-supported imaging system connected to a light microscope (OlympusAX70) with an objective with ×400 magnification. IgA⁺ B cells were then quantified by Image-Pro Plus 5.1 (USA) image analysis software. For each group, the cell count was measured in six chickens with five slices in each chicken and each slice was measured five visions and averaged.

Determination of Ig contents in the duodenum and jejunum by ELISA

At 14, 28, and 42 days of age during the experiment, six broiler chickens in each group were humanely

Table 1: Components of the basal diets for broilers (%)

Ingredient [1] (%)	Met deficient diet		Control diet	
	Starter diet (1 to 21 day)	Grower diet (22 to 42 day)	Starter diet (1 to 21 day)	Grower diet (22 to 42 day)
Ground yellow corn	56	59.5	56	59.5
Soybean meal	37	32.85	37	32.85
Soybean oil	3.66	4.7	3.66	4.7
Ground limestone	0.57	0.5	0.57	0.5
Dicalcium phosphate	1.8	1.6	1.8	1.6
Salt	0.3	0.3	0.3	0.3
Choline Chloride	0.1	0.1	0.1	0.1
DL-met	0.0	0.0	0.24	0.12
Bentonite	0.24	0.12	0.0	0.0
Micronutrients [2]	0.33	0.33	0.33	0.33
Calculated nutrients level (%)				
ME, MJ/kg	12.39	12.79	12.39	12.79
CP	21.17	19.72	21.17	19.72
Lys	1.19	1.08	1.19	1.08
Met	0.26	0.28	0.50	0.40
Met + Cys	0.62	0.62	0.86	0.74
Ca	0.85	0.77	0.85	0.77
Nonphytate P	0.44	0.40	0.44	0.40

[1] Ingredient and nutrient composition are reported on as-fed basis. [2] For the diet of day 1-21 and day 22-42, provided per kg of diet: vitamin A (all-*trans* retinol acetate), 12,500 IU; cholecalciferol, 2,500 IU; vitamin E (all-*rac*-α-tocopherol acetate), 18.75 IU; vitamin K (menadione Na bisulfate), 5.0 mg; thiamin (thiamin mononitrate), 2.5 mg; riboflavin, 7.5 mg; vitamin B_6, 5.0 mg; vitamin B_{12}, 0.0025 mg; pantothenate, 15 mg; niacin, 50 mg; folic acid, 1.25 mg; biotin, 0.12 mg; Cu ($CuSO_4 \times 5H_2O$), 10 mg; Mn ($MnSO_4 \times H_2O$), 100 mg; Zn ($ZnSO_4 \times 7H_2O$), 100 mg; Fe ($FeSO_4 \times 7H_2O$), 100 mg; I (KI), 0.4 mg; Se (Na_2SeO_3), 0.2 mg

sacrificed. The intestinal tract was removed from each bird and divided into duodenum and jejunum. A 4-cm long tissue section was collected from the ascending part of the duodenum and the middle section of the jejunum, and thoroughly cleaned by gentle squeezing. The mucosa was carefully scraped from the luminal face of the intestinal samples and stored at -80°C prior to ELISA. The Ig contents in the intestine were determined using ELISA as described by Wu *et al.* (2012), and the results were analyzed by the standard curve and expressed as μg per ml. Briefly, the mucosa sample was diluted and added to blank wells or test wells (40 μL for each well). Then, the solution was incubated for 30 min at 37°C, the liquid was discarded, dried by swing, and washed 5 times and patted dry. The HRP-Conjugate reagent (50 μL) was added to each well (except for the blank well) followed by incubation and washing as carried out in the previous step. Chromogen solution A (50 μL) and chromogen solution B were added to each well, and kept in the dark for 15 min at 37°C. The reaction was stopped by adding 50 μL stop solution to each well, and the absorbance was measured at 450 nm. For the corresponding density, calculation was obtained according to the standard curve.

Statistical analysis

Significant differences among the two groups were analyzed by analysis of variance, and results were presented as means±SD. The analysis was performed by conducting Independent-Samples t-tests using SPSS 16.0 for windows. A value of $P<0.05$ was considered as significant.

Results

Change in the number of IgA⁺ B cells in the duodenum crypt

As shown in Figs. 1a and b, IgA⁺ B positive cells in the duodenum crypt were stained with brown as revealed by immunohistochemical results. The number of IgA⁺ B cells in the met deficiency group was significantly lower ($P<0.01$) than that of the control group at 28 and 42 days of age (Fig. 1c).

Change in the number of IgA+ B cells in the jejunum crypt

The results of immunohistochemical analysis showed brown stained IgA⁺ B positive cells in the jejunum crypt, as shown in Figs. 2a and b. The number of IgA⁺ B cells in the met deficiency group was significantly lower ($P<0.05$ or $P<0.01$) than that of the control group at 28 and 42 days of age (Fig. 2c).
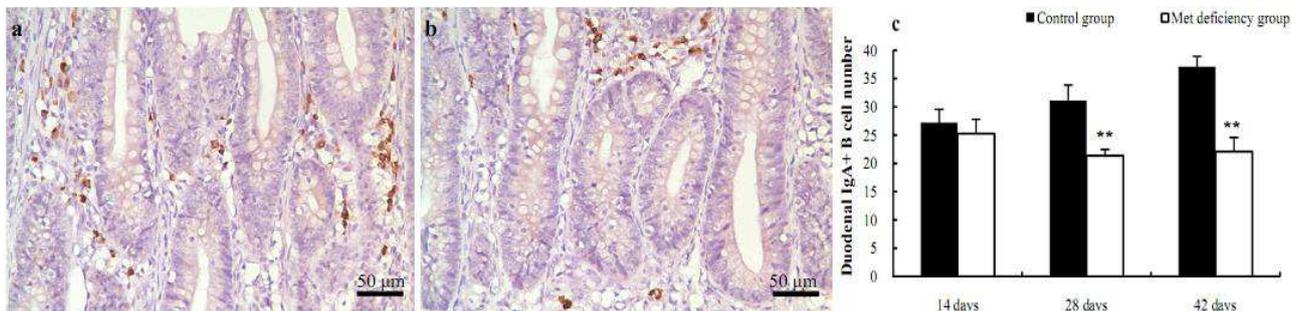
Fig. 1: Change in the number of IgA⁺ B cells in the duodenum crypt. **a:** The IgA⁺ B cells in the duodenum crypt in the control group at 42 days of age, many IgA⁺ B cells can be seen. **b:** The IgA⁺ B cells in the duodenum crypt in the met deficiency group at 42 days of age. Number of the IgA⁺ B cells has decreased (bar=50 μm). **c:** Number of IgA⁺ B cells in the control and met deficiency groups at 14, 28 and 42 days of age. Data presented with means±SD (n=6×6). ** $P<0.01$, compared with the control group
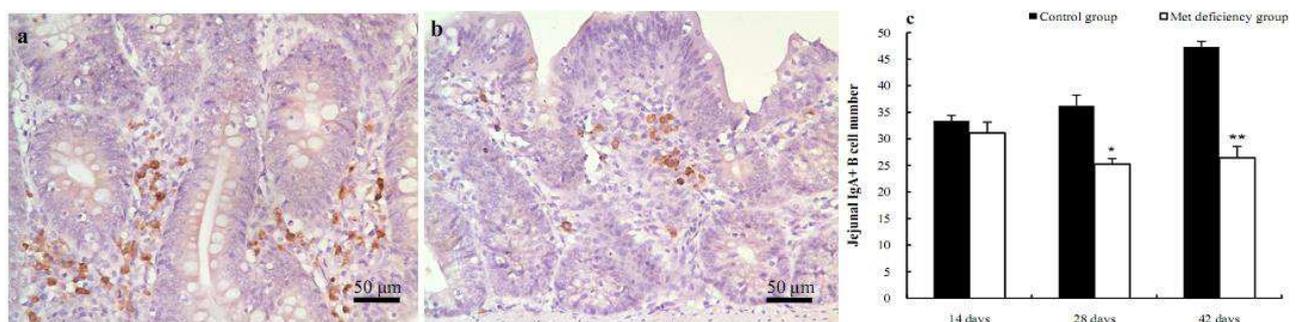
Fig. 2: Change in the number of IgA⁺ B cells in the jejunum crypt. **a:** The IgA⁺ B cells in the jejunum crypt in the control group at 42 days of age, many IgA⁺ B cells can be seen. **b:** The IgA⁺ B cells in the jejunum crypt in the met deficiency group at 42 days of age. Number of the IgA⁺ B cells has decreased (bar=50 μm). **c:** Number of IgA⁺ B cells in the control and the met deficiency groups at 14, 28 and 42 days of age. Data presented with means±SD (n=6×6). * $P<0.05$, compared with the control group, and ** $P<0.01$, compared with the control group

Change in the sIgA, IgA, IgG and IgM contents in the duodenum

As shown in Figs. 3a, b, c and d, the sIgA, IgA, IgG, and IgM contents in the duodenum of the met deficiency group were significantly lower (P<0.05 or P<0.01) than the control group at 28 or 42 days of age.

Change in the sIgA, IgA, IgG and IgM contents in the jejunum

In comparison with those of the control group, the sIgA, IgA, IgG, and IgM contents in the jejunum of the met deficiency group were significantly lower (P<0.05 or P<0.01) at 14, 28 or 42 days of age (Figs. 4a, b, c and d).
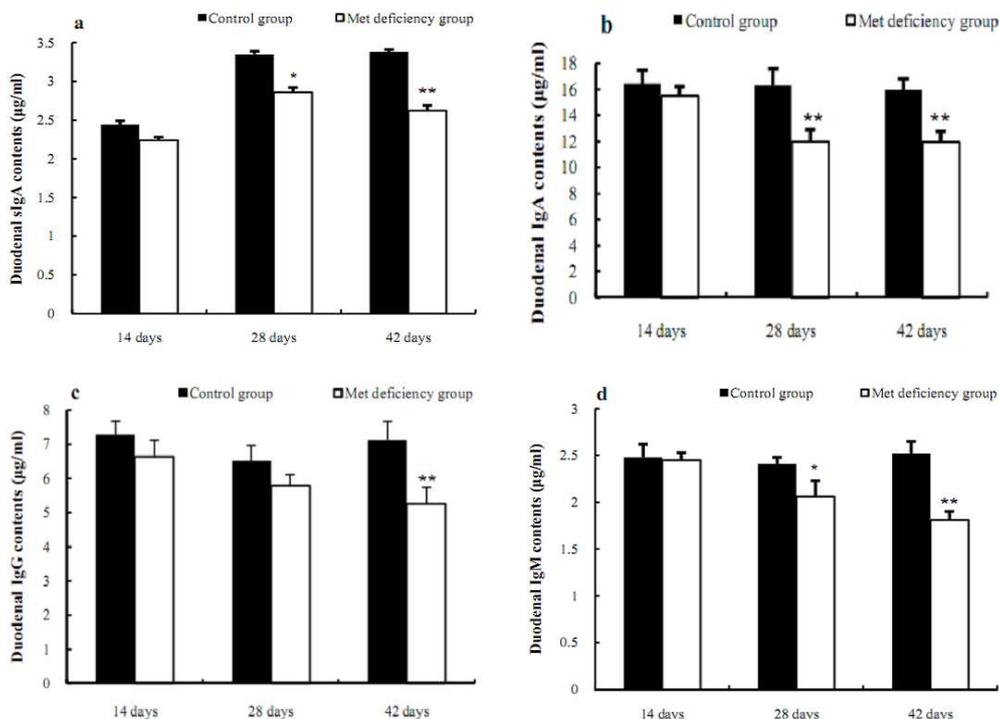
Fig. 3: Change in the sIgA (**a**), IgA (**b**), IgG (**c**), and IgM (**d**) contents (μg/ml) in the duodenum. Data presented with means±SD (n=6×6). * P<0.05, compared with the control group, and ** P<0.01, compared with the control group
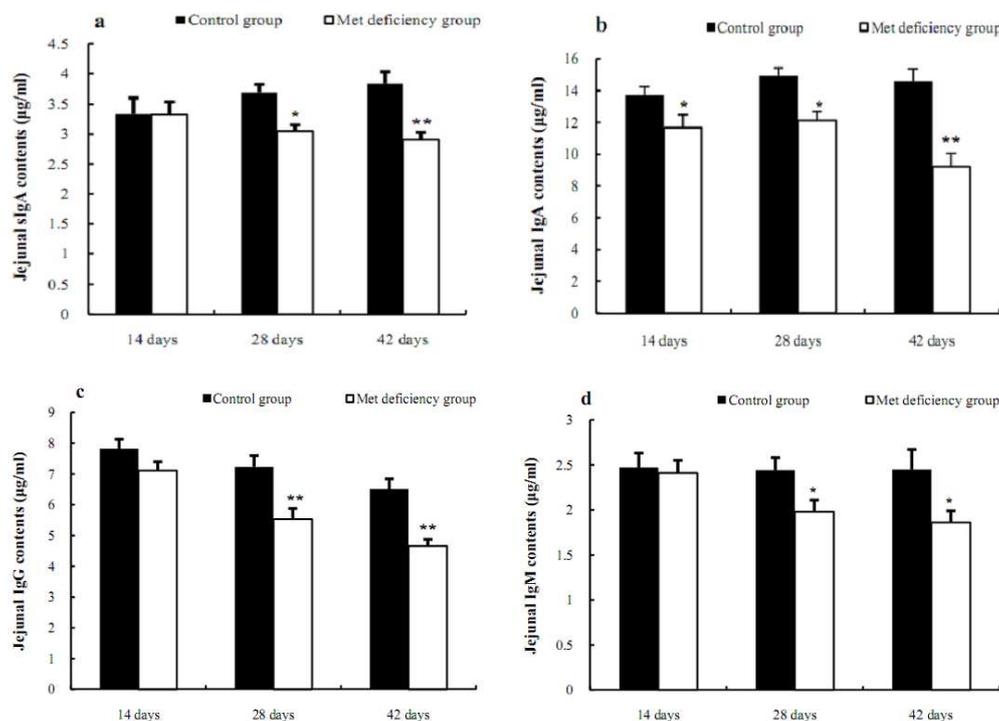
Fig. 4: Change in the sIgA (**a**), IgA (**b**), IgG (**c**), and IgM (**d**) contents (μg/ml) in the jejunum. Data presented with means±SD (n=6×6). * P<0.05, compared with the control group, and ** P<0.01, compared with the control group

Discussion

Sulfur amino acids (SAA), particularly met, are essential for the maintenance of intestinal functions such as digestion, nutrient absorption and metabolism, and the immune surveillance (Fang *et al*., 2010; Tang *et al*., 2016; Zhong *et al*., 2016). In the present study, we aimed to determine the effects of met deficiency on IgA+ B cells and Igs in the intestine. In this study, our focus was to observe the changes in the number of IgA+ B cells and the contents of four Igs (sIgA, IgA, IgG, and IgM) in the duodenum and jejunum, induced by met deficiency. We found that met deficiency had negative effects on IgA+ B cells and the Ig content of the intestine, which is consistent with a previous study showing the positive influence of met supplementation on plasma immunoglobulin concentrations in young turkeys (Kubińsk *et al*., 2016). In addition, the above measured parameters showed significant differences on days 28 and 42, while no significant differences were observed at 14 days, which might be due to their plumage in addition to fast growth, or the large demand of broilers for sulphur-rich amino acids, especially met (Rama *et al*., 2003). It is well known that the mucosal immune system of the intestine contains the onslaught of antigens through B cells/antibodies and mucosal T cells, used for preventing damages from leakage of undesirable substances or compounds (Clem, 2011).

The chicken Ig gene can rearrange and be expressed on the surface of B cell membrane during embryonic development (Ratcliffe and Ivanyi, 1981; Benatar *et al*., 1992; Reynaud *et al*., 1992). Among three major chicken immunoglobulin classes IgG (IgY), IgM and IgA (Mockett, 1986), IgA is predominantly present in most intestinal plasma cells (derived from B cells), enabling transport across epithelia and movements of secretory IgA (sIgA) into the gut lumen (Lindner, 2012). The secretion of IgA antibody onto mucosal surfaces is enabled by IgA class switching (Brandtzaeg *et al*., 2001). IgA plays an essential role in protecting mucosal surfaces against toxins, viruses and bacteria (Macpherson *et al*., 2001), especially sIgA (Schroeder, 2010). As a result, sIgA is considered to be pivotal in maintaining homeostasis in mucosal tissues and may also influence the composition of intestinal microbiota (Lammers *et al*., 2010). Dietary met has been reported to significantly affect the content of serum IgA of 2-month-old New Zealand meat rabbits (Zhang and Li, 2008). Similar to previous reports, in the present study the quantities of IgA+ B cells, sIgA and IgA contents in the duodenum and the jejunum were significantly reduced in the met deficiency group, indicating the suppressive effect of met deficiency on IgA+ B cells and IgA (sIgA) production in the intestine. It is well known that met residues act as catalytic antioxidants that can protect not only adjacent proteins but also other macromolecules (Luo and Levine, 2009), so we hypothesize that met deficiency may decrease antioxidant capacity and further induce IgA+ B cell apoptosis. We also suspect that met deficiency may affect IgA+ B cell proliferation or differentiation, and

lastly inhibit the secretion of specific antibodies. Furthermore, tight connections were found between IgA+ B cells and humoral immune responses. The reduction of IgA+ B cell numbers as well as the restriction of sIgA and IgA production would eventually affect the humoral immune function in the intestinal mucosal immunity.

In our previous study, we found that met deficiency is able to suppress the contents of serum IgM and IgG significantly (Wu *et al*., 2012), hence in this study, we planned to investigate whether met deficiency in the intestine can affect IgM and IgG. IgM is the first antibody generated during a primary antibody response in chicken (Schwarz *et al*., 2010), the first immunoglobulin expressed during B cell development, and the major Ig expressed on the surface of chicken B lymphocytes (Perez-Carbajal *et al*., 2010). In this research, IgM content decreased in the met deficiency group, which suggests that dietary met deficiency could lead to the impairment of primary immune responses. Moreover, IgG, secreted by B cells, is the main antibody isotype found in blood and extracellular fluids, enabling it to control body tissue infections (Hirano *et al*., 1986). IgG contributes directly to immune responses including the neutralization of toxins and viruses (Lindner *et al*., 2012). Functionally, IgG is generated mainly in secondary antibody responses (Shimizu *et al*., 1992). The present study showed that IgG content decreased in the met deficiency group. A similar study reported that dietary met could lead to the alteration of serum IgM and IgA contents in 2-month-old New Zealand meat rabbits (Zhang and Li, 2008). Another study found that it positively influenced blood IgG levels in weaning piglets (Hou *et al*., 2001).

From the results and discussions of the present study, it is concluded that dietary met deficiency reduces the population of IgA+ B cells and the contents of sIgA, IgA, IgG, and IgM in the duodenum and jejunum, implying that the humoral immune function in the intestinal mucosal immunity is impaired by met deficiency in broilers. Met deficiency drops of sIgA, IgA, IgG, and IgM contents may be induced through the reduction and activation of B cells.

Acknowledgements

Conflict of interest

The authors declare that there is no conflict of interest.

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