Effects of dietary supplement of vitamin B6 on growth performance and non-specific immune response of weaned rex rabbits

Gongyan Liu, Chaoran Sun, Hongli Liu, Fan Li, Yanli Zhu and Fuchang Li

ABSTRACT
A sixty-day experiment was carried out to evaluate the effect of a dietary supplement of vitamin B6 on growth performance and non-specific immune response of weaned to three month-old growing Rex rabbits. Two hundred weaned healthy Rex rabbits with similar body weight were randomly assigned to one of five dietary groups, and the dietary groups supplemented of different vitamin B6: 0, 5, 10, 20, and 40 mg/kg. Dietary vitamin B6 had effects on ADG and ADFI (P < .05), the highest ADG values and the lowest diarrhea ratio were found in 20 mg/kg group. Vitamin B6 had effects on the relative weight of thymus and spleen (P < .05), especially in the 10 and 20 mg/kg groups. Additionally, vitamin B6 had significant effects on serum IgA, IL-6 and IFN-γ titres and on slgA titres in the duodenum and ileum (P < .05). Splenic IL-6 and IFN-γ mRNA expression levels increased with vitamin B6 (P < .05). Furthermore, vitamin B6 had increased M cell count in the appendix (P < .05). The findings revealed that vitamin B6 affects the immune performance of rabbits and the recommended vitamin B6 supplemental level was 10–20 mg/kg for weaned to three month-old growing Rex rabbits.

Abbreviations: ADG: average daily gain; ADFI: average daily feed intake; F/G: feed to gain ratio; IgG: immunoglobulin G; IgA: immunoglobulin A; IgE: immunoglobulin E; IL-6: interleukin-6; IFN-γ: interferon-γ; slgA: secretory immunoglobulin A; pilgR: polymeric immunoglobulin receptor; GAPDH: Glyceraldehyde-3-phosphate dehydrogenase; M: cell; Microfold: cell; OD: optical density; ELISA: enzyme-linked immunosorbent assays

Introduction
Rabbit production is a major component of farm economies in developing countries, which contribute to food and fur. Rex rabbit is a breed generally recognized by its velvety fur and is raised for fur and meat production. Weaned rabbits are generally afflicted by digestive disorders, which contribute to reduced growth performance and health. Infectious diseases of the digestive system currently account for 70% of all rabbit diseases (Carabaño et al. 2008). Antibiotics are frequently used to prevent or to treat infectious diseases in rabbits has increased the feeding costs. Moreover, the long-term and extensive use of antibiotics has lead to the appearance of worrying bacterial drug resistance and stressed the problem of food residues. So, treatment in rabbit farming has stimulated the search for alternative solutions. Therefore, researchers have explored alternative methods to treat rabbits with digestive disorders.

Among those, nutritional management has become a priority, and adequate nutrition can minimize the risk of digestive disorders (Gidenne et al. 2010). Pyridoxal 5'-phosphate (PLP), the coenzyme form of vitamin B6, participates in amino acid, glucose, and lipid metabolism and regulates the immune system. It has been shown that experimentally induced vitamin B6 deficiency in terrestrial animals was associated with reduced immunocompetence. Dietary deficiency of vitamin B6 significantly decreases cell-mediated immunity, such as percentage and total number of lymphocytes, the mitogen responses of lymphocytes in human (Meydani et al. 1991), T-cell-mediated cytotoxicity in rats (Ha et al. 1984) and humour-mediated immunity, such as IL-2 secretion in human (Meydani et al. 1991) and IgE, IgG production in rats (Inubushi et al. 2000). The rabbit has been thought to be independent of dietary sources of B vitamins since it consistently consumes a special portion of its excreta, rich in vitamins and proteins, termed ‘soft’ or ‘night’ feces (Hove and Herrdon 1957). Bräunlich (1974) reported that vitamin B6 deficiency causes inflammation around the eyes and nose, scaly thickening of the skin around the ears, alopecia in the forelegs and skin desquamation. However, there is controversy about the effects of high-dose vitamin B6 on immune responses in terrestrial animals (Ha et al. 1984; Inubushi et al. 2000). Ha et al. (1984) believed that seven times extensive the optimal requirement vitamin B6 in diets had no effect on immunocompetence in rats. On the contrary, Inubushi and Okada (2000) pointed out that ten times excess the optimal requirement vitamin B6 in diets was able to suppress antibody productions in rats.
Appreciable amounts of water-soluble vitamins are supplied to the rabbit through caecotrophy, and caecotrophy can meet rabbit requirements for maintenance and average levels of production (NRC 1977; Harris et al. 1983). However, fast-growing fryers and high-producing does may respond to additional supplementation of B vitamins, such as pyridoxine (Vitamin B6) (Maertens 1996; Xiccato 1996; Lebas 2004). The current information about the effects of dietary supplement of vitamin B6 on immune functions of organ systems is largely obtained from other animals, very little information is available for rabbits. Besides, few recent detailed studies have been conducted on the requirements of rabbits for vitamin B6. The objective of this study was to investigate the effects of dietary supplement of vitamin B6 on the growth performance, immune organs development, serum immune markers and intestinal mucosal immunity of weaned to 3 month-old growing Rex rabbits. In addition, the appropriate vitamin B6 supplemental level was determined for weaned (28-day) to 3 month-old growing Rex rabbits.

Materials and methods

Chemical analysis of experimental diets

The experimental diets (Table 1) used in this study was formulated to meet the recommended nutrient requirements of growing rabbits (NRC 1977). The supplemental values recommended of vitamin B6 in the literature vary from 0.5 mg/kg for lactating does and growing rabbits (Mateos and Piquer 1994), 39 mg/kg for growing-fattening (NRC 1977). Therefore, the following five different concentrations of vitamin B6 were supplemented into the diets: 0 (control), 5, 10, 20, and 40 mg/kg (as-fed basis). The vitamin B6 form was pyridoxine hydrochloride (98%, Jiangxi Tyson Pharmaceutical Co., Ltd., China). The five diets were passed through a roller mill prior to being mixed and granulated (3–4 mm in diameter and 10–15 mm in length), and stored in the dark.

The Association of Official Analytical Chemists (AOAC International (2005) procedures were used to determine the content of dry matter (934.01), crude protein (954.01), crude fibre (978.10) and ash (942.05) in feeds. Crude protein content (6.25×N) and ether extract were determined using a Kjeltc Auto 1030 Analyser and a Soxtec 1043, respectively (FOSS Tecator AB, Höganäs, Sweden). The mineral profile (Ca, P) of the diets was analysed by ICP-OES (Spectro Cirus Vision EOP) after microwave digestion (999.10). Lysine and methionine of the feed were analysed using an automatic amino-acid analyzer (Basic L-8900, Japan). All dietary chemical analyses were performed in duplicate.

Animals and experimental design

In this study, 200 healthy 28 days weaned growing Rex rabbits of similar body weight (684 ± 50 g) were randomly assigned to one of the five diets, with 40 animals per dietary group. The experimental procedures were approved by the Committee of Ethics in Research of Shandong Agricultural University and performed in accordance with the Guidelines for Experimental Animals of the Ministry of Science and Technology (Beijing, China). The 60-day feeding trial included a seven day adjustment period and a 53-day experimental period. The experimental rabbits were housed singly in a cage (60×40×40 cm) and had ad libitum access to food and water, and no antibiotics were added to the food or drinking water during the experiment. The animals were housed in a semi-controlled closed building during the experimental period at 18–25°C.

Sample collection and preparation

At the end of the 60-d feeding trial, 40 rabbits (8 rabbits per group, 4 males and 4 females, with body weight close to the average group body weight) were led by cardiac puncture. Blood samples were collected, and the animals were sacrificed by exsanguination from the carotid artery. The blood samples were centrifuged at 1500×g for 10 min, and the resulting serum was stored at −20°C. The digestive tract, including duodenum, jejunum, ileum, and colon (upper 1 cm segment), was removed, suspended in 5 mL phosphate buffer solution (PBS; Beijing Solarbio Science and Technology Co., Ltd., China), and washed for 5 min by vortexing (Zhu et al. 2013). The mucus was collected by centrifugation at 5000 g for 30 min at 4°C, and the resulting supernatant was stored at −20°C for sIgA detection. Splenic and intestinal samples were collected, frozen in liquid nitrogen, and stored at −80°C, for mRNA expression analysis.

Measurements and analyses

Growth performance

Individual weight was measured at the beginning and end of the trial and the ADG was calculated. The ADFI was calculated according to total of food intake divided by total experimental days. The F/G was then calculated. Diarrhea ratio and mortality per group during the experiments were calculated in accordance with the following formula: Diarrhea ratio (%) = 100 * (number of rabbits with diarrhea * average diarrhea days)/ (number of experimental rabbits * experimental days); Mortality (%) = 100 * (number of rabbits with death/ number of experimental rabbits). Results of growth performance were given as
the means of healthy rabbits which were alive without diarrhea during the trial were used for calculating ADG, ADFI, F/G. The ADG, ADFI, F/G, diarrhea ratio and mortality calculations did not include the seven days adjustment period.

**Immune organs development**

Twelve hours prior to slaughter, rabbits were fasted and weighed (Live weight before slaughter). Following slaughter, the thymus, spleen, and liver were carefully removed and weighed, their proportion to the live weight before slaughter was calculated.

**Immune markers**

Serum IgG, IgA, IgE, IL-2, IL-6, and IFN-y titres were measured by ELISA kit (Shang Hai Lengton Bioscience Co., China) according to the manufacturer’s instructions. The concentration in the samples was determined from the standard curve after measuring the optical density of the samples.

sIgA titres in the intestinal mucus supernatant was measured by ELISA as described by Sheela et al. (2003). Ninety-six-well-microtiter plates (Corning Costar, Corning, NY, USA) were coated with a goat anti-rabbit IgA antibody (Alpha Diagnostic Intl., San Antonio, USA) in coating buffer (50 mM sodium carbonate–bicarbonate buffer, pH 9.6) overnight at 4°C. After washing, the mucus samples were added in triplicate, and rabbit IgA serum (Accurate Chemical and Scientific Corp. Westbury, USA) was added as standards. After incubation for 1 h, the plates were incubated with a peroxidase-conjugated anti-rabbit IgA antibody (Alpha Diagnostic Intl. Inc., San Antonio, USA) in PBS containing 0.05% Tween 20 and 1% BSA. After another wash, tetramethyl benzidine (TMB) (Tiangen, Beijing, China) and 0.22% H2O2 in 0.04 M citric acid buffer (pH 5.0) were added. The reaction was stopped after 20 min by adding 100 ml of 0.5 M sulphuric acid. The absorbance was read at 490 nm on a 96-well plate reader (Bio-Rad, Hercules, California). The concentration of sIgA in the mucus samples was calculated using the standard curve.

**mRNA expression**

Total RNA was extracted from splenic and intestinal samples by a single-step isolation procedure using Trizol (Invitrogen, USA). The RNA concentrations were determined by measuring absorbances at 260 nm. Two micrograms of total RNA was used to synthesize cDNA using the Transcriptor First Strand cDNA Synthesis kit (Roche Diagnostics GmbH Mannheim, Germany), the resulting cDNA samples were stored at -20°C.

Semi-quantitative RT–PCR was performed to determine expression levels of IL-6, IFN-γ, and pIgR mRNA expression levels. GAPDH was used as a reference gene. All quantitative PCR primers (Table 2) were designed by Primer Premier 5 software and synthesized by SANGON Biological Engineering Co., Ltd (Shanghai, China). PCR amplification was performed using Fast Start Universal SYBR Green Master (Roche Diagnostics GmbH Mannheim, Germany). The 20 μL PCR reaction mixture consisted of 2 μL cDNA, 10 μL SYBR Premix Ex Taq™ (2x), 0.5 μL PCR forward primer (10 μM), 0.5 μL PCR reverse primer (10 μM), 0.4 μL ROX reference dye II (50x), and 6.6 μL ddH2O. The number of cycles set for the linear amplification of cDNA was 40. The amplified products were analysed by electrophoresis in 1.0% agarose gels and stained with ethidium bromide.

**Intestinal M cell**

The intestinal appendix samples were rinsed in physiological saline and fixed in paraformaldehyde solution for one week. Subsequently, the tissue samples were embedded in paraffin through a series of steps that included incubation in graded alcohol, methyl benzoate, and benzole. Sections (5 mm thick) were cut and placed on poly-L-lysine slides. Immunohistochemical analysis was performed using the PV-9002 Blink-2 plus Polymer HRP Detection System for mouse primary antibody (ZSGB-BIO, Beijing, China). Briefly, the paraffin sections were deparaffinized twice in xylene for 30 min each time and washed in 100%, 95%, 90%, 85%, 80%, and 70% ethanol and distilled water for 10 min. The samples were boiled in citrate buffer (pH 6.0) for 20 min to restore the antigenic properties. The sections were allowed to cool to room temperature and kept in 3% hydrogen peroxide (H2O2) in methanol for 30 min. The sections were incubated overnight with a monoclonal mouse anti-vimentin antibody (1:100, CloneV9, ZSGB-BIO, Beijing, China) at 4°C and subsequently with a Polymer Helper (ZSGB-BIO, Beijing, China) and polyclonal HRP-conjugated anti-mouse IgG antibody at 37°C for 30 min. The antigen–antibody complex was visualized using diaminobezidin (DAB; ZSGB-BIO, Beijing, China). Gill’s hematoxylin was applied for background staining. All washing steps were performed with 0.1 M PBS (pH 7.4), and the incubations were performed in a humidified chamber. Rabbit kidney sections were used as positive controls for anti-vimentin staining. The negative control consisted of goat serum instead of primary antibodies. The results were observed under an optical microscope.

**Statistical analyses**

The data (except for diarrhea ratio and mortality) were analysed by ANOVA and Duncan’s test using the GLM Procedure of SAS 9.1.3 statistical software. The data were expressed as mean and root mean square error (R-MSE). P < .05 was considered to be significant. The data of IL-6, IFN-γ, and pIgR were normalized to the GAPDH expression levels and relative expression levels were calculated using the 2−ΔΔCt method (Livak and Schmittgen 2001). 10 vimentin-positive sections were selected from the same group to evaluate the immunohistochemistry results, a total of 30 fields of view (three per section) were

### Table 2. Primer sequence.

| Gene    | Genbank accession | Specific primers                  | Product size (bp) |
|---------|-------------------|-----------------------------------|-------------------|
| GAPDH   | NM_001082253      | F:5'-TGCCACCCACTCTCCTAGCTTC-3'   | 118               |
| IL-6    | NG_011640         | F: 5'–CGGATCCGACATGAAGCTC-3'     | 128               |
| IFN-γ   | NM_008337         | F:5'-CTGGCTCCTTGTGGTGTTAC-3'     | 120               |
| pIgR    | NM_174143         | F:5'-AGCATTAGCGTGGCTACTCA-3'     | 130               |

Note: GAPDH, Glyceraldehyde-3-phosphate dehydrogenase; IL-6, Interleukin-6; IFN-γ, interferon-γ; pIgR, polymeric immunoglobulin receptor.
Results

Effect of dietary supplement of vitamin B6 on growth performance

The growth performance of the experimental rabbits are presented in Table 3. Vitamin B6 had effects on ADG (P = .034) and ADFI (P = .048), no difference was observed for F/G (P > .05). Further, the highest ADG values and the lowest diarrhea ratio were found in 20 mg/kg group.

Effect of dietary supplement of vitamin B6 on immune organs development

The effects of dietary vitamin B6 on immune organs development are summarized in Table 4. Dietary vitamin B6 affected the relative weight of thymus (P = .0362) and spleen (P = .0478), especially in the 10 and 20 mg/kg groups, respectively.

Effect of dietary supplement of vitamin B6 on immune markers

Dietary vitamin B6 did not affect serum IgG (P = .1848) or IgE (P = .5622) titres; However, the vitamin improved IgA titres (P = .0240; Table 5), especially in the 10 mg/kg group. Vitamin B6 increased serum IL-2 (P = .0353) and IFN-γ (P = .0142) titres.

Effect of dietary supplement of vitamin B6 on slgA titres

Vitamin B6 had significant effects on the slgA titres in the duodenum (P = .0130) and ileum (P = .0453); duodenum slgA titres decreased in the 20 mg/kg group. No significant effects were observed on jejunal and colonic slgA titres (P = .0984 and P = .0783, respectively; Table 6).

Table 3. Effects of dietary supplement of vitamin B6 on growth performance of growing Rex rabbits.

| Items                | Dietary vitamin B6 supplemental level (mg/kg; as-fed basis) |
|----------------------|-------------------------------------------------------------|
|                      | 0               | 5              | 10             | 20             | 40             | R-MSE      | P-value     |
| Initial body weight  | 685.5           | 682.7          | 685.4          | 684.3          | 682.4          | 99.66      | 0.867       |
| Final body weight    | 1305.6          | 1440.2a        | 1528.4b        | 1569.4c        | 1509.2d        | 150.34     | 0.012       |
| Average daily gain   | 11.7ab          | 14.3abc        | 15.9abc        | 16.7abc        | 15.6abc        | 2.47       | 0.034       |
| Average daily feed   | 62.5b           | 64.4bc         | 67.5a          | 69.5a          | 65.0abc        | 3.85       | 0.048       |
| Feed/Gain            | 5.35            | 4.41           | 4.18           | 4.18           | 4.78           | 0.70       | 0.090       |
| Diarrhea ratio       | 12.45           | 9.21           | 7.32           | 5.23           | 5.25           | –          | –           |
| Mortality (%)        | 10.00           | 7.50           | 5.00           | 2.50           | 2.50           | –          | –           |

1Diarrhea ratio (%) = 100 * (number of rabbits with diarrhea / number of experimental rabbits)
2Mortality (%) = 100 * (number of rabbits with death / number of experimental rabbits)
R-MSE, root mean square error

Effect of dietary supplement of vitamin B6 on mRNA expression

As shown in Figure 1, dietary vitamin B6 increased splenic IL-6 and IFN-γ mRNA expression levels (P < .05). However, there was no effects on intestinal plgR mRNA expression levels (P > .05).

Effect of dietary supplement of vitamin B6 on M cell count

The immunohistochemical results revealed that, with increasing vitamin B6 levels, OD value was increased (P < .05; Figure 2). The OD value positive correlates with the number of M cells. Thus, vitamin B6 can increase the M cell number of the appendix.

Discussion

Effect of dietary supplement of vitamin B6 on growth performance

Vitamin B6 is an essential nutrient required to maintain normal physiological functions of animals, including pyridoxine (PN), pyridoxal (PL) and pyridoxamine (PM), with equivalent activity in mammals. PN deficiency produces retarded growth, dermatitis, convulsions, anaemia, scaly skin, alopecia, diarrhoea and a fatty liver, among other symptoms. Vitamin B6 deficiency in the laying hen causes an immediate anorexia, loss of body weight, greatly reduced body fat stores and severe effects upon primary and secondary sex characteristics resulting in severely reduced hatchability culminating in complete cessation of egg production (Weiss and Scott 1979). In the rabbit, PN deficiency can cause inflammation around the eyes and nose, scaly thickening of the skin around the ears, alopecia in the forelegs and skin desquamation (Bräunlich 1974). Woodworth et al. (2000) suggested that added PN in the diet improves weaning pig growth performance, and the optimal PN levels for post-weaning piglets would range between 7 and 8 mg/kg. Indian catfish were fed semi-purified diets containing 0, 1.7, 3.4, 6.5, 13.5 and 27.2 mg/kg of vitamin B6 for 15 weeks, the highest weight gain was the diet supplemented with 3.4 mg/kg (Mohamed 2001). Supplemental values recommended 0.5 mg/kg for lactating does and growing rabbits (Mateos and Piquer 1994), 39 mg/kg for growing-fattening (NRC 1977) and up to 400 mg/kg for growing angora rabbits (Schlolaut 1987). The premixes included in the survey contained between 0 and 4 mg/kg PN in Spanish and Portuguese. Most of
the commercial premixes (25 out of 29) contained between 1 and 2 mg/kg PN and no clinical symptoms have ever been detected in the field using these low levels (Mateos and Piquer 1994). In this study, vitamin B6 had effects on ADG, and the highest value was found in 20 mg/kg group. It may also be attributed to vitamin B6 can enhance protein synthesis as described by Albrektsen et al. (1993) where more protein was also be attributed to vitamin B6 can enhance protein synthesis and reduces thymus weight (Ha et al. 1984). Shimoi et al. (1992) reported that animals fed a vitamin B6-deficient diet with 20 or 40 mg/kg vitamin B6 was significantly improved in its disease resistance (Hardy et al. 1979). Our findings that vitamin B6 increased serum IgA titre, which might be due to PLP is involved in IgA synthesis. Cytokines are important signal molecules of the immune system. Antigen-specific immune markers in serum titres (Inubushi et al. 2000). However, challenged with a virulent strain of Vibrio banguillarum, the fish fed the high protein diet with 20 or 40 mg/kg vitamin B6 was significantly improved in its disease resistance (Hardy et al. 1979). Our findings that vitamin B6 increased serum IgA titres, which might be due to PLP is involved in IgA synthesis.

Effect of dietary supplement of vitamin B6 on serum immune markers

Nurtritional status is the most important factor influencing immune defense mechanisms of animals, deficient or excess levels of nutrients can alter the immune system. Lu et al. (2018) reported that inclusion of increased citrus pulp could improve immunization and hepatic antioxidant status of growing rabbits. Pourhossein et al. (2015) found that Citrus sinensis peel extract significantly improves the serum levels of IgM, and IgG in broiler chickens. Liu et al. (2015) reported that dietary vitamin B6 supplemental level had influence on serum total antioxidant capacity, glutathione peroxidase and total superoxide dismutase. Evaluation of the plasma oxidative status for different animal species could become an important parameter of the health conditions of animals, Meineri et al. (2017) have evaluated the parameters of plasma oxidative status in rabbits under physiological conditions. It is reported that vitamin B6 deficiency decreases the titres of IL-2, T-, and B-lymphocytes in healthy individuals (Meydani et al. 1991). Additionally, lack of vitamin B6 decreases serum IgE, IgG1, and IgG2a titres (Inubushi et al. 2000). However, challenged with a virulent strain of Vibrio banguillarum, the fish fed the high protein diet with 20 or 40 mg/kg vitamin B6 was significantly improved in its disease resistance (Hardy et al. 1979). Our findings that vitamin B6 increased serum IgA titres, which might be due to PLP is involved in IgA synthesis. Cytokines are important signal molecules of the immune system. Antigen-specific immune markers in serum titres (Inubushi et al. 2000). However, challenged with a virulent strain of Vibrio banguillarum, the fish fed the high protein diet with 20 or 40 mg/kg vitamin B6 was significantly improved in its disease resistance (Hardy et al. 1979). Our findings that vitamin B6 increased serum IgA titres, which might be due to PLP is involved in IgA synthesis. Cytokines are important signal molecules of the immune system. Antigen-specific immune markers in serum titres (Inubushi et al. 2000). However, challenged with a virulent strain of Vibrio banguillarum, the fish fed the high protein diet with 20 or 40 mg/kg vitamin B6 was significantly improved in its disease resistance (Hardy et al. 1979). Our findings that vitamin B6 increased serum IgA titres, which might be due to PLP is involved in IgA synthesis. Cytokines are important signal molecules of the immune system. Antigen-specific immune markers in serum titres (Inubushi et al. 2000). However, challenged with a virulent strain of Vibrio banguillarum, the fish fed the high protein diet with 20 or 40 mg/kg vitamin B6 was significantly improved in its disease resistance (Hardy et al. 1979). Our findings that vitamin B6 increased serum IgA titres, which might be due to PLP is involved in IgA synthesis. Cytokines are important signal molecules of the immune system. Antigen-specific immune markers in serum titres (Inubushi et al. 2000). However, challenged with a virulent strain of Vibrio banguillarum, the fish fed the high protein diet with 20 or 40 mg/kg vitamin B6 was significantly improved in its disease resistance (Hardy et al. 1979). Our findings that vitamin B6 increased serum IgA titres, which might be due to PLP is involved in IgA synthesis. Cytokines are important signal molecules of the immune system.

Table 4. Effects of dietary supplement of vitamin B6 on immune organs development of growing Rex rabbits (g/kg live weight before slaughter).

| Items | Dietary vitamin B6 supplemental level (mg/kg; as-fed basis) | R-MSE | P-value |
|-------|----------------------------------------------------------|-------|---------|
| Thymus relative weight | 0.28a 0.38ab 0.45a 0.31b 0.33b 0.517 0.0362 |       |         |
| Spleen relative weight | 2.26b 2.29ab 2.39ab 2.62a 2.30b 0.487 0.0478 |       |         |
| Liver relative weight | 26.13 24.28 23.53 25.91 24.29 3.498 0.5102 |       |         |

a,b,cDifferent letters in the same row denote significance (P < 0.05). R-MSE, root mean square error.

Table 5. Effects of dietary supplement of vitamin B6 on serum immune active compounds of growing Rex rabbits (μg/mL serum).

| Items | Dietary vitamin B6 supplemental level (mg/kg; as-fed basis) | R-MSE | P-value |
|-------|----------------------------------------------------------|-------|---------|
| Serum IgG titre | 19.64 19.83 20.50 23.91 21.75 3.893 0.0240 |       |         |
| Serum IgA titre | 132.74c 135.25c 162.78a 148.54b 140.04bc 43.330 0.0240 |       |         |
| Serum IgE titre | 13.17 14.62 14.82 15.49 15.04 3.650 0.5622 |       |         |
| Serum IL-2 titre | 28.69 32.44 32.92 34.31 33.10 5.584 0.0501 |       |         |
| Serum IL-6 titre | 250.28b 251.73ab 267.59ab 288.56a 298.61a 49.129 0.0353 |       |         |
| Serum IFN-γ titre | 210.12b 211.38ab 221.11a 222.83a 232.16a 43.282 0.0142 |       |         |

a,b,cDifferent letters in the same row denote significance (P < 0.05). R-MSE, root mean square error.

Table 6. Effects of dietary supplement of vitamin B6 on intestinal mucus sIgA titres of growing Rex rabbits (μg/mL intestinal mucus supernatant).

| Items | Dietary vitamin B6 supplemental level (mg/kg; as-fed basis) | R-MSE | P-value |
|-------|----------------------------------------------------------|-------|---------|
| Duodenum sIgA titre | 24.73b 26.73b 27.79b 30.48a 28.71b 1.319 0.0130 |       |         |
| Jejunum sIgA titre | 28.00 28.62 29.05 29.14 28.68 1.177 0.0984 |       |         |
| Ileum sIgA titre | 30.37c 34.25b 34.81b 36.03a 35.49ab 0.775 0.0453 |       |         |
| Colon sIgA titre | 28.46 28.72 28.93 30.75 29.12 1.615 0.0738 |       |         |

a,b,cDifferent letters in the same row denote significance (P < .05). R-MSE, root mean square error.
B-lymphocytes differentiate into mature sIgA-producing plasma cells, in a process that requires the presence of cytokines. IL-2 is a peptide activation factor of T-lymphocytes that promote the proliferation and differentiation of T- and B-lymphocytes. IL-6 enhances the secretion of IgA, and IgA can directly promote the synthesis of more B lymphocytes. In this study, dietary vitamin B6 affected serum IL-6 and IFN-γ titres, increased serum IL-2 titres, and affected splenic IL-6 and IFN-γ mRNA expression levels. These results contrast with those reported by Kwak et al. (2002) in young women where B6 status was positively related to lymphocyte proliferation and IL-2 production. Vitamin B6 has been associated to immune response through the B6 dependent transsulfuration pathway in which the intermediary amino acids homocysteine is directed towards cysteine and eventually, the glutathione peroxidase system (Mosharov et al. 2000).

B-lymphocytes differentiate into mature sIgA-producing plasma cells, in a process that requires the presence of cytokines. IL-2 is a peptide activation factor of T-lymphocytes that promote the proliferation and differentiation of T- and B-lymphocytes. IL-6 enhances the secretion of IgA, and IgA can directly promote the synthesis of more B lymphocytes. In this study, dietary vitamin B6 affected serum IL-6 and IFN-γ titres, increased serum IL-2 titres, and affected splenic IL-6 and IFN-γ mRNA expression levels. These results contrast with those reported by Kwak et al. (2002) in young women where B6 status was positively related to lymphocyte proliferation and IL-2 production. Vitamin B6 has been associated to immune response through the B6 dependent transsulfuration pathway in which the intermediary amino acids homocysteine is directed towards cysteine and eventually, the glutathione peroxidase system (Mosharov et al. 2000).

**Effect of dietary supplement of vitamin B6 on sIgA titres and M cell count**

Total sIgA titres in the intestinal mucus supernatant were quantified by ELISA kit, and the results revealed that sIgA titres in the ileum were higher than those in the duodenum, jejunum, or colon. Dietary vitamin B6 levels increased sIgA titres in the duodenum and ileum, in agreement with the findings obtained by Hanson and Lanning (2008). Additionally, sIgA assists M cells in presenting antigens to lymphoid follicles (Neutra et al. 1999). pIgR is a complete transmembrane secretion segment that binds to poly-IgA molecule ‘J’ chain, involved in poly-IgA transport and secretion of IgA, and may play an important role in the removal of pathogens and toxins (Gurevich et al. 2003). In this study, dietary vitamin B6 had no significant effects on pIgR mRNA expression levels in the ileum.

The relatively constant expression of vimentin by M cells in the appendix was significantly affected by dietary supplement of vitamin B6. The appendix forms part of the gut-associated lymphoid tissues and is lined by a specialized tissue known as the follicle-associated epithelium, which primarily consists of M cells and enterocytes (Shaykhiev and Bals 2007). M cells play a crucial role in the resistance to infections. Besides, M cells produce vimentin (Gebert and Hszch 1992), which is an intermediate filament generally present in cells of mesenchymal origin (Beyaz et al. 2010). Vimentin immunoreactivity has been determined in both the perinuclear cytoplasm and cytoplasmic regions surrounding epithelial lymphocyte of mature M cells. M cells migrate towards the intestinal mucosa mainly to develop immunocompetence against subsequent contact with antigens (Carabaño et al. 2008). In this study, M cell morphology was visualized by immunohistochemical staining and dietary B6 can improve M cell counts.
Conclusions

In summary, vitamin B6 clearly plays an important role in stimulating the non-specific immune responses of growing Rex rabbits under the experimental conditions. Using a ration mainly consisting of corn, wheat bran, and peanut vene, the appropriate vitamin B6 supplemental level for weaned (28-day) to 3-month old growing Rex rabbits was 10 to 20 mg/kg.

Disclosure statement

No potential conflict of interest was reported by the authors.

Funding

This study were supported by the earmarked fund for Modern Agricultural Industry Technology System Special Funding [CARS-44-B-1] and Funds of Shandong ‘Double Tops’ Program.

References

Albrektsen S, Waagbo R, Sandnes K. 1993. Tissue vitamin B6 concentrations and aspartate amino transferase (Asp T) activity in Atlantic salmon (salmo salar) fed graded levels of vitamin B6. Fisk Dr Skr Ser Ernaring. 6:21–34.

AOAC International. 2005. Official methods of analyses, 18th ed. Rockville, MD: Association of Official Analytical Chemists.

Beyaz F, Ergün E, Bayraktaroğlu AG, Ergün L. 2010. The identification of intestinal M cells in the sacculus rotundus and appendix of the angora rabbit. Vet Res Commun. 34:255–265.

Bräunlich K. 1974. Vitamin B6. Basel: Hoefle & Wettstein AG.

Bråunlich K. 1974. Vitamin B6. Basel: Hoefle & Wettstein AG.

Braunlich K. 1974. Vitamin B6. Basel: Hoefle & Wettstein AG.

Gebert A, Hsz G. 1992. Vimentin antibodies stain membranous epithelial cells in the rabbit bronchus-associated lymphoid tissue (GALT). Histochem Cell Biol. 98:271–273.

Gidenne T, Garcia J, Lebas F, Licois D. 2010. Nutrition and feeding strategy: interactions with pathology. In: De Blas C, Wiseman J, editor. The nutrition of the rabbit. Wallingford Oxon: CAB International Publishing; p. 179–199.

Gurevich P, Zusman I, Moldavsky M. 2003. Secretory immune system in human intrauterine development: immuno pathomorphological analysis of the role of secretory component (IgGf/SC) in immunoglobulin in transport. Int J Mol Med 12:289–297.

Ha C, Miller LT, Kerkvliet NI. 1984. The effect of dietary vitamin B6 deficiency on cytotoxic immune responses of T cells, antibodies, and natural killer cells, and phagocytosis by macrophages. Cell Immunol. 85(2):318–329.

Hanson NB, Lanning DK. 2008. Microbial induction of B and T cell areas in rabbit appendix. Dev Comp Immunol. 32:980–991.

Hardy RW, Halver JE, Brannon EL. 1979. Effect of dietary protein level on the pyridoxine requirement and disease resistance of chinook salmon. Proceedings of the World Symposium on Fish Nutrition and Fish Feed Technology. 2:53–60.

Harris DJ, Cheeke PR, Patton NM. 1983. Effect of supplemental vitamins on fryer rabbit performance. J Applied Rabbit Res. 6:29–131.

Hove EL, Herndon JF. 1957. Vitamin B6 deficiency in rabbits. J Nutr. 61(1):127–136.

Inubushi T, Okada M, Matsui A, Hanba J, Murata E, Katunuma N. 2000. Effect of dietary vitamin B6 contents on antibody production. Biofactors. 11(1-2):93–96.

Kwak HK, Hansen CM, Leklem JE, Hardin K, Shultz TD. 2002. Improved vitamin B-6 status is positively related to lymphocyte proliferation in young women consuming a controlled diet. J Nutr. 132:3308–3313.

Lebas F. 2004. Reflections on rabbit nutrition with special emphasis on feed ingredients utilization. In: Becerril CM, Pro A, editors. Proceedings of the 8th world rabbit congress, puebla. Montecillo: Colegio de Postgraduados; p. 686–736.

LiLivak KJ, Schmittgen TD. 2001. Analysis of relative gene expression data using real-time quantitative PCR and the 2-ΔΔCT method. Methods. 25:402–408.

Liu G, Sun C, Shang S, Wu Z, Wang C, Li F. 2015. Effects of dietary vitamin B6 supplemental level on digestion and metabolism, and antioxidant ability of 3 to 5-month old growing Rex rabbits. Chinese J Animal Nutri. 27(11):3468–3477.

Lu J, Long X, He Z, Shen Y, Yang Y, Pan Y, Zhang J, Li H. 2018. Effect of dietary inclusion of dried citrus pulp on growth performance, carcass characteristics, blood metabolites and hepatic antioxidant status of rabbits. J Appl Anim Res. 46(1):529–533.

Maertens L. 1996. Nutrition du lapin. connaissances actuelles et acquisitions recentes. Cunicultura. 23:33–35.

Mateos G, Piquer J. 1994. Diseño de programas alimenticios para conejos: aspectos teóricos y formulación práctica. Boletín de Cunicultura; p. 175–180.

Meineri G, Giacobini M, Forneris G. 2017. Evaluation of physiological parameters of the plasma oxidative status in rabbits. J Appl Anim Res. 45(1):315–319.

Meydani SN, Ribaya MJ, Russell RM, Sahyoun N, Morrow FD, Gershoff SN. 1991. Vitamin B6 deficiency impairs interleukin 2 production and lymphocyte proliferation in elderly adult. Am J Clin Nutr. 53(5):1275–1280.

Mohamed JS. 2001. Dietary pyridoxine requirement of the Indian catfish (heteropneustes fossilis). Aquaculture. 194:327–335.

Mosharof E, Cranford MR, Banerjee R. 2000. The quantitatively important relationship between homocysteine metabolism and glutathione synthesis by the transsulfuration pathway and its regulation by redox changes. Biochem. 39:13005–13011.

Neutra MR, Mantis NJ, Frey A, Giannasca PJ. 1999. The composition and function of M cell apical membranes: implications for microbial pathogenesis. Semin Immun. 11:171–181.

NRC. 1977. Nutrient requirements of domestic animals, no.9. nutrient requirements of rabbits, 2nd revised edn. Washington, DC: National Academy of Science, National Research Council.

Pourhossein Z, Qotbi AAA, Seidavi A, Laudadio V, Centoducati G, Tufarelli V. 2015. Effect of different levels of dietary sweet orange (citrus sinensis) peel extract on humoral immune system responses in broiler chickens. Anim Sci J. 86:105–110.

Schlolut W. 1987. Nutritional needs and feeding of German angora rabbits. J Appl Rabbit Res. 10:111–121.

Shakhyrie R, Bals R. 2007. Interactions between epithelial cells and leukocytes in immunity and tissue homeostasis. J Leukoc Biol. 82(1):53–58.

Sheela RR, Babu U, Mu J, Elankumaran S, Bautista DA, Raybourne RB, Heckert RA, Song W. 2003. Immune responses against salmonella enterica serovar enteritidis infection in virally immunosuppressed chickens. Clin Diagn Lab Immunol. 10:670–679.

Shimoi K, Akiwa E, Mori N, Sano M, Nakamura Y, Tomita I. 1992. Bio-antimutagenic activities of vitamin B6 in E. coli and mouse peripheral blood cells. Mutat Res-Fund Mol M. 266(2):205–213.

Stanley EL. 2009. Statistical evaluation of methods for quantifying gene expression by autoradiography in histological sections. BMC Neurosci. 10:1–15.

Trakatellis A, Dimitriadou A. 1992. Effect of pyridoxine deficiency on immunological phenomena. Postgrad Med. 68:570–577.

Weiss FG, Scott ML. 1979. Influence of vitamin B6 upon reproduction and upon plasma and egg cholesterol in chickens. J Nutri. 109:1010–1017.

Woolworth JG, Goodband RD, Nelssen JL, Tokach MD, Musser RE. 2000. Added dietary pyridoxine, but not thiamin, improves weanling pig growth performance. J Anim Sci. 78:B88–93.

Xiccato G. 1996. Nutrition of lactating does. In: Lebas F, editor. Proceedings of the 11th world rabbit congress. toulouse. Lempdes: Association Francaise de Cuniculture; p. 175–180.

Zhu Y, Wang C, Wang X, Li B, Sun L, Li F. 2013. Effects of dietary fiber and starch levels on the non-specific immune response of growing rabbits. Livest Sci. 155:285–293.